

Carina Sofia Barradas Maranga

Early mitochondrial modifications in YACI28 transgenic model of Huntington's disease

Dissertação de Mestrado em Biologia Celular e Molecular.





UNIVERSIDADE DE COIMBRA

Carina Sofia Barradas Maranga

Early mitochondrial modifications in YAC128 transgenic model of Huntington's disease

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de mestre em Biologia Celular e Molecular, realizada sobre a orientação da Professora Doutora Ana Cristina Rego (Faculdade de Medicina da Universidade de Coimbra e Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e co-orientada pela Professora Doutora Emília Duarte (Departamento de Ciência da Vida, Universidade de Coimbra)

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Image on the cover was kindly provided by Luana Naia and Catarina Carmo (*Mitochondrial Dysfunction and Signaling in Neurodegeneration group*). Confocal image obtained with a 63x objective, NA=1.4 on a Zeiss LSM 710 inverted microscope. Cortical neurons (11 DIV) isolated from YAC128 mice embryos were transfect with mitochondria-targeted DsRed in order to visualize their mitochondrial network. Mitochondria are displayed with a Fire LUT.

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Whitey Durham

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Abbreviations

- ADP adenosine diphosphate
- ALCAR Acetyl-L-carnitine
- ALA- Alpha-lipoic acid
- ARE Antioxidant response element
- ATP adenosine triphosphate
- **BDNF** Brain-derived neurotrophic factor
- BSA Bovine serum albumin
- CAG Cytosine-Adenine-Guanine
- **CBP** cAMP-response-element-binding-protein-binding protein
- EGTA Ethylene glycol tetraacetic acid
- FCCP Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
- **GABA** γ -aminobutyric acid
- GP Globus pallidus
- GPe Globus pallidus external segment
- **GPi** *Globus pallidus* internal segment
- **GPx** Glutathione peroxidase
- GRed Glutathione reductase
- GSH Glutathione, reduced form
- GSSG Glutathione, oxidized form
- HAP 1 Huntingtin-associated protein 1
- HD Huntington's disease
- HEAT Huntingtin, elongation factor 3, protein phosphatase 2A and yeast kinase
- HIP Huntingtin-interacting protein
- HO' Hydroxyl radical
- H₂O₂ Hydrogen peroxide
- Htt Huntingtin
- **KA** Kainate
- MA Malonic acid
- MCU Mitochondrial Ca2+ uniporter
- mHtt Mutant huntingtin

- **MIM** Mitochondrial inner membrane
- **MOM** Mitochondrial outer membrane
- mPTP Mitochondrial permeability transition pore
- MLK2 Mixed-lineage kinase 2
- MSN Medium spiny neurons
- mtDNA Mitochondrial DNA
- mTOR- Mammalian target of rapamycin
- **NAD** β -Nicotinamide adenine dinucleotide
- **NADP** β -Nicotinamide adenine dinucleotide 2'-phosphate
- NaF Sodium fluoride
- **NaOH** Sodium hydroxide
- NEM N-ethylmaleimide
- **NES** Nuclear export signal
- NMDA N-methyl-D-aspartate
- **NMDARs** NMDA receptors
- NOX NADPH oxidase
- 3-NP 3-nitropropionic acid
- Nrf2 Nuclear factor-erythroid 2-related factor-2
- NRSF Neuron-restrictive silencer factor
- O2 -- Superoxide anion radical
- O3 Ozone
- OCR Oxygen consumption rate
- **OXPHOS** Oxidative phosphorylation
- PGC-1α Peroxisome proliferator-activated receptor γ coactivator 1-alpha
- Pi Inorganic phosphate
- PSD 95 Postsynaptic density 95
- **QA** Quinolinic acid
- **REST** Repressor-element-1 transcription factor
- RCS Reactive chloride species
- **RNS** Reactive nitrogen species
- ROS Reactive oxygen species
- **RSS** Reactive sulfur species

- SDS Sodium dodecyl sulphate
- **SDS-PAGE** SDS polyacrylamide gel electrophoresis
- SH3 Src homology-3
- SNpr Substantia nigra pars reticulata
- **SOD** Superoxide dismutase
- SP1 Specificity protein 1
- STS Staurosporine
- TAFII130 TBP-associated factor
- *t*-BHP *tert*-butyl hydroperoxide
- **TBP** TATA-binding protein
- Tfam Mitochondrial transcription factor A
- TUNEL Deoxynucleotidyl transferase-mediated dUTP nick-end labeling
- **Ub** Ubiquitin
- VDAC voltage-dependent anion-selective channel
- YAC Yeast artificial chromosome
- $\Delta \Psi_m$ Mitochondrial membrane potential

Abstract

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by psychiatric, motor and cognitive symptoms, strongly affecting the striatum and the cerebral cortex. HD is caused by a CAG expansion in the *HTT* gene encoding for mutant huntingtin (mHTT), a misfolded protein that interferes with several cellular processes, including mitochondrial function and redox regulation leading to oxidative stress. We hypothesized that defects in mitochondrial function and evidences of oxidative stress can occur in early stages of HD. Thus, the objective of our study was to analyze the mitochondrial parameters and oxidative stress in cortical and striatal isolated mitochondria from YAC128 mice (transgenic mice expressing full-length mHTT with 128 glutamines) *versus* age-matched wild-type (WT) littermates at pre-symptomatic and symptomatic stages.

Our results demonstrated a significant decrease in both mitochondrial coupling and respiratory chain complexes (CX) I-IV activities in pre-symptomatic (3 mo) YAC128 cortical mitochondria, whereas a significant increase in these parameters was observed in striatal mitochondria from YAC128 mice at this stage. Conversely, at symptomatic stages (12 mo), no major differences in OCR or complexes I-IV activities were observed either in cortex or striatal mitochondria.

Both YAC128 and WT cortical mitochondria exhibited a significant decrease in mitochondrial respiration from 3 to 9 mo; however, a tendency for a recovery in OCR parameters was observed between 9 and 12 mo. In striatal mitochondria from YAC128 mice an age-dependent decrease in all parameters was observed. Regarding CX activities, our results showed a decrease in cortical mitochondria from WT mice upon aging, whereas little age-dependent changes were observed in YAC128 cortical mitochondria; conversely, a decrease was observed in YAC128 mouse striatal mitochondria. Moreover, our data showed a decrease in Ca²⁺ handling in cortical and striatal mitochondria from pre-symptomatic YAC128 mice, whereas no changes were detected in both areas of symptomatic mice. Cortex-derived mitochondria also exhibited a significant increase in hydrogen peroxide (H₂O₂) production in pre-symptomatic YAC128 mouse mitochondria obtained from the striatum. At 3 mo enhanced levels of H₂O₂ could be accounted for by a significant decrease in mitochondrial glutathione peroxidase activity, suggesting insufficient capacity to reduce H₂O₂. Decreased glutathione reductase protein levels were also found in mitochondrial fractions from 3 mo YAC128 striatum, although the activity of this enzyme or GSH and GSSG levels were unchanged. Nevertheless,

in cortical mitochondria from 3 mo YAC128 mice an increase in GSH levels was observed. In addition, cortical mitochondria from 12 mo YAC128 mice exhibited decreased catalase levels and an increase in superoxide dismutase (SOD)2 activity (analyzed through acetylated SOD2 at Lys68) in despite of decreased SOD2 protein levels, suggesting compensatory effects that translated into unchanged H₂O₂ production.

Altogether, these data evidence mitochondrial modifications occurring in pre-symptomatic YAC128 mice, namely decreased mitochondrial function in the cortex and enhanced organelle activity in striatum, accompanied by early oxidative stress. Interestingly, striatal mitochondrial are highly affected during aging, sustaining the oxidant status, which may implicate enhanced susceptibility of this brain region in HD.

Keywords: Huntington's disease, YAC128 mice, Oxidative stress, O2 consumption, Calcium handling

Resumo

A doença de Huntington (DH) é uma doença neurodegenerativa autossómica dominante caracterizada por sintomas psiquiátricos, motores e cognitivos, afetando preferencialmente o estriado e o córtex cerebral. DH é causada por uma expansão do trinucleótido CAG no gene *HTT*, que codifica para a huntingtina mutada (mHTT), uma proteína que interfere com vários mecanismos celulares, incluindo a função mitocondrial e a regulação redox, causando stresse oxidativo. Colocámos então a hipótese de que os defeitos na função mitocondrial e as evidências de *stress*e oxidativo possam ocorrer numa fase inicial da HD. Assim, o objetivo do nosso estudo foi avaliar os parâmetros mitocondriais e o *stresse* oxidativo em mitocôndrias corticais e estriatais isoladas do murganho YAC128 *versus* murganhos controlo da mesma idade nas fases pré-sintomática e sintomática. Os nossos resultados demonstram uma diminuição significativa no acoplamento mitocondrial e nas atividades dos complexos I-IV da cadeia respiratória em mitocôndrias derivadas do córtex do YAC128 pré-sintomático (3 meses), tendo-se verificado um aumento significativo destes parâmetros em mitocôndrias estriatais em DH. Contudo, na fase simptomática (12 meses) não se observaram diferenças no consumo de oxigénio ou atividade dos complexos I-IV nas mitocôndrias corticais ou estriatais.

Ambas as mitocôndrias do cortex dos murganhos YAC128 e WT exibiram uma diminuição significativa na respiração mitocondrial dos 3 aos 9 meses; porém, observou-se uma tendência para a recuperação dos parâmetros de consumo de oxigénio entre os 9 e os 12 meses de idade. Nas mitocôndrias estriatais do murganho YAC128 foi observada uma diminuição dependente da idade em todos os parâmetros. Relativamente às atividades dos complexos, os nossos resultados mostram uma diminuição nas mitocôndrias do córtex do murganho WT com o envelhecimento, enquanto pequenas alterações dependentes da idade foram observadas nas mitocôndrias do córtex do murganho YAC128. Porém, foi observada uma diminuição nas mitocôndrias do a captação de Ca²⁺ em mitocôndrias corticais e estriatais de murganhos YAC128 pré-sintomáticos, não se tendo observado diferenças em ambas as áreas nos murganhos sintomáticos. Nas mitocôndrias derivadas do córtex observou-se ainda um aumento significativo na produção de peróxido de hidrogénio (H₂O₂) nos murganhos YAC128 pré-sintomáticos; níveis aumentados de H₂O₂ foram detetados em ambas as mitocôndrias estriatais de murganhos YAC128 pré-sintomáticos. Aos 3 meses este efeito poderá estar relacionado com a

diminuição significativa na atividade da glutationa peroxidase mitocondrial, sugerindo uma capacidade insuficiente para reduzir o H_2O_2 .

Uma diminuição nos níveis proteicos da glutationa redutase foi também descrita nas frações mitocondriais do estriado do murganho YAC128 com 3 meses de idade, porém a atividade da enzima ou os níveis de GSH e GSSG encontravam-se inalterados. Ainda assim, observou-se um aumento dos níveis de GSH nas mitocôndrias corticais do murganho YAC128 de 3 meses. Para além disso, as mitocôndrias do córtex do YAC128 com 12 meses mostraram uma diminuição dos níveis de catalase e um aumento da atividade da SOD2 (avaliado pela acetilação de SOD2 no resíduo Lys68) apesar dos níveis de SOD2 diminuídos, sugerindo efeitos compensatórios sem que se detetassem diferenças significativas na produção de H₂O₂.

Em suma, os nossos resultados evidenciam modificações mitocondriais precoces no murganho YAC128 (pré-sintomático), nomeadamente uma diminuição na função mitocondrial no córtex e aumento na atividade do organelo no estriado, acompanhado de stresse oxidativo. Curiosamente, as mitocôndrias do estriado são aparentemente mais afetadas durante o envelhecimento, mantendo o estado oxidativo, o que pode implicar uma suscetibilidade elevada desta região cerebral na DH.

Palavras-chave: Doença de Huntington, ratinhos YAC128, *stresse* oxidativo, consumo de oxigénio, captação de cálcio.

CHAPTER I -INTRODUCTION

1.1HUNTINGTON'S DISEASE

"The hereditary chorea ... is confined to certain and fortunately a few families, and has been transmitted to them, an heirloom from generations away back in the dim past. It is spoken of by those in whose veins the seeds of the disease are known to exist, with a kind of horror, and not at all alluded to except through dire necessity, when it is mentioned as 'that disorder.' It is attended generally by all the symptoms of common chorea, only in an aggravated degree, hardly ever manifesting itself until adult or middle life, and then coming on gradually but surely, increasing by degrees, and often occupying years in its development, until the hapless sufferer is but a quivering wreck of his former self... There are three marked peculiarities in this disease: 1. Its hereditary nature. 2. A tendency to insanity and suicide. 3. Its manifesting itself as a grave disease only in adult life... When either or both the parents have shown manifestations of the disease, and more especially when these manifestations have been of a serious nature, one or more of the offspring almost invariably suffer from the disease, if they live to adult age. But if by any chance these children go through life without it, the thread is broken and the grandchildren and great-grandchildren of the original shakers may rest assured that they are free from the disease..." (Huntington, 1872)

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder firstly described by George Huntington in 1872. After publication in *The Medical and Surgical Reporter*, the disorder was named Huntington's chorea and later HD, since not all patients developed chorea (Douglas, 2000). In 1983, Gusella identified a polymorphic DNA marker associated with a mutation on the tip of the short arm of chromosome 4 showing genetic linkage to the HD gene (Gusella, 1983). Only a decade later, in one of the first successful linkage analyses, the anonymous DNA fragment was identified as an unstable expanded CAG trinucleotide repeat in a novel gene, the *IT15* (interesting transcript 15) gene, presently known as the *HTT* gene. This new gene containing 67 exons was located between the regions D4S127 and D4S180 on chromosome 4p16.3 and spanning over a 200 kb genomic region; the protein product of about 348 kDa, containing 3144 amino acids, was named huntingtin (The Huntington's Disease Collaborative Research Group., 1993). In HD, increase number of CAG repeats encode for a polyglutamine (polyQ) expansion at the N-terminus of mutant huntingtin (mHTT in humans).

Normal huntingtin is encoded by the *HTT* gene containing up to 35 CAG repeats. Alleles with 35-39 repeats show incomplete penetrance, which means that some individuals will develop the disease (particularly at old age) while others do not. Repeats higher than 39 invariably lead to HD (Novak & Tabrizi, 2010). Alleles with 40–50 repeats give rise to the most common adult-onset form of HD in which the mean age of onset is around 40 years of age, with death occurring 15-20 years later. Paternal transmission is related to longest repeats (higher than 60) responsible for the severe juvenile HD (JHD) cases. Indeed, there is an inverse correlation between the age of onset and the number of

CAG repeats, which is unequivocal for the longest CAG repeats (The Huntington's Disease Collaborative Research Group, 1993; Rego and de Almeida, 2005, for review). Therefore, HD was added to the group of polyQ genetic disorders. in which mHTT causes a gain and/or loss of protein function that affects different brain regions, mainly the striatum and cortex, leading to many clinical features, as shown in **Table 1**.

Polyglutamine disorder CAG	Protein product/ locus	Normal and pathogenic repeat number	Primary target of neuropathology	Main clinical features
Dentatorubro- pallidoluysian atrophy (DRPLA)	Atrophin 1 12p12	3-35 49-88	Cerebellum (dentate nucleus), red nucleus, globus pallidus (external segment), subthalamic nucleus	Ataxia, seizures, choreoathetosis, dementia
Huntington's disease (HD)	Huntingtin 4p16.3	6-35 36-121	Caudate nucleus (medium spiny neurons), putamen, globus pallidus- external segment	Chorea, dystonia, cognitive deficits, psychiatric problems
Spinal and bulbar muscular atrophy (SBMA; Kennedy disease)	Androgen receptor Xq11-q12	9-36 38-62	Motor neuron in anterior horn cells of the spinal cord and brainstem	Motor weakness, swallowing, gynecomastia, decreased fertility
Spinocerebellar ataxia 1 (SCA1)	Ataxin 1 6p23	6-38 39-83	Cerebellum, red nucleus, inferior olive, pons, anterior horn cells and pyramidal tracts	Ataxia, slurred speech, spasticity, cognitive impairments
Spinocerebellar ataxia 2 (SCA2)	Ataxin 2 12q24	14-31 32-77	Cerebellar Purkinje cells, cytoplasmatic inclusions	Ataxia, polyneuropathy, decreased reflexes, infantile variant with retinopathy
Spinocerebellar ataxia 3 (SCA3)	Ataxin 3 14q24.3-q31	12-40 54-86	Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord	Ataxia, parkinsonism, spasticity
Spinocerebellar ataxia 6 (SCA6)	Ca ²⁺ channel 19p13	4-19 20-30	Cerebellar Purkinje cells, dentate nucleus, inferior olive, cytoplasmatic inclusions	Ataxia, dysarthria, nystagmus, tremors
Spinocerebellar ataxia 7 (SCA7)	Ataxin 7 3p21.1-p12	4-35 37-200	Cerebellum, brain stem, macula, visual cortex	Ataxia, blindness, cardiac failure in infantile form
Spinocerebellar ataxia 17 (SCA17)	TATA-binding protein (TBP) 6q27	29-42 47-55	Cerebellum, cortex (diffuse atrophy), caudate and putamen. NII	Ataxia, cognitive decline, seizures, and psychiatric problems

TABLE 1 : POLYQ DISORDES GENETIC FEATURES, AFFECTED PROTEINS, BRAIN REGION AND MAIN CLINICAL SYMPTOMS

(Adapted from Rego & de Almeida, 2005 and Orr & Zoghbi)

1.1.1 Clinical features

HD is more common in people of northern European origin. In 2011, HD prevalence was described as 5.70 per 100,000 individuals in Europe, North America and Australia, being only 0.40 per 100,000 people in Asia (Pringsheim et al., 2012). HD prevalence in UK is about 10:10,000. In Portugal, the prevalence of HD is estimated to range from 2-5 per 100,000 people (Costa et al., 2003).

The life course of an HD patient can be divided into 1) a risk stage, defined by the presence of the CAG expansion repeats in the HTT gene within the range of 35-39 CAGs; 2) a preclinical stage and 3) a clinical stage, characterized by a decrease in independence, severe health deterioration, and a significant increase in the need for care (Roos, 2010). The symptoms and signs of HD consist of psychiatric, cognitive and motor disturbances, progressive dementia and weight loss. Psychiatric and cognitive problems can be the first signs occurring many years before motor signs become visible. The most common psychiatric symptoms are depression, anxiety, apathy or obsessive-compulsive behavior, which can be confused with schizophrenia and with paranoid hallucinations. Cognitive impairment, described as reduction in thought processing and deterioration of executive functions and memory is one of the main features of HD appearing, in some cases, before motor dysfunction (Roos, 2010). The characteristic motor changes are divided in two different categories, involuntary movements (chorea) and impaired voluntary movements (loss of postural reflexes, rigidity, bradykinesia, dystonia and akinesia). Depression and aggression are HD features and almost all patients show severe weight loss and endocrine dysfunction, along with sleep and circadian rhythm disturbances (Novak & Tabrizi, 2010; Roos, 2010). In JHD, symptoms begin before the age of 20 years; in these cases disease progression is faster and the symptoms are more severe than the adult onset cases, being characterized by behavior disturbances, learning difficulties at school and impairment in motor behavior. Chorea only appears in the second decade of the disease and epileptic seizures are very frequent in these patients.

HD diagnosis is based on a combination of motor symptoms, psychiatric or cognitive changes, detailed family history and genetic testing showing CAG repeat expansion in the *HTT* gene (Roos, 2010).

1.1.2 Neuropathology

HD is characterized by striatal (caudate nucleus and putamen) degeneration and astrogliosis. In order to assess the severity of HD degeneration, Vonsattel and coworkers described macroscopic and microscopic criteria, divided into five different grades (0-4). In grade 0, first examination did not exhibit signals of neurodegeneration; however, histological examination revealed 30-40% of neuronal loss in the head of caudate nucleus. Grade 1 patient's brains show atrophy in the tail and sometimes in the body of the caudate nucleus; in addition, neuronal loss and astrogliosis appear in the head, tail and body of this region. In grade 2, HD brains evidence striatal atrophy that becomes more severe in grade 3. In grade 4, HD patients show a combination between severe striatal atrophy and 95% of neuronal loss (Vonsattel et al., 1985; Gil & Rego, 2008). Despite the higher susceptibility of striatum, atrophy and neuronal loss also appears to be present in the cerebral cortex and other regions with a decrease in 40% of brain weight (Gusella, 2001). For example, volumetric losses occur in striatum (60%), cortex (20%), cerebral white matter (30%), globus pallidus (55%) and thalamus (30%) (Reiner, 2011).

The main cell targets of HD are the medium-sized projection spiny neurons (MSNs), which correspond to 95% of the striatal neuronal population and use γ -aminobutyric acid (GABA) as neurotransmitter. It is hypothesized that the loss of these neurons with subsequent loss of downstream inhibitory pathway is the cause of uncontrolled movements, characteristic of this disorder (Gil & Rego, 2008; Gusella, 2001). Modulation of movement by basal ganglia occurs through two different pathways, the direct pathway, where D1 dopamine (DA) receptors are expressed, and the indirect pathway expressing D2 receptors. In the direct pathway, GABAergic MSNs exert direct inhibitory actions on neurons of the substantia nigra pars reticulata (SNpr) and the internal segment of globus pallidus, which are also GABAergic cells. This inhibition of the SNpr leads to a disinhibition of glutamatergic neurons in the thalamus and consequently locomotor activation. The indirect pathway has the opposite effect, through reduction of locomotor activity and movement. MSNs project to the lateral or external segment of the globus pallidus and this external division sends inputs to the subthalamic nucleus (STN). STN neurons activate SNpr/ and the internal segment of globus pallidus, projecting to the thalamus. Therefore, "indirect pathway" modulates the disinhibitory actions of direct pathway; the imbalance between these two pathways can culminate in perturbations of locomotor activity, as the characteristic HD involuntary movements in the beginning of disease.

In HD patients, MSNs that project to the external segment of the globus pallidus degenerate (indirect pathway) and in the absence of inhibitory input the external globus pallidus becomes abnormally active as outlined in **Figure 1**. Then, the activity reduces in turn the excitatory output of the STN to the internal segment of globus pallidus (direct pathway), leading to a significant reduction of inhibitory outflow to the thalamus and thus to the frontal cortex (Calabresi, 2014; Centonze, 2007; Purves, 2003). Along disease progression both indirect and direct pathways are affected, which may even lead to bradykinesia.



FIGURE 1: ALTERATIONS OF DIRECT AND INDIRECT PATHWAY IN HUNTINGTON'S DISEASE

Medium-sized projection spiny neurons of the indirect pathway degenerate, causing a decrease in the projection (thinner arrow) from the caudate/putamen to the globus pallidus (external segment). Tonic inhibition from globus pallidus to the subthalamic nucleus is increased (larger arrow). The excitatory output of the subthalamic nucleus to the globus pallidus (internal segment) is diminished, causing less tonic inhibition from globus pallidus to the thalamus. The decrease of this inhibitory outflow leads to an increased excitation of frontal cortex (large arrow) and consequently inappropriate motor activity. Adapted from Purves, 2003.

1.1.3 Huntingtin protein

1.1.3.1 Wild type Huntingtin

Huntingtin is a protein composed mainly of repeat units of about 50 amino acids termed HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, target of rapamycin 1) repeats (Figure 2). These repeats are segments of two antiparallel α -helices, each one containing a helical hairpin configuration. Wild-type HTT was shown to be present in both cytoplasm and nucleus, being associated with organelles like mitochondria, Golgi apparatus, endoplasmic reticulum, and with synaptic vesicles and components of the cytoskeleton. HTT also interact with many proteins, for example huntingtin-associated protein 1 (HAP1), huntingtin-interacting protein 1 (HIP1) and -2 (HIP2) (Gil & Rego, 2008; Ross & Tabrizi, 2011).

The main functions of HTT are not completely understood. HTT was shown to interact with postsynaptic density 95 (PSD95) through its Src homology-3 (SH3) sequence (proline-x-x-proline), regulating the anchoring of *N*-methyl-D-aspartate (NMDA) and kainate (KA) receptors to the postsynaptic membrane (Sun et al., 2001). HTT also promotes the expression of brain-derived neurotrophic factor (BDNF) by interaction with the transcriptional factor complex repressor-element-1 transcription factor (REST)/neuron-restrictive silencer factor (NRSF) in the cytoplasm, thus preventing its translocation into the nucleus (Zuccato et al., 2003). HTT is also responsible for the vesicular transport of BDNF along the microtubules involving HAP1 and dynactin (Gauthier et al., 2004). In addition, HTT interacts with HAP1 and mixed-lineage kinase 2 (MLK2) to promote the expression of NeuroD, a transcription factor that is crucial for the development of the dentate gyrus in the hippocampus (Marcora et al., 2003). HTT has been also described as a pro-survival protein, since it prevents the activation of caspase 8 through interaction with HIP1 (Gervais et al., 2002). Therefore, HTT seems to have many important functions, such as in protein trafficking, transcriptional regulation, vesicle transport and anchoring to the cytoskeleton, clathrin-mediated endocytosis, postsynaptic signaling and anti-apoptotic function (reviewed by Gil & Rego, 2008 and Landles & Bates, 2004).



FIGURE 2 : A SCHEMATIC DIAGRAM OF THE HTT AMINO ACID SEQUENCE

(Q)n indicates the polyQ tract, followed by the polyproline sequence, (P)n, and the red squares indicate the three main clusters of HEAT repeats. The arrows indicate the caspase cleavage sites and their amino acid positions. B identifies the regions cleaved preferentially in the cerebral cortex, C indicates those cleaved mainly in the striatum, and A indicates regions cleaved in both. Green and orange arrowheads point to the approximate amino acid regions for protease cleavage. NES is the nuclear export signal. The red and blue circles indicate posttranslational modifications: ubiquitination (UBI) and/or SUMOylation (SUMO) (red), and phosphorylation at serine 421 and serine 434 (blue). The glutamic acid (Glu)-, serine (Ser)-and proline (Pro)-rich regions are indicated (serine-rich regions encircled in green). AA indicates number of amino acids. (Cattaneo, Zuccato, & Tartari, 2005 authorized by Nature Reviews)

1.1.2.2 Mutant Huntingtin

It has been described that polyQ domain may cause changes in the conformation of HTT leading to protein aggregation and appearance of neuronal intranuclear or perinuclear inclusions. As reviewed by Gil & Rego (2008), the formation of mHTT aggregates can occur by two different mechanisms: 1) the polar zipper model, which defends that interaction between mHTT and other proteins results in polar zipper structures via hydrogen bonds; and 2) the transglutaminase model, which suggests that transglutaminases (involved in the crosslinking of glutamine residues) are increased and interact with other molecules of mHTT and normal HTT, allowing the precipitation of protein complexes.

Several authors hypothesized that abnormalities observed in HD could be caused by loss of HTT function or gain of mHTT function. According to the first theory (loss of function), toxic effects might be due to the sequestration of normal HTT by mHTT, aggravating the neurodegeneration present in HD (Cattaneo et al., 2001). Dragatsis and colleagues demonstrated that deletion of Htt in adult mice causes neurological features of HD (Dragatsis et al, 2000).

Hayden's group created a targeted disruption in exon 5 of *Hdh*, the murine homologue of the HD gene. They observed that homozygotes for the Hdh^{ex5} mutation exhibited embryo lethality, while the heterozygotes survived to adulthood and had a significant increase in motor activity, neuronal loss and cognitive deficits (Nasir et al., 1995; O'Kusky et al., 1999); these data suggested the

importance of HTT in neuronal function. It was also evidenced that the presence of murine Htt can protect yeast artificial chromosome (YAC) mice model against pro-apoptotic effects of human mHTT, meaning that wild-type HTT may have an anti-apoptotic role (Leavitt et al., 2001). Therefore, loss of function may also contribute to the pathology.

In the second hypothesis, the toxic gain of function by expanded polyQ might disrupt several intracellular pathways and/or sequestration of many important components of these pathways into the aggregates. For example, the mutant protein interacts with other nuclear proteins and transcription factors such as cAMP response element binding protein binding protein (CBP), TATAbinding protein (TBP), TBP-associated factor (TAFII130), and specificity protein 1 (Sp1) (Gil & Rego, 2008). Another feature of toxic aggregates is the impairment in axonal transport. As described above, HTT interacts with HAP1, promoting axonal and retrograde transport, which means that polyQ compromises the transport of vesicles, organelles, motor proteins and other components essentials to cells. mHTT leads to synaptic dysfunction by altering the availability of several synaptic proteins through impairment of gene transcription or their sequestration in HTT inclusions and inhibition of neurotransmitter receptors activity (reviewed in Gil & Rego, 2008). The mutant protein also interferes with PSD95 binding, causing alteration in the function of NMDA and KA receptors (Sun et al., 2001).

mHTT is a substrate for several caspases, namely caspase-3 cleavage at amino acids 513 and 552, for caspase-2 at amino acid 552, and for caspase-6 at amino acid 586 (Figure 2), because of the misfolded nature of the protein (Wellington et al., 1998). Graham and co-authors (2006) generated YAC mice expressing expanded htt containing selective mutations of the caspase-3 and caspase-6 cleavage sites and proved that mutation of the caspase-6, but not caspase-3, cleavage site in mhtt provided protection from neuronal dysfunction and neurodegeneration in vivo, meaning that caspase-6 cleavage site has an important role in HD neurotoxicity. Concordantly, others studies showed that caspases activation and autophagy pathways are involved in HD pathogenesis. Studies using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) identified apoptotic cells in HD striatum (Gil & Rego, 2008). In 2002, Wellington and his team showed that fragments of mHTT are present in mouse and human HD brains and that these cleavage products are increased in cortical projections neurons, leading to corticostriatal dysfunction and excitotoxicity as an early event in HD (Wellington et al., 2002). Hence, calpain inhibitors may be a good target for protection against cell death in HD brains. A study performed in 2008 showed that calpain inhibitors decrease the cleavage fragments, leading to lower levels of mHTT along with less toxicity and neuronal loss (Williams et al., 2008). Moreover, mHTT sequesters the mammalian target of rapamycin (mTOR) into the aggregates, thus inhibiting its kinase activity. Since the mTOR is a negative regulator of autophagy and this cellular mechanism is responsible for recycling organelles, removing misfolded or aggregated proteins, mHTT will enhance these clearance pathway to eliminate toxic HTT fragments. Furthermore, mouse and fly models of HD treated with rapamycin and analogs induced autophagy and efficient reduction of HTT aggregates, suggesting that inhibition of mTOR can be a potential therapeutic target for HD. Thus, autophagy is an initial step to fight against toxic aggregates present in HD, however along the course of the disease might be impaired after prolonged huntingtin-expression or increased aggregate formation (Ravikumar et al., 2004).

Summarizing, cytotoxic forms of HTT can cause dysfunction of many cellular processes, including altered transcription and intracellular signaling, intracellular transport, the secretory pathway, endocytic recycling, and mitochondrial and synaptic function, as show in **Figure 3**. Due to the relevance of mitochondria in HD, mitochondrial impairment will be fully explained in **section 1.2**.



FIGURE 3 : PATHOGENETIC CELLULAR MECHANISMS IN HUNTINGTON'S DISEASE

1-HTT translation produces the full-length huntingtin protein as well as an HTT exon 1 and other fragments (the result of aberrant splicing). 2- Full-length native huntingtin is cleaved through proteolysis to generate additional protein fragments. 3- HTT exon 1 and other fragments translocate into the nucleus. 4- Protein aggregates and fragments are retained in the nucleus by oligomerization and aggregation, leading to the formation of inclusions, a process that causes transcriptional dysregulation. 5- Huntingtin fragments oligomerize and aggregate in the cytoplasm. 6- The aggregation of huntingtin is exacerbated through the disease-related impairment of the proteostasis network, which also leads to global cellular impairments. 7- The aberrant forms of huntingtin results in impairment of several cellular processes, such as synaptic dysfunction, mitochondrial toxicity and energy imbalance and a decreased rate of axonal transport. PRD, proline-rich domain; Ub, ubiquitin. From Bates et al., 2015 authorized by Nature Reviews.

1.2 MITOCHONDRIAL DYSFUNCTION IN HD

1.2.1 The mitochondria

Mitochondria are dynamic organelles essential for several cellular functions, namely production of adenosine triphosphate (ATP), Ca²⁺ homeostasis, metabolism of amino acids, fatty acids and steroids, formation of reactive oxygen species (ROS) and apoptosis regulation. Morphologically, they are surrounded by a double-membrane, consisting of mitochondrial inner and outer membranes (MIM and MOM, respectively), separated by the intermembrane space. Mitochondria contain their own DNA, which encodes tRNA, rRNA and some mitochondrial proteins. Neurons are highly dependent on mitochondrial function since they require a high energy demand to perform many cellular processes, as axonal transport of organelles and macromolecules, neurotransmitter vesicle loading and release, and maintenance of transmembrane ionic gradients (Kim et al., 2010; Martin, 2012). The mitochondrial respiratory chain (MRC) is composed by four multimeric protein complexes (complexes I - IV) and ATP synthase (Figure 4). The main role of this chain is the generation of a proton gradient (due to complex I, III and IV activities) coupled to electron transfer, O_2 consumption and to ATP production. Complex I is also named ubiquinone oxidoreductase or NADH dehydrogenase, complex II is succinate-ubiquinone oxidoreductase or succinate dehydrogenase, complex IIIubiquinol-cytochrome c reductase and the complex IV, cytochrome c oxidase. Electrons are donated to complex I from NADH, and then transferred to coenzyme Q, which carries them to complex III. FADH₂ generated by complex II also donates electrons to coenzyme Q. Complex III transfers electrons to cytochrome c, which relays them to complex IV. In this complex, the combination of hydrogen ions (H⁺) and molecular oxygen (O₂) generate water (H₂O). H⁺ are pumped across the MIM from the matrix to the intermembrane space through complexes I, III and IV. This generates an electrochemical gradient that results in the mitochondrial transmembrane potential ($\Delta\Psi$ m) and a proton motive force (Δ pH) and that is used by complex V to transport protons back to the matrix and thus generate ATP from ADP (adenosine diphosphate) and inorganic phosphate (Pi).

Mitochondrial oxidative phosphorylation is a major cellular source of ROS, as approximately 1-2% of O₂ consumed during physiological respiration is converted into superoxide anion (O₂^{•-}), by leakage of electrons from electron transport chain (complexes I and III are the major sites) to O₂ (Stowe & Camara, 2009).

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ROS produced within mitochondria can be converted into hydrogen peroxide (H_2O_2) in the matrix by superoxide dismutase 2 (SOD2) or in the intermembrane space by SOD1. In the mitochondrial matrix or cytosol, H_2O_2 can be detoxified into H_2O by glutathione peroxidase (GPx); catalase, which is mainly located in peroxisomes, can also detoxify H_2O_2 into H_2O and O_2 as shown in **figure 4**.



Figure 4: MITOCHONDRIAL OXIDATIVE PHOSPHYLATION AND ROS GENERATION.

The movement of electrons across complex I, complex III and complex IV is also coupled to H⁺ pumping across the inner mitochondrial membrane from the matrix to the intermembrane space. This generates an electrochemical gradient that is used by complex V (also known as ATP synthase) to generate ATP from ADP and inorganic phosphate (Pi). Complexes I and III are the major sites of superoxide anion generation. Superoxide anion can be converted in H_2O_2 in the matrix by superoxide dismutase 2 (SOD2) or in the intermembrane space by SOD1. In the matrix, H_2O_2 is converted in H_2O by glutathione peroxidase (GPx) and in the cytoplasm by the peroxisomal enzyme catalase (West, Shadel, & Ghosh, 2011 authorized by Nature Reviews).
Strong evidences suggest that CAG expansion triggers defects on mitochondrial Ca²⁺ uptake capacity, mitochondrial dynamics, altered mitochondrial biogenesis, impairment of mitochondrial transmembrane potential, decreased ATP generation and increased ROS production, as summarized in **figure 5**, and described in more detail in the next sections.



FIGURE 5: IMPAIRMENT OF MITOCHONDRIAL FUNCTIONS IN HD.

Mutant huntingtin (mHTT) inhibits PGC1- α , increases the open probability of the mitochondrial permeability transition pore (PTP), causes impairment of mitochondrial transport, respiratory chain defects, and acts at the level of mitochondrial dynamics, causing fragmentation. Increase of oxidative stress, decrease of mitochondrial membrane potential and ATP production are mitochondrial alterations shown in HD (V. Costa & Scorrano, 2012 authorized by The EMBO Journal).

1.2.2 Mitochondrial respiration impairment in HD

Mitochondrial-related bioenergetic defects are one of the main contributing factors to neuronal dysfunction in HD; accordingly the MRC activity is responsible for neuronal homeostasis and its impairment could potentiate neurodegeneration. Functional respiratory complexes are required to generate (Δ pH) and $\Delta\Psi_m$, which fuels ATP production, Ca²⁺ handling and ROS generation/detoxification (e.g. Nicholls & Budd, 2000). Regarding this, it is still unclear whether mHTT directly affects oxidative metabolism and the potential mechanisms involved.

Previous results demonstrated a significant decrease in HD caudate mitochondria respiration, analyzed through Clark oxygen electrode (Brennan et al., 1985). Increased mitochondrial complex II respiration was observed in striatum of symptomatic transgenic mice expressing the first 171 amino acids of htt with 98 CAG repeats (D9-N171-98Q) along with a reduction in pre-symptomatic mice, compared to their age-matched controls (Kim et al., 2011).

In accordance, other authors found a reduction in complex II activity and expression of subunits constituents in R6/1 and N171-82Q transgenic mice, and in a rat model using lentiviral vectors coding the N-terminal 171 amino acids of mHtt with 82 glutamines (lenti-171-82Q) (Damiano et al., 2013). The expression of two important constituents of mitochondrial complex II, the 30 kDa iron-sulfur (Ip) subunit and the 70 kDa FAD (Fp) subunit (Benchoua et al., 2006), and complex II activity were shown to be impaired in postmortem brain extracts of HD patients (Gu et al., 1996). In addition, 3-NP and malonate, respectively, irreversible and reversible inhibitors of complex II commonly used as chemical models of HD, were shown to increase striatal lactate levels and neurodegeneration (Ramaswamy et al., 2007). Other studies revealed that not only complex II, but also complexes I, III, IV and V activities are significantly decreased in HD striatum and putamen (Benchoua et al., 2006; Brennan et al., 1985; Sawa et al., 1999; Tabrizi et al., 1999), and in mitochondrial platelets from both pre-symptomatic and symptomatic HD carriers (Silva et al., 2013). Other authors did not detect differences in mitochondrial complex activities in heterozygous transgenic mice expressing full-length mutant huntingtin (FLmHTT), when compared with WT mice, and in striatal cells established from HdhQ7 (wild-type) and HdhQ111 (mutant huntingtin knock-in) mouse embryos (Guidetti et al., 2001; Milakovic & Johnson, 2005). However, Milakovic and Johnson demonstrated that striatal HD cells exhibited a decrease in mitochondrial respiratory rates; surprisingly, these differences were not observed in isolated mitochondria from the same cells (Milakovic, Quintanilla, & Johnson, 2006). No impairment on mitochondrial respiration were observed in Hdh150 knock-in heterozygotes, compared with WT mice (Oliveira et al., 2007). In agreement, Gouarné and co-authors described no differences in respiration of striatal neurons fueled with glucose and pyruvate from BACHD rats (HD transgenic rat model expressing FL-mHTT and all regulatory elements integrated from a bacterial artificial chromosome), compared to WT littermates (Gouarné et al., 2013). Recently, Hamilton et al. (2015) also demonstrated that 2 and 10 mo YAC128 mice have no differences in respiration when compared with WT mice; in this work the authors did not find any differences in respiratory activity of total brain synaptic and nonsynaptic isolated mitochondria and cultured cortical and striatal neurons from YAC128 versus WT mice.

A decrease in $\Delta \Psi_m$ is widely described in different HD models. When subjected to apoptotic stress induced by cyanide (complex IV inhibitor), mitochondria from HD patients lymphocytes were shown to depolarize more when compared with lymphocytes from control subjects (Sawa et al., 1999). In

addition, mitochondria isolated from lymphoblasts of HD individuals presented a decrease in $\Delta \Psi_m$ compared with controls (Panov et al., 2002). IGF-1 ammeliorated O₂ consumption and $\Delta \Psi_m$ in HD lymphoblasts (Naia et al., 2014). HdhQ111 cells also showed a decrease in the Ca²⁺ threshold necessary to trigger opening of mPTP (Choo et al 2004). Using HD cybrids, an *ex vivo* HD model, we also described a significant decrease in $\Delta \Psi_m$ compared with CTR cybrids (Ferreira et al., 2010). Moreover, 3-NP treatment caused significant loss in $\Delta \Psi_m$ in HdhQ111 cells (Ruan et al., 2004) and in Wistar rats (Mehrotra et al., 2015). Cyclosporine A (CsA), an inhibitor of mPTP, and ruthenium red, an inhibitor of the mitochondrial calcium uniporter (MCU), rescued HdhQ111 cells from 3-NP-induced cell death; mitochondrial cofactors, namely <u>acetyl-L-carnitine</u> (ALCAR) and <u>alpha-lipoic acid</u> (ALA) was prevented the loss of $\Delta \Psi_m$, suggesting their therapeutic efficacy in the management of HD.

Summarizing, these data show mitochondrial dysfunction in different models of HD, which could be related to calcium deregulation, as discussed in the next section.

1.2.3 Calcium deregulation in HD

Excitotoxicity is one of the main neuropathological features of neurodegenerative disorders, including HD. In the 80's, Choi and his team had an important role in the research of "glutamate excitotoxicity". Accordingly with these authors, exposure of cortical neurons to glutamate caused excitotoxicity which was dependent on Ca²⁺ entry through NMDA receptors (NMDAR) (Choi, 1987). Quinolinic acid (QA), an agonist of NMDARs that mimics HD pathology, triggered the loss of striatal neurons in rodents; in addition, 3-NP caused striatal lesions that could be rescued by NMDAR antagonists, suggesting that overactivation of NMDAR signaling is one of the causes for neurodegeneration in HD (Sepers & Raymond, 2014). Indeed, glutamate is the main neurotransmitter in cortico-striatal projections activating NMDAR located in post-synaptic of striatal MSNs. Therefore, defects in Ca²⁺ signaling in neurons are another event involved in HD pathology (e.g. Sánchez et al., 2008). By using YAC128 mouse model, Zhang and co-authors showed that pulses of glutamate induced loss of $\Delta \Psi_m$ and abnormalities in Ca²⁺ signaling, which were prevented by antagonists of NMDARs, evidencing the relationship between excitotoxicity, Ca²⁺ defects and degeneration of MSNs (Zhang et al., 2008)

Mitochondrial Ca²⁺ accumulation plays an important role in buffering cytosolic Ca²⁺ levels that increase in response to neuronal activity. Ca²⁺ entry into the mitochondrial matrix occurs through the mitochondrial Ca²⁺ uniporter (MCU), a Ca²⁺ channel present at the MIM (Chalmers & Nicholls, 2003).

Although Ca²⁺ is regularly required for the activity of enzymes (e.g. dehydrogenases) present in the mitochondrial matrix, when mitochondrial Ca²⁺ is excessive, the opening of mPTP along with the release of cytochrome c can occur, leading to the activation of cell death pathways (e.g. Nicholls, 2009), thus mitochondrial Ca²⁺ uptake capacity is dependent of the sensitivity of mitochondria to the damaging effect of Ca²⁺. There is no consensus regarding the effect of mHTT on mPTP function.

While some authors hypothesized that mHTT induce facilitation of mPTP induction, leading to defects in mitochondrial Ca²⁺ uptake (Gellerich et al., 2008; Milakovic et al., 2006; Panov et al., 2002; Quintanilla., 2013; Seong et al., 2005), other authors did not find evidences for Ca²⁺ uptake impairment in HD (Brustovetsky et al., 2005; Oliveira et al., 2007; Pellman et al., 2015). Panov et al reported bioenergetic abnormalities and a decrease in Ca²⁺ accumulation by isolated mitochondria exposed to mHtt and a direct interaction of the mutant protein with mitochondria, highly suggesting that the impairment in mitochondrial function is a direct effect of mHTT (Panov et al., 2002). Mitochondria from HdhQ111 cells, treated with high Ca²⁺ concentrations also showed reduced mitochondrial Ca²⁺ uptake capacity, decreased respiration and $\Delta \Psi_m$ in comparison with mitochondria from wild type cells, being $\Delta \Psi_m$ ameliorated by mPTP inhibitors (Milakovic et al., 2006). Using brain mitochondria isolated from transgenic HD rats expressing mHTT with 51 glutamine repeats, Gellerich and co-authors reported a decreased Ca²⁺ uptake capacity and diminished Ca²⁺ threshold for mPTP induction (Gellerich et al., 2008). In support of these findings, Quintanilla et al observed that HdhQ111 cells treated with thapsigargin (a SERCA antagonist that raises cytosolic calcium levels) showed decreased mitochondrial Ca²⁺ uptake and also decreased calcein fluorescence intensity in mutant cells, indicating that Ca^{2+} overload opens the mPTP. Mitochondrial defects in both HD models were attenuated by CsA, suggesting an important role of mPTP in mitochondrial injury (Quintanilla., 2013). Conversely, Brustovetsky et al did not find an increased susceptibility to Ca²⁺ in mitochondria from HD mice (Q50, Q92, Q111, and R6/2 mice) compared with mitochondria from WT animals (Brustovetsky et al., 2005). Moreover, real-time measurements of Ca^{2+} in intact HdhQ7/Q111 cells and primary striatal neurons revealed deficits in mitochondrial- dependent Ca²⁺ handling in cells expressing FLmutant HTT (Oliveira et al., 2006). One year later, Oliveira et al described an increase in Ca^{2+} loading capacity in mouse forebrain mitochondria from R6/2 (12–13 weeks) and YAC128 mice (12 mo), when compared with respective WT animals (Oliveira et al., 2007).

In accordance with this, Pellman et al demonstrated that both synaptic and non-synaptic mitochondria isolated from YAC128 mice increased Ca²⁺ handling in comparison with mitochondria from wild-type littermates and that the increase observed in 12 month-old YAC128 mice was

correlated with an increase in the amount of mHTT associated with mitochondria (Pellman et al., 2015). These studies failed to find a correlation between mPTP induction and mitochondrial Ca²⁺ uptake capacity in HD pathogenesis. Taken together, there are discrepancies in the findings related to mHTT in inducing defects in mitochondrial Ca²⁺ uptake that in turn could be related to the use of different approaches and HD models.

1.2.4 Evidence of oxidative stress in HD

Free radicals are defined as chemical species that contain one unpaired electron and can be included in four classes together with oxidant species that are not free radicals: ROS, reactive nitrogen species (RNS), reactive sulfur species (RSS) and reactive chloride species (RCS). ROS are the main reactive compounds generated in living systems, being the unstable $O_2^{\bullet-}$, H_2O_2 , hydroxyl radical (HO[•]), singlet oxygen ($^{1}O_2$) and ozone (O_3) the most commons. In healthy cells, the production of ROS is controlled by antioxidants, both enzymatic (catalase, glutathione peroxidase and reductase, or superoxide dismutases) and non-enzymatic (ascorbic and lipoic acid, polyphenols and carotenoids, reduced glutathione) (Uttara et al., 2009). Thus, classically, oxidative stress is described as a process that results from an imbalance between production of ROS and elimination through antioxidants, leading to DNA mutations, changes or loss in protein function and/or lipid peroxidation (Kirkinezos & Moraes, 2001; Sosa et al., 2013).

Oxidative stress has been shown in HD. Decreased catalytic activity of the mitochondrial enzymes (mitochondrial creatine kinase, citrate synthase and F_1F_0 ATP synthase) due to their oxidation was observed in *post-mortem* samples of HD patients (Sorolla et al., 2010). In the R6/2 transgenic mice, a correlation between oxidative damage and HD severity was also shown (Perluigi et al., 2005). In our group, higher ROS levels were shown in HD cybrids upon exposure to 3-NP and staurosporine (STS), when compared to control cybrids (Ferreira et al., 2010). Another study showed that NADPH oxidase (NOX) activity, which produces ROS, was increased in *post-mortem* cortex and striatum of HD patients and pre-symptomatic individuals (Valencia et al., 2013). Synaptosomes from HD140Q/140Q mice at 3, 6 and 12 months of age were isolated from the same brain regions and results evidenced high NOX activity with increase in age (Valencia et al., 2013). To affirm that polyQ aggregation is directly involved in oxidative stress, Hang *et al* studied the relation between polyQ aggregates, ROS production and cell death; they found that polyQ oligomerization results in early generation of H₂O₂ and disruption of glutathione and the antioxidant defense system (Hands et al., 2011). As mentioned before in this

work, Ca^{2+} handling defect is one of the main features of mitochondrial dysfunction; thus Wang *et al* (2013) proposed a relation between disrupted Ca^{2+} signaling and oxidative stress. Their data demonstrated that YAC128 derived embryonic fibroblasts showed elevated O_2^{*-} generation, when compared to WT cells; similar results were observed for HD model mice and fibroblast cells from HD patients (Wang et al., 2013). Moreover, they found that mitochondrial Ca^{2+} caused mtDNA damage through excessive oxidative stress in HD cells (Wang et al., 2013). In 2014, Ribeiro *et al* analyzed the role of insulin and IGF-1 in HdhQ111 cells and showed a reduction in mitochondrial ROS production (mitochondrial O_2^{*-}) induced by mHtt, and restoration of mitochondrial SOD activity, meaning that insulin and IGF-1 treatment may ameliorate ROS production (Ribeiro et al., 2014). In the literature, several biomarkers of oxidative stress have been described in both HD human blood or brain and HD mouse models, namely: 1) oxidized DNA and mtDNA; 2) increase in lipid peroxidation markers; 3) increase in protein oxidation markers, such as carbonyl and sulfhydryl levels (for review Cunha-Oliveira et al., 2012).

In HD, antioxidant defenses are also affected. In the striatum and cortex of patients with HD an induction of antioxidant defense proteins were observed, such as peroxiredoxins -1, -2, and -6, peroxidases -1, -6 and an increase in mitochondrial SOD1/2 and catalase activity, when compared with controls (Sorolla et al., 2008). Moreover, in 19 week-old R6/1 mice an increase in total SOD and Cu/Zn-SOD activities was described, followed by a decrease in 35 week-old R6/1 mice (Santamaría et al., 2001). These results suggested that in the initial phases of the disease, antioxidants are increased possibly representing a compensatory mechanism to protect cells from oxidative damage; however, defense mechanisms become insufficient, later resulting in decreased enzymatic activities. A recent study showed that GPx activity ameliorates symptoms of HD in mammalian cell and fly models of the disease, although it did not affect autophagy (Mason et al., 2013). Importantly, expression of endogenous antioxidant proteins is regulated via transcription by the promoter antioxidant response element (ARE), which is activated through binding of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Zhang et al., 2013). Nrf2^{-/-} mice showed hypersensitivity to 3-NP, resulting in motor deficits and striatal lesions. Moreover, increased ARE-mediated gene expression in Nrf2expressing animals through tBHQ attenuated tissue damage and preserved motor function (Shih et al., 2005). These data revealed the importance of Nrf2 in mitochondrial dysfunction and oxidative stress. The transcriptional co-activator peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC-1alpha) is involved in many cell functions, namely in the regulation of genes related with ROS detoxifying systems, such as the expression of antioxidant enzymes (e.g. GPx, SOD2) (St-Pierre et al., 2006). Thus, PGC-1 α controls mitochondrial metabolism and minimize the impact of ROS on cell physiology. Studies performed in HD patients and in HD models showed reduced expression of PGC-1 α target genes and TFAM (Chaturvedi et al., 2009; Cui et al., 2006; Weydt et al., 2006). Of relevance, abnormal PGC-1 α results in mitochondrial impairment (Kim et al., 2010); on the other hand, knockdown of mHTT resulted in PGC-1 α increase (St-Pierre et al., 2006). Therefore, PGC-1 α is an important regulator of mitochondrial biogenesis, function and oxidative damage that is inhibited by mHTT, leading to decreased ROS defenses and increased oxidative stress.

Taken together, these results evidence that oxidative stress is a HD feature and that overexpression of antioxidant proteins might be a future therapeutic strategy to fight against this disease.

1.3 HD MODELS

To study the neuropathological features of HD different models can be used such as human and mouse cell lines, primary neuronal cultures from selective brain areas, and human *post-mortem* brain samples as well as animal models.

1.3.1 Animals models

Animals models can be divided in two classes: non-genetic models and genetic models as reviewed by Ramaswamy et al. (2007). The non-genetic models are models in which the pathology is induced by toxins administration. The most used non-genetic models are rodents and primates submitted to intraperitoneal administration of excitotoxic agents such as QA, 3-NP and malonate. QA causes cell death by activating NMDAR, while 3-NP and MA cause cellular energy depletion (Ramaswamy et al., 2007). However, these models have a number of limitations, including the acute nature of the lesions.

In genetic models there is genetic incorporation of full-length or only a fragment of human mHTT into the genome (transgenic models) or the introduction of CAG repeats into the *Htt* gene locus of rodents (knock-in models) (**Table 2**). There are different types of genetic models, the mammalian models and the non-mammalian models, such as *Caenorhabditis elegans* and *Drosophila*

melanogaster that have mutations into the endogenous Htt locus or expression of truncated N-terminal fragments of mHTT.

One of the most common transgenic mouse models is the R6/1 and/or R6/2, firstly described by the group of Gillian Bates, in which the 5' end of the *HTT* gene (exon 1) carries a repeat expansion of 115 CAGs in exon 1 for R6/1 and 150 CAGs for R6/2 (Mangiarini et al., 1996). These mouse models present a reduction in brain weight and volume, neuronal atrophy, formation of huntingtin aggregates, decreased dopamine levels and other HD-like neuropathological features (Ferrante, 2009). These mice show motor impairment concomitantly with cognitive defects, as analyzed through animal behavioral tests (Ramaswamy et al., 2007).

Creation of knock-in mouse models involves replacing of a portion of the mouse *Htt* gene (which has 7 CAGs) by a mutant human copy having an expanded CAG region; in this case mice have only two copies of the *Htt* gene, a wild type allele and a mutant allele, controlled by mouse promoter. The ST*Hdh* cell line were the first cell model derived from knock-in mouse striatum expressing an *Htt* gene with 111 or 92 CAG repeats at exon 1 (HDhQ111 and HDhQ92) (Wheeler et al., 2000). Another knock-in mouse used in research is a chimeric mouse/human exon 1 containing 140 CAG repeats inserted in the murine *Htt* gene (CAG140). They present behavioral symptoms, neuropathological abnormalities, nuclear aggregates and inclusion bodies (Menalled et al., 2003).

TABLE 2: MOUSE MODELS OF HD

Rodent Model	CAG repeats	Construct	Neuropathology	Survival
CAG 140	140	Inserting CAG repeats into the mouse <i>Htt</i> gene	Nuclear and neuropil; aggregates with diffuse huntingtin activity; neuronal loss.	Normal life span
HdhQ111	111	Replacing exon 1 of the mouse huntingtin <i>Htt</i> gene with a mutant human exon 1	Diffuse huntingtin activity; nuclear inclusions; neuropil aggregates; astrogliosis.	Normal life span
HdhQ72	72	Replacing exon 1 of the mouse huntingtin <i>Htt</i> gene with mutant human exon 1	No neuronal loss or reactive astrogliosis; HTT aggregates by 28 weeks and forms nuclear aggregates by 96 weeks	Normal life span
N171-82Q mouse	82	First 171 a.a. YAC of human HTT randomly inserted into the mouse genome	Gross brain atrophy; hyperventricular enlargement; striatal neuron atrophy and loss; huntingtin aggregates.	19-26 weeks
R6/1 mouse	115	First 90 amino acids of human HTT randomly inserted into the mouse genome	Reduced brain volume; neuronal atrophy but not neuronal loss is present; huntingtin aggregates; reduced dopamine levels.	12+months
R6/2 mouse	144– 150	First 90 amino acids of human HTT randomly inserted into the mouse genome	Significant brain weight loss; gross brain atrophy; neuronal loss; progressive huntingtin aggregate formation; astrogliosis; reduced dopamine levels.	12-18 weeks
YAC128	128	Yeast artificial chromosome expressing the entire human HTT protein	Neuronal and volume loss; increased nuclear HTT staining; brain atrophy.	Normal life span

(Adapted from Pouladi et al., 2013 and Ramaswamy et al., 2007)

In 1999 the N171-82Q transgenic mouse model was generated; these mice express an Nterminal fragment of human HTT with 82, 44 and 18 CAG repeats, resulting in a later onset of symptoms. These mice present motor impairment, loss of coordination, tremors, hypokinesia and abnormal gait (Schilling et al., 1999). Similarly to the R6/1 and R6/2 mouse, the N171-82Q mouse model has two wild type huntingtin copies and one mutated copy of the truncated human *HTT* gene. The main neuropathological features of the mice are gross brain atrophy, loss of brain weight, hyperventricular enlargement, striatal neuron atrophy and loss, aggregates of HTT and cognitive impairment (Ferrante, 2009). In the next subsection we will focus on the transgenic models expressing FL-mHTT, namely the YAC 128 mouse model that was used in the present study.

1.3.1.1 Full-length human HD gene transgenic mouse models

In 1999 Hayden and colleagues developed the yeast artificial chromosome (YAC) transgenic mouse model containing human genomic DNA spanning the full-length HTT gene, including all regulatory elements. The HD clones were created to contain CAG sizes similar to those seen in adult onset (46 CAG repeats, YAC46) and juvenile onset (72 CAG repeats, YAC72), moreover a wild type YAC model was generated retaining 18 CAG repeats (Hodgson et al., 1999). YAC46 and YAC72 develop electrophysiological abnormalities, nuclear translocation and aggregation of HTT. Slow et al established a new YAC containing 128 CAG repeats. These mice mimics the changes observed in the disorder, showing brain atrophy, volumetric loss in the striatum followed by striatal neuronal loss, motor deficits, cognitive impairment, HTT aggregation and a biphasic profile combining hyperactivity and hypokinesis. Compared with the other mouse models, the YAC128 is considered to resemble HD disease in humans since a significant reduction in striatal neurons was observed at older mice and mice show a progressive development of the disease (Slow et al., 2003). Van Raamsdonk et al showed that brain weight was decreased and lesser volume of the striatum in YAC128 mice, when compared with wild-type mice, suggesting that the small global changes in brain weight might be explained by larger changes in select regions of the brain. YAC128 show impaired motor learning on the rotarod, impaired memory in the open-field habituation test, cognitive deficits in swimming T-maze test, difficulties in shifting strategy and decreased sensorimotor gating in prepulse inhibition (PPI) test. In summary, they reported mild cognitive deficits in YAC128 mice that precede motor onset and progressively worsen with age, as shown in Figure 6. This HD transgenic mice display enhanced sensitivity to different excitotoxins (Graham et al., 2009). YAC128 is generated on FVB/N background strain based on its advantages to use for research with transgenic mice (Taketo et al., 1991).

In conclusion, YAC128 mice model is considered a good model to assess the efficacy of potential treatments for HD in preserving and or restoring cognitive function.



FIGURE 6: ABNORMALITIES IN YAC128 MICE FROM 2 TO 18 MONTHS OF AGE.

At 2 months of age, YAC128 mice show motor learning deficits on the rotarod and strategy shifting deficits in the reversal phase of the swimming T-maze test. Hyperkinesia appears in 3 month-old YAC128 mice. At 4 months, YAC128 mice begin to show motor deficits on the rotarod. Between 8 and 9 months, YAC128 mice show a decrease in activity, habituation and cognition in swimming tests. At 9 month-old, neuropathology becomes apparent with a decrease in striatal volume and brain weight in the YAC128 mice, relatively to WT mice. At 12 months, YAC128 mice show cortical atrophy, striatal neuronal loss, decreased in PPI and habituation to acoustic startle. Finally, at 18 months, macroaggregates become apparent in the animal (Van Raamsdonk et al 2005 authorized by Human Molecular Genetics).

1.4 MAIN GOALS

An association between expression of mHTT and several pathological mechanisms, such as excitotoxicity, transcription deregulation, mitochondrial dysfunction (*e.g.* mitochondrial calcium-handling) and oxidative stress, the later associated to unbalanced antioxidant activity and free radical generation, has been widely described. There are several evidences that oxidative stress and mitochondrial dysfunction have a major role in HD-associated neurodegeneration, considerably affecting the striatum and the cortex, at a stage HD symptoms are already established. However, the effect of mHTT on mitochondrial function and their contribution for early HD pathogenesis and disease progression, before gross neurodegeneration, is still not entirely clear. Therefore, analysis of pre-symptomatic alterations reveal to be extremely relevant, since neuronal dysfunction but not death are expected at this stage.

Thus, in this novel study we aimed to evaluate changes in mitochondrial function and evidences of oxidative stress in early stages of HD pathogenesis. For this purpose, and to better understand the progression of the disease, HD transgenic mouse model, the YAC128 mice, were evaluated in both pre-symptomatic, at 3 months of age, and symptomatic stages, at 9 and 12 months of age. To achieve this goal, we thoroughly analyzed mitochondrial bioenergetics, including mitochondrial coupling and electron flow, mitochondrial calcium handling, and evaluated H₂O₂ production and the antioxidant status of mitochondria, namely the glutathione redox system and antioxidant proteins profile in cortical and striatal mitochondria isolated from YAC128 *versus* age-matched WT mice.

CHAPTER II – MATERIALS AND METHODS

2.1 Materials

Amplex[®] Red and Calcium Green-5N were obtained from Molecular Probes, Life Technologies (Eugene, OR, USA). PVDF membrane and BioRad Protein Assay solution were purchased from BioRad Laboratories, Inc. (Munich, Germany). Bovine serum albumin (BSA) was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., TX, USA) and ECF substrate and Percoll solution were purchased from GE Healthcare (GE Healthcare Bio-Sciences, PA, USA). Adenosine 5' diphosphate (ADP) potassium salt, antimycin A, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), glutathione reductase (GRed), L-Glutathione reduced (GSH), L-Glutathione oxidized (GSSG), L-Glutamic acid, L-Malic acid, L-Ascorbic acid, 2,3-naphthalenedicarboxaldehyde (NADPH), N-ethylmaleimide (NEM), oligomycin, ortho-phthaldialdehyde (OPA), polyethyleneimine (PEI), phenylmethanesulfonyl fluoride (PMSF), protease inhibitors, rotenone, sodium pyruvate, succinic acid and tert-butyl hydroperoxide (t-BHP) were from Sigma Chemical Co. (St Louis, MO, USA). All other reagents were of analytical grade. PCR reagents, such as Taq polymerase, proteinase K, primers and DNA loading dye were acquired from Invitrogen[®] (Carlsbad, CA, USA). Primary and secondary antibodies used in this study are listed in **Table 3**.

TABLE 3 : PRIMARY AND SECONDARY ANTIBODIES

Primary Antibodies	Host	Dilution	Reference/supplier
Catalase	Rabbit	1:200	Abcam ab15834 (Abcam, Cambridge, UK)
CX II (70 kDa subunit)	Mouse	1:10000	Molecular Probes A11142 (Molecular Probes— Invitrogen (Eugene, OR, USA)
GAPDH	Mouse	1:2500	Chemicon MAB374
Glutathione peroxidase	Rabbit	1:1000	Abcam ab 108427 (Abcam, Cambridge, UK)
Glutathione reductase	Rabbit	1:2000	Abcam ab16801 (Abcam, Cambridge, UK)
HSP60	Mouse	1:1000	Millipore MAB3514 (Merck Millipore, Darmstadt, Germany)
mHTT	Mouse	1:200	Millipore MAB2166 (Merck Millipore, Darmstadt, Germany)
Polyglutamine	Mouse	1:5000	Milipore MAB 1574 - 1C2 (Merck Millipore, Darmstadt, Germany)
Superoxide dismutase 2 (SOD2)	Rabbit	1:5000	Abcam ab13533 (Abcam, Cambridge, UK)
SOD2 (acetyl K68)	Rabbit	1:500	Abcam ab 137037 (Abcam, Cambridge, UK)
TATA binding protein (TBP)	Mouse	1:1000	Abcam ab51841 (Abcam, Cambridge, UK)
Tubulin	Mouse	1:1000	Sigma T-6199 (Sigma, St. Louis, MO, USA)
Secondary Antibodies	Host	Dilution	Reference/supplier
Anti-Rabbit (H+L), Alkaline Phosphatase Conjugated	Goat	1:10000 (WB)	Thermo Scientific Pierce #31340 (Thermo Fisher Scientific, Rockford, IL, USA)
Anti-Mouse (H+L), Alkaline Phosphatase Conjugated	Goat	1:10000 (WB)	Thermo Scientific Pierce #31320 (Thermo Fisher Scientific Rockford, IL, USA)

Table represents the primary antibodies used, the species where they were produced, the dilution used and the supplier

2.2 Methods

2.2.1 Animals

In this study both male and female HD transgenic hemizygous YAC128 (line 53), expressing full-length human mHTT with ~128 CAG repeats, and non-transgenic wild-type (WT) littermate mice with 3, 9 and 12 months of age were used. All animals were generated from our local colony, with breeding couples gently provided by Dr. Michael Hayden (University of British Columbia, Vancouver, Canada). YAC128 mice were maintained on FVB/N background and compared with WT littermate mice (FVB/N strain). Mice were housed in groups with *ad libitum* access to chow food and water under standard conditions (12 h light/dark cycle, 22°C). All studies were performed according to the Helsinki Declaration and EU guidelines (86/609/EEC). Mice were weighted and then sacrificed by cervical dislocation and decapitation. Brains were removed from the skull and washed once in phosphate saline buffer (PBS) containing (in mM): 137 NaCl, 2.7 KCl, 1.8 KH₂PO₄, 10 Na₂HPO₄·2H₂O, pH 7.4; followed by striatum and cortex dissection. For some experiments tissues were immediately subjected to mitochondria isolation by Percoll gradient in order to obtain functional mitochondria; alternatively, samples were frozen in liquid nitrogen until use.

2.2.2 Genotyping

All animals were genotyped using an ear-tip DNA polymerase chain reaction (PCR) standard procedure by using LyA1 (CCT GCT CGC TTC GCT ACT TGG AGC), LyA2 (GTC TTG CGC CTT AAA CCA ACT TGG), RyA1 (CTT GAG ATC GGG CGT TCG ACT CGC), RyA2 (CCG CAC CTG TGG CGC CGG TGA TGC), actin foward (GGA GAC GGG GTC ACC CAC AC), and actin reverse (AGC CTC AGG GCA TCG GAA CC) primers. PCR protocol consisted in 35 cycles, with the following temperatures and times for each step: denaturation—94 °C for 30 s; annealing—63 °C for 30 s; and elongation—72 °C for 30 s. PCR products were analyzed on 1.7 % agarose gels followed by staining with GreenSafe (NZYtech, Lisboa, Portugal) at 100V for 40minutes with Tris-acetate-EDTA running buffer and visualized under a UV trans-illuminator, Gel Doc XR system (BioRad[®], Hercules, USA).

2.2.3 Mitochondrial isolation by percoll gradient

Cortical or striatal mitochondria derived from neurons and glial cells were isolated using discontinuous percoll density gradient centrifugation, according to Wang et al., 2011 with some minor modifications. Fresh cortex and striatal tissues were immediately collected in ice-cold isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.2/KOH, plus 1 mg/mL fatty acid free bovine serum albumin (BSA) and homogenized with 10 - 12 up and down strokes in Dounce All-Glass Tissue Grinder (Kontes Glass Co., Vineland, NJ, USA) using pestle A (0.07-0.12 mm- clearance) followed 10 - 12 up and down strokes with pestle B (0.02-0.056 mm-clearance).

After a brief centrifugation at 1100 xg for 2min at 4°C, the supernatant was mixed with freshly made 80% Percoll prepared in 1 M sucrose, 50 mM HEPES, 10 mM EGTA, pH 7.0, then carefully layered on the top of freshly made 10% Percoll (prepared from 80% Percoll) and further centrifuged at 18 500 xg for 10 min at 4°C. The cloudy myelin containing top fraction was removed leaving the mitochondria enriched pellet in the bottom of the tube. Supernatant was discarded and the pellet was ressuspended in 1mL of washing buffer (250 mM Sucrose, 5 mM HEPES-KOH, 0.1 mM EGTA, pH 7.2) and centrifuged again at 10,000 xg for 5 min at 4°C. Finally, the mitochondrial pellet was ressuspended in ice-cold washing buffer and the amount of protein quantified by the Bio-Rad Protein Assay method. Isolated mitochondria were kept on ice until used for further functional analysis. Alternatively samples were frozen at -80°C.

The purity grade of mitochondria isolated by percoll gradient, was evaluated by western blotting by using complex II (CXII) and HSP60 as mitochondrial markers, and tubulin and GAPDH as cytosolic markers. As shown in **Figure 7A** isolated mitochondrial samples are enriched in CXII and HSP60. Although present, tubulin and GAPDH appear in mitochondrial extracts. As previously described by Carré et al (2002) the presence of tubulin in isolated mitochondria are due to tubulin binding to voltage-dependent anion channel (VDAC) in the OMM.

2.2.3 Mitochondrial and cytosolic extracts

Cortex or striatum tissues (freshly isolated or freezed in liquid nitrogen) were homogenized (20 strokes up and down, 280 rpm) at 4°C in sucrose buffer containing (in mM) 250 sucrose, 20 HEPES, 100 KCl, 1.5 MgCl₂, 1 EGTA, 1 EDTA, pH 7.5/KOH, supplemented with 1 mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF), 100 nM okadaic acid, 5 mM sodium fluoride (NaF) and $1 \mu g/mL$ protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain). Lysates were then centrifuged at 560 xq for 12 min at 4°C. The resulting pellet (containing nuclei and debris) was discarded and the supernatant was centrifuged at 11,900 x g for 20 min at 4°C. The resulting supernatant (cytoplasmic fraction) was collected. The mitochondrial pellet was further ressuspended in supplemented TNC buffer containing: 10 mM Tris Acetate pH 8, 0.5% Nonidet P40, 5 mM CaCl₂ with 1 µg/mL protease inhibitor cocktail). Protein content in both cytosolic and mitochondrial fractions was determined by the Bio-Rad Protein Assay method, based on the method of Bradford and samples stored at -80°C until use. The purity grade of mitochondrial and cytosolic fractions, obtained by subcellular fractionation, was evaluated by western blotting by using anti-CX II and anti-HSP60 as mitochondrial markers and tubulin and GAPDH as cytosolic markers. As shown in Figure 7B an enrichment in CX II and HSP60 was present in mitochondrial extracts (ME) whereas an enrichment in both anti-tubulin and anti-GAPDH labeling was observed in cytosolic fractions (CE).



FIGURE 7: Purity grade of isolated mitochondria and subcellular fractions

Representative Western blots showing the abundance of protein markers in isolated mitochondria (IM) from 6mo WT mice cortex (A), cytoplasmic (CE) and mitochondrial (ME) extracts from cortex of WT mice (B) using antibodies against CX II and HSP60 (mitochondrial markers) and against tubulin and GAPDH (cytoplasmic markers). Western blot were performed as described in Materials and Methods section.

2.2.4 Sample preparation and Western blotting

Mitochondrial and cytosolic extracts were denatured with 6x concentrated loading buffer (containing 300 mM Tris-HCl pH 6.8, 12% SDS, 30% glycerol, 600 mM DTT, 0.06% bromophenol blue) at 95°C for 5 min. When isolated mitochondria from cortex and striatum were desired for western blotting (for Htt and mHtt labeling and for purity grade analysis), samples were boiled in 1% SDS for 10 min and then denatured with 6x loading buffer as described above. Equivalent amounts of protein (15 µg-50 µg) were separated in 6-15% SDS-PAGE gel electrophoresis (accordingly to the molecular weight of proteins of interest) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were further blocked with 5% skim milk or BSA in TBS-Tween: 25 mM Tris-HCl, 150 mM NaCl, pH 7.6,)/0.1% Tween-20 (v/v), during 1 h at room temperature and further incubated overnight at 4°C with gentle agitation with primary antibodies in 1% BSA in TBS-Tween. Membranes were incubated with anti-mouse or anti-rabbit IgG secondary antibodies prepared in 1% BSA in TBS-Tween for 1 h, at room temperature. In order to normalize the amount of protein per lane anti-CX II and anti- α tubulin were used as loading controls for mitochondrial and cytosolic extracts, respectively. Immunoreactive bands were visualized by alkaline phosphatase activity after incubation with ECF reagent on BioRad ChemiDoc Touch Imaging System (BioRad, Hercules, USA) and quantified using Image Lab analysis software (BioRad, Hercules, USA).

2.2.5 Oxygen consumption rate evaluation by SeaHorse analyzer

Oxygen consumption rate (OCR) was measured in fresh cortical and striatal mitochondria isolated from YAC128 versus WT mice (5 µg of protein per well), attached to 24-well XF culture plates pre-coated with polyethyleneimine (PEI, 1:15000 dilution from a 50% solution in mitochondrial assay solution (MAS) (70 mM sucrose, 220 mM mannitol,10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA and 0.2% (w/v) fatty acid-free BSA, pH 7.2) by using Seahorse XF24 flux analyzer (Rogers et al., 2011). For this purpose, the multiwall plate was centrifuged at 2,000 xg for 20 minutes at 4°C. In order to evaluate the mitochondria attachment efficiency, the plates were analyzed under light microscopy using 20X magnification to ensure consistent adherence to the wells (data not shown). After an incubation period of 8 minutes at 37°C, the multiwell plate was transferred to the Seahorse XF24 flux analyzer. Typical mix and measurement cycle times for the assays are illustrated in **supplementary table I** and are similar for all experiments unless otherwise noted.

Two different experiments were performed: 1) the levels of respiratory coupling ('coupling experiment') and 2) the sequential determination of CX I-IV-dependent respiration ('electron flow experiment') in YAC128 and WT mice striatal and cortical mitochondria. These experiments were performed by using different substrates, modulators and inhibitors, as described in figure legends and in the results section. For the coupling experiments, mitochondrial OCR were analyzed in MAS containing: 10 mM succinate plus 2 μ M rotenone as substrates and basal respiration followed before the addition of modulators and inhibitors; mitochondria were energized by the injection of ADP (4 mM), which increased OCR; respiration drived by ATP synthesis was then prevented by addition of oligomycin (2.5 μ g/mL, inhibitor of ATP synthase). The addition of the uncoupler FCCP (4 μ M) caused an increase in OCR reflecting the maximal respiratory chain activity as well as the maximal substrate oxidation rate; finally, antimycin A (4 μ M, CX III inhibitor) was add to fully block the respiratory chain and the residual OCR.

In order to follow and interrogate each complex of the electron transport chain, another set of experimentswas performed. For this purpose, isolated mitochondria were analyzed in uncoupled state by using MAS containing 10 mM pyruvate, 2 mM malate plus 4 μ M FCCP. Next, addition of rotenone (2 μ M; inhibitor CX I) led to respiration inhibition; further, succinate injection (10 mM; CX II substrate) allowed mitochondria to respire by increasing OCR. Electron flow was then evaluated by adding antimycin A (4 mM; CX III inhibitor) causing OCR inhibition as expected. Finally, injection of ascorbate and TMPD (1 mM and 100 μ M, respectively), which act as electron donors to cytochrome *C*/CX IV, increased OCR values.

2.2.5.1 SeaHorse data analysis

For the respiratory coupling experiments, basal and maximal OCR, oxygen consumed for ATP generation through the CX V, respiratory control ratio (State3/State 4) and Proton (H⁺) leak which indicates passive H⁺ leakage across the MIM, were calculated as shown in **table 4**. For the electron flow experiments, CX I – IV activities were sequentially determined **(table 5)**. The results were normalized per μ g protein.

Respiratory Coupling Experiment				
Parameter	Parameter Rate Measurement Equation			
Basal Respiration	Last rate measurement before first injection			
Maximal Respiration	Last rate measurement after FCCP injection			
ATP Production	Last rate measurement before oligomycin injection minus minimum rate measurement			
AIFFIOUUCUON	after oligomycin injection			
H+ Look	Minimum rate measurement after oligomycin injection minus measurements after			
H LEUK	antimycin A			
Respiratory control ratio	Measurement before ADP injection divided for measurement before oligomycin			
(State3/State 4)				

TABLE 4: ANALYSIS OF RESPIRATORY COUPLING EXPERIMENT PARAMETERS

TABLE 5: ANALYSIS OF ELECTRON FLOW EXPERIMENT PARAMETERS

Electron Flow Experiment				
Parameter	Rate Measurement Equation			
CX I activity	Last rate measurement of basal minus minimum rate measurement after rotenone injection			
CX II activity	Last rate measurement of rotenone minus maximal rate measurement after succinate injection			
CX III activity	Last rate measurement of succinate minus minimum rate measurement after antimycin A injection			
CX IV activity	last rate measurement of antimycin A minus maximal rate measurement after asc/TMPD injection			

2.2.6 Measurement of glutathione levels

Levels of reduced and oxidized glutathione (GSH and GSSG, respectively) were determined in YAC128 and WT striatal and cortical mitochondria by using a fluorimetric assay, according to a previously described method (Hissin and Hilf, 1976). GSH levels were measured in samples (20 µg) after the addition of *ortho*-phthalaldehyde (OPA) (1 mg/mL methanol) and phosphate buffer (100 mM NaH₂PO₄, 5 mM EDTA, pH 8.0). After 15 min of incubation at room temperature in the dark, the end point fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. For GSSG determinations, 25 µg of samples were mixed during 5 min with *N*ethylmaleimide (NEM) (5 mg/mL methanol) which form adducts with GSH. Then, the mixture was incubated for 15 min in 100 mM NaOH plus OPA (1 mg/mL in methanol), and the fluorescence measured with excitation at 350 nm and emission at 420 nm. Both GSH and GSSG levels were quantified in a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). The results were calculated and plotted as RFU per mg protein.

2.2.7 Measurement of glutathione peroxidase (GPx) and glutathione reductase (GRed) activities

Glutathione Peroxidase (GPx) and Glutathione Reductase (GRed) activities were measured in YAC128 and WT striatal and cortical isolated mitochondria, spectrophotometrically, at 340 nm, through the kinetic analysis of NADPH oxidation, as described previously (Paglia and Valentine, 1967) with some modifications. Briefly, the activity of GPx in samples (15 µg) was measured upon a 5 min incubation with phosphate buffer containing 0.25 M KH₂PO₄, 0.25 M K₂HPO₄ and 0.5 mM EDTA, pH 7.0, 10 mM GSH and GRed (1 unit) in the dark at 30°C. After the addition of 2.5 mM NADPH and 12 mM *tert*-Butyl hydroperoxide the absorbance were registered for 5 minutes. For the activity of GRed, samples (20-25 µg) were ressuspended in phosphate buffer containing 0.2 M K₂HPO₄ and 2 mM EDTA, pH 7.0, plus 2.5 mM NADPH and activity recorded after the addition of 20 mM GSSG. GRed and GPx activities were determined using a Microplate Spectrophotometer SpectraMax Plus384 (Molecular Devices, USA). Results were presented per mg protein.

2.2.8 Mitochondrial Ca²⁺ handling capacity

Mitochondrial Ca^{2+} uptake was measured fluorimetrically in the presence of the Ca^{2+} -sensitive fluorescent dye Calcium Green 5N (150 nM), using excitation and emission wavelengths of 506 nm and 532 nm, respectively, accordingly to Pellman et al (2015) with some minor modifications. Calcium Green is a cell-impermeant visible light-excitable Ca^{2+} indicator that exhibits an increase in fluorescence emission intensity upon binding Ca^{2+} ; thus a decrease in the Calcium Green fluorescence is function of external Ca^{2+} concentration which indicates the capacity of mitochondria to handle Ca^{2+} .

Briefly, 5µg of YAC128 and WT striatal or cortical isolated mitochondria was added to the standard incubation medium contained 125 mM KCl, 0.5 mM MgCl₂, 3 mM KH₂PO₄, 10 mM Hepes, pH 7.4, 10 µM EGTA, supplemented either with 3 mM pyruvate plus 1 mM malate or 3 mM succinate plus 3 mM glutamate, 0.1 mM ADP and 1 µM oligomycin. After a basal fluorescence record a first 10 µM CaCl₂ pulse was added and fluorescence measured during 10 min followed by a second addition of a second 10 µM CaCl₂ pulse, for additional 5 min. Data are expressed as calcium handling capacity, which reflects the rate of decrease of Calcium Green-5N fluorescence (per min).

2.2.9 Mitochondrial H₂O₂ production

 H_2O_2 production by YAC128 and WT striatal and cortical mitochondria was measured by using the Amplex[®] Red (10-acetyl-3,7-dihydroxyphenoxazin)-horseradish peroxidase method according to Bhattacharya et al., (2009) and O'Malley et al., (2006) with some alterations. In the presence of peroxidase, the Amplex[®] Red reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Briefly, 5 µg of mitochondria was ressuspended in reaction buffer (100 mM sucrose, 100 mM KCl, 2mM KH₂PO₄, 5mM HEPES, 10 µM EGTA) containing Amplex[®] Red reagent (10 µM), horseradish peroxidase (0.5 units per mL), supplemented with 3mM succinate and fluorescence recorded for 15 min. When desired, the effect of antimycin A (2 µM) was tested. Fluorescence was followed at an excitation wavelength of 550 nm and an emission wavelength of 580 nm, using a microplate reader Spectrofluorometer Gemini EM (Molecular Devices, USA). Experiments were performed at 30°C during a 10 min experiment.

2.2.10 Statistical analysis

Data were analyzed by using Excel and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software and results expressed as the mean ± SEM of the number of experiments performed in replicates of the number of animals indicated in the figure legends. Comparisons among multiple groups were performed by two-way ANOVA followed by the Bonferroni's post-hoc test. Student's *t*-test was also performed for comparison between two Gaussian populations, as described in figure legends. Significance was defined as p<0.05.

CHAPTER III - RESULTS

3.1 Characterization of the experimental model and isolated mitochondria

In this work, both pre-symptomatic (3-month old, mo) YAC128 mice and symptomatic YAC128 mice, with 6, 9 and 12 months of age, and age-matched WT littermates were used to prepare isolated cortical and striatal functional mitochondria by subcellular fractionation of these two brain areas. Animals of both genders used in our experiments were subjected to genotyping analysis (Fig. 8A). In addition YAC128 and WT mice were weighted before sacrifice. Results depicted in Figure 8B show that YAC128 mice exhibited a significant gain of body weight with the course of disease, which was significantly increased in 6, 9 and 12 mo mice, but not in pre-symptomatic 3 mo YAC128 mice, when compared to the respective age-matched WT mice (Fig. 8B). Moreover, at 9 and 12 mo YAC128 mice showed an increase in brain weight when compared with 3 and 6 mo YAC128 mice. In order to evaluate if mHTT was retained in mitochondria isolated by percoll gradient, western blotting was performed. Unfortunately, we did not test striatal mitochondria due to the low yield of this brain area. Our results show that both wild-type HTT and mHTT were recognized by monoclonal anti-HTT antibody 2166 (mAb 2166, Millipore), and by monoclonal anti-polyQ antibody 1C2 (mAb 1574, Millipore). By using the polyQ-specific mAb 1C2, a ~350 kDa band (representing mHTT), was detected in 3, 6, 9, 12-month old YAC128 mice, but not in WT mice, indicating that mHTT is associated with isolated mitochondria (Fig. 8C). After probing with anti-HTT mAb 2166, we detected a ~350 kDa band, corresponding to wild-type HTT, in both cortical WT and YAC128 mitochondria (3, 6, 9 and 12mo), and also detected a band with a slightly higher molecular weight that represents mHTT in YAC128 mouse mitochondria (Fig. 8D). Of note, using mAb 2166, only wild-type HTT was easily detected in isolated mitochondria from both WT and YAC128 mice, whereas the labeling of mHTT was much lower in YAC128 mouse mitochondria, in accordance with Hamilton et al (2015). These data are consistent with the previous findings obtained in an early study, demonstrating mHTT associated with mitochondria (Panov et al., 2002).



FIGURE 8: CHARACTERIZATION OF EXPERIMENTAL ANIMALS AND ISOLATED MITOCHONDRIA OBTAINED BY PERCOLL GRADIENT

A) Representative genotyping data of ear tissue from WT and YAC128 mice. **B**) Body weight of WT and YAC128 mice at 3, 6, 9, 12 months of age. **C)** mHtt was detected with anti-polyQ 1C2 antibody. **D)** Detection of wild-type mouse huntingtin (Htt) and mutant huntingtin (mHtt). mHtt were detected by western blotting with 2166 antibody, which recognizes both Htt and mHtt. Western blotting was performed using 50 µg cortical mitochondria and resolved by SDS-PAGE in 6% acrylamide gel as described in Material and Methods. In **C** and **D**, CX II (70 kDa) was used as a mitochondrial marker. Data are presented the mean +/- SEM of 10 to 30 mice from each genotype. Statistical analysis: * p<0.05; **** p<0.0001 by two-way ANOVA followed by Bonferroni post-hoc test when comparing between genotypes; ####p<0.0001 by two-way ANOVA followed by Bonferroni post-hoc test when comparing to 3mo YAC128 mice; ^{&&&}p<0.001; ^{&&&&&}p<0.0001 by two-way ANOVA followed by Bonferroni post-hoc test when comparing to 6mo YAC128 mice.

3.2 Pre-symptomatic YAC128 mitochondria exhibit decreased oxygen consumption rate (OCR) in cortex, but increased OCR in striatum

To examine the effect of mHTT on oxidative metabolism of early pre-symptomatic YAC128 (3 mo) versus WT mice, the respiratory activity of cortical and striatal mitochondria were evaluated through the analysis of mitochondrial oxygen consumption rate (OCR; pmol/min/ μ g protein) using the SeaHorse XF24 flux analyzer. Coupling experiments (Fig. 9) performed under conditions of CX II feeding and CX I inhibition with succinate and rotenone, respectively, showed decreased basal respiration in cortical mitochondria of YAC128 mice (Fig. 9Ai). Moreover, a decrease in maximal respiration (Fig. 9Aii), achieved after FCCP stimulus, which completely depolarize the organelle, along with decreased H^+ leak, which represents a passive permeability to protons not coupled to ATP synthesis, were observed in YAC128 cortical mitochondria. Interestingly, an increase in State3/State4 (a respiratory control index) (Fig. 9Av) that indicates the tightness of the coupling between respiration and phosphorylation, accomplished the increase in ATP production in YAC128 mice compared to WT mice (Fig. 9Aiii). In the electron flow experiment cortical mitochondria were evaluated in conditions of CX I inhibition in an uncoupled state (Fig. 9B and D). In these conditions, cortical mitochondria from pre-symptomatic YAC128 mice showed a significant decrease in CX II (Fig. 9B ii), CX III (Fig. 9B iii) and CX IV (Fig. 9B iv) activities compared with cortical mitochondria derived from age-matched WT mice, being CX IV the most affected. We did not observe any difference in CX I activity (Fig. 9B i) in YAC128 compared with WT mitochondria.

Interestingly, we observed almost opposite effects in striatal mitochondria from YAC128 and WT mice, namely an increase in basal respiration (Fig. 9C *i*), maximal respiration (Fig. 9C *ii*), ATP production (Fig. 9B *iii*) and H⁺ leak (Fig. 9B *iv*) were evidenced. Concerning state3/state4 respiration levels, no differences between genotypes were verified (Fig. 9B *v*). Moreover, the increase in mitochondrial respiration was in accordance with the increase in CX I – IV activities, as shown in Figure 9D *i-iv*.

Data demonstrate increased susceptibility of cortical mitochondria in initial/pre-symptomatic stages of YAC128 mice (3 mo), when compared to HD striatal mitochondria.



FIGURE 9: OCR IN CORTICAL AND STRIATAL MITOCHONDRIA ISOLATED FROM 3 MO YAC128 VERSUS WT MICE

A, **C**) Levels of respiratory coupling in cortical (**A**) and striatal (**C**) mitochondria in YAC128 *versus* WT mice. Experiments were performed by using MAS containing 10 mM succinate plus 2 mM rotenone; mitochondrial inhibitors and substrates were sequentially injected into different ports: 4 mM ADP; 2.5 µg/mL oligomycin, 4 µM FCCP, and 4 µM antimycin A, **A and Ci**) Basal OCR, **A and C.ii**) Maximal OCR, **A and C.iii**) ATP production, **A and C.iv**) H⁺ leak, **A and C.v**) Respiratory control ratio (State3/State 4). **B**, **D**) CX I -IV-dependent respiration in cortical (**B**) and striatal (**D**) mitochondria derived from YAC128 *versus* WT mice. Experiments were performed in MAS containing 10 mM pyruvate, 2 mM malate and 4 mM FCCP. The following compounds were then added sequentially: 2 mM rotenone, 10 mM succinate, 4 mM antimycin A and 1mM ascorbate/ 100mM TMPD. **B and D.i**) CX I activity. **B and D.ii**) CX II activity. Data are expressed in pmol/min/ug protein and presented the mean +/- SEM of 5 mice from each genotype performed in duplicates or triplicates. Statistical analysis: tp<0.05; ttp<0.01; tttp<0.001; ttttp<0.001; ttttp<0.

3.3 Symptomatic cortical and striatal YAC128 mitochondria exhibit no differences in OCR

In order to understand if mitochondria bioenergetics were also compromised in symptomatic mice, we analyzed mitochondrial respiratory activity in symptomatic cortical and striatal mitochondria from YAC128 (9 and 12mo) *versus* age-matched WT mice using the Seahorse XF24 flux analyzer. In 9mo, no differences in respiratory coupling in cortical YAC128 *versus* WT mitochondria (Fig. 10A) were observed; moreover, unchanged CX I - III activities (Fig.10B), along with an increase in YAC128 CX IV activity (Fig.10B *iv*) were evidenced.

An increase in basal respiration (Fig. 10C *i*), maximal respiration (Fig. 10C *ii*) and state3/state4 (Fig. 10C *v*) levels were still detected in YAC128 mouse striatal mitochondria at 9 month of age, although no differences were observed in CX I-IV activities at this age (fig.10D). When the same parameters were analyzed in 12 mo YAC128 and WT mice, no differences in coupling experiments (Fig.11A and C) or electron flow experiments (Fig.11B and D) between genotypes or brain areas (cortex and striatum) were observed.



FIGURE 10: OCR IN CORTICAL AND STRIATAL MITOCHONDRIA ISOLATED FROM 9 MO YAC128 VERSUS WT MICE

A, **C**) Levels of respiratory coupling in cortical (**A**) and striatal (**C**) mitochondria in YAC128 *versus* WT mice. Experiments were performed by using MAS containing 10 mM succinate plus 2 mM rotenone; mitochondrial inhibitors and substrates were sequentially injected into different ports: 4 mM ADP; 2.5 μg/mL oligomycin, 4 μM FCCP, and 4 μM antimycin A, **A and Ci**) Basal OCR, **A and C.ii**) Maximal OCR, **A and C.iii**) ATP production, **A and C.iv**) H⁺ leak, **A and C.v**) Respiratory control ratio (State3/State 4). **B**, **D**) CX I -IV-dependent respiration in cortical (**B**) and striatal (**D**) mitochondria derived from YAC128 *versus* WT mice. Experiments were performed in MAS containing 10 mM pyruvate, 2 mM malate and 4 mM FCCP. The following compounds were then added sequentially: 2 mM rotenone, 10 mM succinate, 4 mM antimycin A and 1mM ascorbate/ 100mM TMPD. **B and D.i**) CX I activity. **B and D.ii**) CX II activity **B and D.iii**) CX III activity. **B and D.iii**) CX II activity. Data were obtained using SeaHorse XF24 Analyzer and show the mean +/- SEM of 2-4 mice from each genotype performed in duplicates or triplicates. Statistical analysis: [†]p<0.05 ; [#]p<0.01 by Student's *t*-test.


FIGURE 11: OCR IN CORTICAL AND STRIATAL MITOCHONDRIA ISOLATED FROM 12 MO YAC128 VERSUS WT MICE

A, **C**) Levels of respiratory coupling in cortical **(A)** and striatal **(C)** mitochondria in YAC128 *versus* WT mice. Experiments were performed by using MAS containing 10 mM succinate plus 2 mM rotenone; mitochondrial inhibitors and substrates were sequentially injected into different ports: 4 mM ADP; 2.5 µg/mL oligomycin, 4 µM FCCP, and 4 µM antimycin A, **A and Ci**) Basal OCR, **A and C.ii**) Maximal OCR, **A and C.iii**) ATP production, **A and C.iv**) H⁺ leak, **A and C.v**) Respiratory control ratio (State3/State 4). **B**, **D**) CX I -IV-dependent respiration in cortical (**B**) and striatal (**D**) mitochondria derived from YAC128 *versus* WT mice. Experiments were performed in MAS containing 10 mM pyruvate, 2 mM malate and 4 mM FCCP. The following compounds were then added sequentially: 2 mM rotenone, 10 mM succinate, 4 mM antimycin A and 1mM ascorbate/ 100mM TMPD. **B and D.i**) CX I activity. **B and D.ii**) CX II activity **B and D.iii**) CX III activity **B and D.iii**) CX III activity and show mean +/- SEM of 3 mice from each genotype performed in duplicates or triplicates.

3.4 Influence of age in bioenergetic parameters

In order to evaluate the effect of age in some parameters analyzed in section 3.3 in both cortex and striatum-derived mitochondria from YAC128 and WT mice, results obtained in **Figures 9**, **10** and **11** were replotted as a function of age (**Figs. 12** and **13**). Our data demonstrate that both YAC128 and WT cortical mitochondria exhibit a significant decrease in mitochondrial respiration until 9 mo; however, a tendency for a recovery in OCR parameters, namely basal (**Fig. 12A** *i*), maximal respiration (**Fig. 12B** *i*), and H⁺ leak (**Fig. 12C** *i*) between 9 at 12 mo was observed. Conversely, in striatum, a tendency for a constant decrease in all parameters with the course of age (3 to 9 mo) was observed; major effects were observed for basal and maximal respiration as well as H⁺ leak in YAC128 mouse mitochondria (**Fig. 12A**-D *ii*). No major differences in ATP production (**Fig. 12C** *i*, *ii*) or State3/State4 (**Fig. 12E** *i*, *ii*), were observed with aging in both areas.

Regarding CX activities, our results showed that CX II, CX III and CX IV activities significantly decrease in cortex from WT mice from 3 to 9 months of age, whereas little age-dependent changes were observed in YAC128 cortical mitochondria (Fig. 13 B-D *i*); conversely, a decrease in CX II, CX III and CX IV activities was observed in YAC128 mouse striatum mitochondria from 3 to 9 months of age (Fig. 13 B-D*ii*).

These data suggest that striatal mitochondria from YAC128 mice are more affected by aging when compared to cortical mitochondria from the same animals.



FIGURE 12: AGE-DEPENDENT VARIATION OF OCR PARAMETERS IN YAC128 AND WT MICE MITOCHONDRIA DERIVED FROM CORTEX AND STRIATUM

Basal respiration (A) maximal respiration (B) ATP production (C) H⁺ leak (D) and state 3/state 4 (E) analysis in (*i*) cortical or (*ii*) striatal isolated mitochondria were replotted from Figs. 9, 10 and 11 as a function of mice age. Statistical analysis: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.001 by two-way ANOVA followed by Bonferroni post-hoc test when comparing between genotypes; #p<0.05; **p<0.05; **p<0.01; **** p<0.001 by two-way ANOVA followed by Bonferroni post-hoc test when comparing between WT mice; *p<0.05; **p<0.01; ****p<0.001; ****p<0.001; **** p<0.001; ****



FIGURE 13: AGE-DEPENDENT VARIATION OF CX ACTIVITIES IN YAC128 AND WT MICE MITOCHONDRIA DERIVED FROM CORTEX AND STRIATUM

CX I (A), CX II (B), CX III (C) and CX IV (D) activities in (*i*) cortical or (*ii*) striatal isolated mitochondria from YAC128 and WT mice were replotted from Figs. 9, 10 and 11 as a function of mice age. Statistical analysis: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.001 by two-way ANOVA followed by Bonferroni post-hoc test, when comparing between genotypes; #p<0.05; **p<0.01; **** p<0.01; **** p<0.01;

RESULTS

3.5 Decreased mitochondrial Ca²⁺ handling in pre-symptomatic cortical mitochondria from YAC128 mice

Since altered mitochondrial Ca²⁺ retention capacity could serve as an indicator of mitochondrial impairment in HD, we next evaluated whether the differences observed in mitochondrial respiratory rate correlates with the effect of mHTT on Ca²⁺ handling in mitochondria isolated from pre-symptomatic (3 mo) and symptomatic (12 mo) YAC128 and WT mice (Fig. 14-legend in page 57). Since no major changes in OCR parameters were observed in mitochondria obtained from 9 mo YAC128 or WT mice, we did not analyze the Ca²⁺ uptake capacity at this age.

Our experiments evidenced that pre-symptomatic, but not symptomatic YAC128 micederived cortical mitochondria, subjected to a first pulse of Ca²⁺, exhibited a lower capacity to handle Ca²⁺ when compared to cortical WT mouse mitochondria (Fig. 14 Aiii). A second bolus of Ca²⁺ did not enable to observe differences between genotypes for both ages. In striatal mitochondria, Ca²⁺ handling achieved similar values in both pre-symptomatic and symptomatic YAC128 mice *versus* WT mice at first pulse (Fig. 14 Biii). In the second pulse, a significant decrease in Ca²⁺ retention occurred in striatal mitochondria from pre-symptomatic YAC128; however, no changes were observed between genotypes for symptomatic mice. Moreover, both cortical and striatal mitochondria from 12 mo YAC218 or WT mice showed decreased mitochondrial Ca²⁺ handling when compared to 3 mo mice, evidencing an age-dependent effect.

In order to evaluate if Ca^{2+} entry occurs through mitochondrial Ca^{2+} uniporter (MCU), a preincubation was performed in the presence of RU360 (10 μ M), an inhibitor of MCU (**Supplementary Fig. 1C**). In these conditions a complete inhibition of mitochondrial Ca^{2+} retention was observed, which is in agreement with the involvement of a selective pathway for Ca^{2+} entry into mitochondria. At the end of the experiments, FCCP was also tested in order to depolarize mitochondria; therefore a significant release of Ca^{2+} from mitochondria was observed (**Supplementary Fig. 1A,B**).

Data show slight but significant defects in Ca²⁺ handling in cortical and striatal presymptomatic YAC128 mice, but no changes in both areas of symptomatic mice.



3.6 Increased mitochondrial H₂O₂ production in cortical and striatal mitochondrial YAC128

ROS are major activators of the mPTP and its induction is the key factor that restricts Ca^{2+} uptake capacity (Chalmers & Nicholls, 2003). Taking this into account and since different levels of ROS production could underlie the differences in Ca^{2+} retention, we examined mitochondrial H_2O_2 production in mitochondria isolated from pre-symptomatic and symptomatic cortical and striatal YAC128 compared to WT mice, using the Amplex Red assay. We observed a significant increase in H_2O_2 production in pre-symptomatic cortical mitochondria from YAC128 mice (Fig. 15A). Moreover, an increase in H_2O_2 production was demonstrated in striatal mitochondria from pre-symptomatic, as well as symptomatic YAC128 *versus* WT mice (Fig. 15B). Antimycin A (CX III inhibitor) was added as a control of the H_2O_2 production experiments in isolated mitochondria. We observed a similar increase in H_2O_2 production upon cortical and striatal mitochondria. We observed a similar increase in H_2O_2 production upon cortical and striatal mitochondria. We observed a similar increase in H_2O_2 production upon cortical and striatal mitochondria form respective of the stressor, which confirms the ROS production (Supplementary Fig. 2).



FIGURE 15: H_2O_2 production in cortical and striatal mitochondria from YAC128 and WT mice.

 H_2O_2 production in mitochondria isolated from cortex (A) and striatum (B) of 3, 9 and 12 month-old YAC 128 and WT mice was evaluated using Amplex Red assay, as described in Materials and Methods section. Data are the mean ± SEM of triplicate experiments from n=10 3mo mice, n=7 9mo mice and n=11 12mo mice. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni post-hoc test: ** p<0.01 compared to WT mitochondria and *t* p< 0.001 by Student's *t*-test when comparing with WT mitochondria.

FIGURE 14: Ca²⁺ HANDLING IN YAC128 AND WT MICE MITOCHONDRIA DERIVED FROM CORTEX AND STRIATUM.

Mitochondria were isolated from the cortex and striatum of 3 and 12 month-old mice. Representative traces of extramitochondrial Ca²⁺ (RFU) measured with Calcium Green-5N fluorescent probe in cortex (A) and striatum (B) from 3 mo (A, B.*i*) and 12 mo (A, B.*ii*) YAC128 *versus* WT mice. Ca²⁺ handling (expressed as a linear reduction of Calcium Green-5N fluorescence/min) from cortical (A.*iii*) and striatal (B.*iii*) mitochondria from 3 mo and 12 mo YAC128 *versus* WT mice. Ca²⁺ retention capacity was measured as described in Materials and Methods section. Two 10 μ M Ca⁺ pulses were applied. Data are mean ± SEM of 5 to 6 mice from each genotype run in quadruplicates. Statistical analysis was performed by Student's t-test: ^t *p*<0.05 compared to WT mice.

RESULTS

3.7 Glutathione redox cycle is affected in pre-symptomatic YAC128 mice

In order to investigate the levels of antioxidant defense systems present in pre-symptomatic and symptomatic YAC128 *versus* WT mice cortex and striatal-derived mitochondria, the mitochondrial levels of GSH and GSSG were determined. We found a significant increase in GSH levels in cortical mitochondria derived from 3 mo YAC128 mice (Fig 15 Ai); however no differences were observed in mitochondria obtained from cortex of 9 and 12 mo mice. In addition, unchanged levels in GSH were detected in striatal mitochondria from the same mice (Fig 16 Ai and ii). Moreover, no significant differences were detected in the levels of GSSG between genotypes, brain area or age (Fig 16 Bi and ii).

We further determined GPx and GRed activities (Fig. 16 C,D). GPx converts 2 molecules of GSH and H₂O₂ into GSSG and H₂O, respectively. GRed uses GSSG and NADPH to regenerate GSH levels. Results depicted in Figure 16 *Cii* showed a significant decrease in GPx activity in pre-symptomatic striatal YAC128 mitochondria in comparison with that derived from WT age-matched mice. However, no differences in GPx activity were observed in symptomatic YAC128 mice (Fig.16 *Cii*). In these mitochondria, unchanged levels of GRed activity were observed in both YAC128 and WT presymptomatic and symptomatic mice (Fig.16 *Dii*). In cortical mitochondria from pre-symptomatic and symptomatic mice and symptomatic changes were detected in either GPx or GRed activities at 3 or 12 months of age (Fig 16 *Ci*, *Di*).



FIGURE 14: MEASUREMENT OF GLUTATHIONE REDOX CYCLE IN CORTICAL AND STRIATAL ISOLATED MITOCHONDRIA FROM YAC128 AND WT MICE

Quantification of GSH levels in cortical (A.i) and striatal mitochondria (A.ii) from 3, 9 and 12 mo YAC128 versus WT mice. Quantification of GSSG levels in cortical (B.i) and striatal mitochondria (B.ii) from 3, 9 and 12 mo YAC128 versus WT mice. GPx activity in cortical (C.i) and striatal mitochondria (C.ii) from 3, 9 and 12 mo YAC128 versus WT mice. GRed activity in cortical (D.i) from 3, 9 and 12 mo and striatal mitochondria (D.ii) from 3 and 12 mo YAC128 versus WT mice. Results are expressed as the mean \pm S.E.M. of 3 to 4 mice from each genotype performed in duplicates. Statistical analysis was performed by Student's t-test: tP<0.05

3.8 Compromised antioxidant protein levels in pre-symptomatic and symptomatic YAC128 mice

Since differences in glutathione redox system were detected in this model, we further analyzed the levels of proteins involved in antioxidant defects, including acetyl-SOD2, SOD2, SOD1, catalase, GRed and GPx by western blotting. We detected a decrease in SOD2 levels (Fig. 17A) and acetyl (K68) SOD2 levels (Supplementary Fig 3A), as well as a decrease acetyl(K68)SOD2/SOD2 ratio in cortical mitochondria from symptomatic (12 mo) YAC128 *versus* WT mice (Fig. 17B), indicating an increase in SOD2 activity despite decreased SOD2 protein levels. However, no changes on SOD2 or Acetyl-SOD2 were observed in striatal mitochondria from YAC128 mice at any age tested. Furthermore, no significant changes could be observed on SOD1 levels, despite a tendency for a decrease in 3 and 9 mo cortical cytoplasmic fractions of YAC128 mice, when compared to WT mice (Supplementary Fig 3B). Despite unchanged activity (Fig. 16D), GRed levels were shown to be significantly decreased in mitochondrial fractions from 3 mo YAC128 mouse striatum (Fig. 17C) only.

In addition, a decrease in catalase levels was observed in cortical cytoplasmic extracts from 12 mo YAC128 mice only. In striatal extracts no differences were detected in catalase levels. (Fig 17D).



FIGURE 15: ANTIOXIDANT PROTEIN LEVELS IN YAC128 VERSUS WT MICE

SOD2 levels (A), ratio between acetylated (K68)SOD2 and total SOD2 (B), GRed levels (C) in cortical and striatal mitochondria fraction from 3, 9 and 12mo YAC128 *versus* WT mice; Catalase levels (D) of cortical and striatal cytoplasmic fraction from 3, 9 and 12mo YAC128 *versus* WT mice. Protein levels were assessed in cytoplasmic and mitochondrial extracts by Western Blotting as described in Material and Methods section. Data is presented as the mean ± SEM of 3-10 mice from each genotype. Statistical significance: ^tp<0.05 by Student's t-test.

<u>CHAPTER IV – DISCUSSION AND</u> <u>CONCLUSIONS</u>

4.1 Discussion

Since the discovery of a link between the mutation in huntingtin and HD pathogenesis, a great number of hypotheses have been raised concerning the role mHTT on mitochondrial function. Indeed, mitochondria have an important role in producing metabolites and ATP, and in maintaining mitochondrial membrane potential for neuronal energy production, required for efficient brain function (Martínez-Reyes et al., 2016).

The understanding of HD pathology has been facilitated by the development of transgenic models, such as the YAC128 mouse model used in the present study. YAC128 mice exhibited a significant weight gain over time and significantly higher body weight than age-matched WT mice. Our results are in accordance with Van Raamsdonk and co-authors (2007), who described a gain weight in transgenic mice expressing full-length human mHTT, such as BACHD and YAC128 mice, compared to WT mice; these authors suggested that this might be related with the FVB/N background strain in which these mice were developed. We also confirmed the presence of mHTT in isolated mitochondria by Percoll gradient. We were able to detect both HTT and mHTT associated with cortical mitochondria derived from YAC128 mice. In accordance with Hamilton et al. (2015), we identified lower amount of mHTT, when compared with the amount of mitochondria-bound-wild-type HTT, which suggests a lower affinity of mHTT compared to HTT for mitochondria. The presence of HTT in mitochondria isolated from WT mice seems to indicate that wild-type HTT may plays a relevant role in the normal regulation of mitochondrial function.

Biochemical studies from HD patients tissues (Brennan et al., 1985, Benchoua et al., 2006; Kim et al., 2010; Sawa et al., 1999; Silva et al., 2013; Tabrizi et al., 1999), HD cell models (Benchoua et al., 2006) and animal models (Lim et al., 2008) revealed decreased activity of MRC complexes I-IV. Conversely, another group of investigators defend that MRC function is not impaired in HD (Gouarn et al., 2013; Guidetti et al., 2001; Hamilton et al., 2015; Hamilton et al., 2016; Milakovic & Johnson, 2005; Milakovic et al., 2006; Oliveira et al., 2007). According to our best knowledge, we described for the first time defects in mitochondrial respiration in pre-symptomatic YAC128 mice model, which may reflect the first stages of HD pathogenesis. Surprisingly, we observed a tissue-specific alteration in MCR activity; in fact, a decrease in MRC activity in cortical mitochondria was observed along with an increase in striatal mitochondrial respiration is compromised in cortical mitochondria from YAC128 mice; moreover, the increase in ATP production and state3/state4 ratio (despite the impairment in respiratory CX II-IV) may be accounted for by reduced H^+ leak, probably resulting from reduced levels and/or activity of uncoupling proteins. In pre-symptomatic striatal mitochondria from YAC128 mice, our results demonstrated an increase in mitochondrial respiration, namely basal and maximal respiration, ATP production and H⁺ leak, and maintained state3/state4 ratio. These results are in accordance with the observed increase in complexes I-IV activities. Damiano et al., (2013) described that brain mitochondria from N171-82Q mice at 2 mo evidenced a significant defect in CX II activity and that a significant neuroprotection of striatal neurons against mHTT neurotoxicity occurred when CX II subunits, Ip and Fp, are overexpressed in the 4 mo rat striatum. Even though our results were obtained in another transgenic animal model, we could hypothesized that the increment observed in OCR parameters and in complex activities in striatal mitochondria from YAC128 mice could be a compensatory mechanism underlying neurotoxicity induced by mHTT. These results are in disagreement with those obtained by Hamilton and colleagues who described a lack of respiratory defects in isolated brain synaptic and non-synaptic mitochondria and also in striatal cultured neurons obtained from 2 and 10 mo YAC128 and WT mice (Hamilton et al., 2015). In this study, OCR of isolated mitochondria were measured through Clark-type oxygen electrode. Important to note that in our studies an experimental protocol was designed to perform SeaHorse analysis in isolated mitochondria. Since XF24 analyzer increases the range of OCRs, the temporal resolution, timescale of measurements and the possibility to run multiple samples simultaneously, makes the difference from conventional Clark-type oxygen electrode procedure (Gerencser et al., 2009; Rogers et al., 2011). Taking this into account, we considered that accuracy of the methodology used in the present work may explain the changes observed in 3 mo YAC128 mitochondria in comparison with lack of defects showed in Hamilton et al., 2015. In 9 mo the increase in basal and maximal respiration detected in striatal mitochondria in YAC128 mice was still visible; however, respiratory activity became similar for transgenic and control mice at later ages. Similar results were obtained for 9 and 12 mo cortical mitochondria derived from YAC128 versus WT mice.

Interestingly, our results show that with aging, the most affected area in WT mice seems to be the cortex. Moreover, the later stages of both genotypes exhibit a tendency for a recovery in OCR parameters. Conversely, in YAC128 mice striatal mitochondria appear to be mostly affected along aging; consistently, the striatum shows selective neurodegeneration in this animal model after 12 months of age. Thus, striatal mitochondria may implicate enhanced susceptibility of this brain region. Together, our results suggest that early changes in brain energy metabolism are followed by

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compensatory shifts to maintain energetic homeostasis when pathological symptoms become apparent.

Ca²⁺ is an important signal for neurotransmitter release and subsequent activation of several pathways (Brini & Carafoli, 2000), however excessive amount of intracellular Ca²⁺ can induce neuronal dysfunction, including mitochondrial depolarization, increased ROS formation, activation of calpains and opening of the mPTP (Damiano et al., 2010). The bioenergetic differences observed in our study could influence the mitochondrial Ca²⁺ loading capacity. Cumulating evidences indicate that impairment of mitochondrial Ca²⁺ homeostasis through mHTT-induced facilitation of PTP induction underlies the susceptibility to selective neuronal death observed in HD (Gellerich et al., 2008; Milakovic et al., 2006; Panov et al., 2002; Quintanilla., 2013; Seong et al., 2005), however, other authors failed to observe defects in mitochondrial Ca²⁺ uptake in HD models (Brustovetsky et al., 2005; Oliveira et al., 2007; Pellman et al., 2015). Our lab showed that HdhQ111 cells use glycolytic ATP masking in this way the mitochondrial role in Ca^{2+} handling. In addition, these cells and primary cultured striatal neurons are dependent on oxidative phosphorylation for Ca²⁺ handling, being the expression of mHTT associated with deficits in this parameter (Oliveira et al., 2006). Moreover, we previously identified an increase in Ca²⁺ loading capacity of forebrain mitochondria isolated from 12-13 weeks R6/2 and 12mo YAC128 mice, but not in those from Hdh150 knock-in mice (Oliveira et al., 2007). Pellman et al. showed evidences of a large Ca^{2+} uptake capacity in YAC128 mice using synaptic and nonsynaptic isolated mitochondria from 2 and 12 mo of YAC128 when compared with YAC18 and FVB/NJ, reflecting a compensatory adaptation to augmented Ca²⁺ influx (Pellman et al., 2015). Therefore, since data obtained with different models and experimental approaches remain controversial, we examined the direct effect of expanded polyQ tract with possible deficits in mitochondrial Ca2+ buffering capacity in 3 and 12 mo YAC128 versus WT mice. Our data shows a significant decrease in Ca²⁺ handling in cortical pre-symptomatic YAC128 mice mitochondria subjected to a bolus of Ca^{2+} which is in agreement with decreased functional respiratory chain described above. Interestingly, the increase in the MRC function observed in YAC128 striatal mitochondria, were not accompanied by changes in mitochondrial Ca²⁺ retention between YAC128 and WT mice, suggesting that the improvement in mitochondrial respiration is not enough to enhance Ca²⁺ retention under these conditions. Analysis of mitochondrial function in symptomatic YAC128 and WT mice showed that Ca²⁺ handling is not affected, which corroborate the unchanged parameters in OCR observed in both cortex and striatum. These data suggest that early changes observed in mitochondrial Ca²⁺ loading capacity are transient in HD mice and that the aging process involves other pathological

mechanisms, such as oxidative stress, apparently turning mitochondrial dysfunction not as relevant for HD-related neurodegeneration at later stages. Future experiments should be carried out to evaluate the effects of Ca^{2+} retention on $\Delta\Psi_m$.

Oxidative stress is described to be an important pathological mechanism in HD that may result from impairment of mitochondrial function, due to interaction of the organelle with mHTT exacerbated under excitotoxic conditions. Since PTP induction, through decreased $\Delta \Psi_m$, is the key factor that restricts Ca²⁺ uptake capacity (Chalmers & Nicholls, 2003) and ROS is one of the main activators of PTP (Bernardi, 1999), we hypothesized that abnormal levels of ROS production could explain the differences in mitochondrial Ca²⁺ uptake. In previous studies in our lab, we observed a significant increase in superoxide and hydroperoxide production in HD cybrids upon treatment with 3-NP and STS compared with control cybrids (Ferreira et al., 2010); moreover, evidences of oxidative stress were found in striatal cells expressing full-length mHTT (Oliveira et al., 2015; Ribeiro et al., 2012). Importantly, very recently, Pellman and colleagues did not find any correlation between Ca²⁺ uptake capacity and ROS generation in mitochondria from YAC128 compared with FVB/NJ mice, being unable to observed any differences in ROS production between genotypes (Pellman et al., 2015). However, in this study, we evidenced an increase in H_2O_2 production in striatal mitochondria from 3, 9, 12 mo YAC128 and in cortical mitochondria from 3 mo YAC128 versus WT mice. Enhanced O2production by isolated mitochondria underlies the hypothesis that it can be due to the damage of MRC, slow respiration and a high NADH/NAD⁺ ratio in the matrix (Murphy, 2009). Thus, we can hypothesize that the increase in H_2O_2 production, observed in pre-symptomatic cortical mitochondria, is a consequence of an impairment in oxidative phosphorylation and complex activities as well as defects in Ca²⁺ handling, as described above. These data confirm defects at an early stage of HD in YAC128 mice cortical mitochondria and give further evidence for age-dependent ROS production in striatal mitochondria derived from YAC128 mice.

The imbalance between ROS and levels of antioxidants is very important to maintain the organelle homeostasis and to prevent against oxidative stress. GSH is a cysteine-containing tripeptide present at a very high concentration in the brain. It is *"the heart of one of the most important cellular antioxidant systems"* and is responsible for the scavenge of ROS and RNS. GSH is oxidized at cysteine-SH groups, resulting in the formation of a disulfide bridge between two molecules, forming GSSG (Couto et al., 2016). Choo and co-authors (2005) described unchanged levels of GSH in tissue homogenates, while an increased in both cortical and striatal mitochondria of R6/2 *versus* WT mice.

In cortical and striatal mitochondria derived from 8- to 12- week-old R6/2 HD mice an increase in GSH and antioxidant protein levels was also described (Tkac et al., 2010). Accordingly, our previous findings, showed a significant increase in GSH and GSSG levels in total and mitochondrial fractions of Q111 cells expressing FL-mHtt (Ribeiro et al., 2012). In cortical mitochondria, we were also able to observe an increase in the levels of GSH, but not in GSSG levels in 3 mo YAC128 mice. However, in striatal mitochondria we did not detect differences in these levels between genotypes or ages. Since GSH and GSSG are substrates for GPx and GRed enzymes respectively, we hypothesized that alterations in enzyme activities might occur. However we could not correlate the increase in GSH with GPx or GRed activities. Notably, apart from the glutathione redox cycle, GSH is also involved in other cellular processes such as sequestration and storage of nitric oxide and cysteine, sulfur assimilation, maturation of iron-sulfur clusters of diverse proteins, S-glutathionylation, operation of several transcriptional factors, also serving as an electron donor for other enzymes as glutathione S-transferases and glyoxalases (Couto et al., 2016) which activities were not measured in this study.

Using erythrocytes from HD patients, Chen et al., (2007) showed an impairment in the antioxidant defenses, namely a decrease in GPx and SOD1 activities. Our data showed unchanged GPx activity in cortical mitochondria in all ages, but decreased GPx activity in striatal mitochondria from 3 mo YAC128 mice. Unfortunately, in our work, we failed to demonstrate GPx protein levels by Western Blotting due to insufficient labeling of the antibody used. The present data argue against the increase in GPx activity observed by Ribeiro et al., (2012) in knock-in striatal cells and also with the increase in mRNA levels of GPx, catalase and SOD1, demonstrated in the same model by Lim et al., (2008). We need to take into account that these studies used a cell line, while our experiments were performed in isolated brain mitochondria. In 2013, Mason and collaborators, using different models, including yeast, mammalian cells and Drosophila, proved the protective role of GPx in ameliorating HD-like symptoms by reducing ROS levels, without adverse effects on autophagy, an important mechanism for removing mutant Htt (Mason et al., 2013). We can correlate the observed decrease in GPx activity in HD striatal mitochondria with the evidences of enhanced H₂O₂ production, suggesting insufficient capacity to reduce H₂O₂ in pre-symptomatic YAC128 mice.

Although Ribeiro et al., (2012) showed an increase in GRed activity in mutant cells (Q111), we did not observed differences in GRed activity between both genotypes on brain areas or mice age. The decreased in protein levels of GRed (although maintaining similar activity under optimal experimental conditions) in striatal mitochondria from 3mo YAC128 compared to WT mice suggest a higher susceptibility to oxidative stress in YAC128 mice, which corroborate the increased H₂O₂

production in this brain area. Since striatum is the most affected brain area in HD, it seems natural that the defects in glutathione redox cycle (namely decrease in GPx activity and GRed protein levels) were only observed in striatum.

To better clarify the regulation of other antioxidants, we analyzed the activated form of SOD2 and its protein levels, as well as the levels of catalase in both brain regions. An increase in SOD2 activity (which converts superoxide into H_2O_2) despite decreased SOD2 levels were demonstrated in mitochondrial extracts of symptomatic cortical YAC128 mice only, suggesting compensatory effects that translate into unchanged H_2O_2 production, described above. We also observed a decrease in catalase levels, a major peroxisomal enzyme responsible for the metabolism of H_2O_2 , in cytoplasmic extracts of 12 mo YAC128 cortex. Even though we could not correlate the changes in catalase levels with ROS production in mitochondria, these observations might be indicative of defects in antioxidant defenses in other relevant organelles.

Overall this study provide novel and important data regarding mitochondrial dysfunction, namely impairment of MRC, defects in Ca²⁺ handling, ROS production and compromised antioxidant defenses in pre-symptomatic transgenic mice, which may influence the age-related effect of mHTT in mitochondria.

4.2 Conclusion

Expression of mHTT has been largely associated with mitochondrial dysfunction, with consequent bioenergetic deficits, increased ROS production and decreased Ca²⁺ buffering capacity causing neurodegeneration of the striatum, and later the cortex. Whether mitochondrial dysfunction is a characteristic of early/pre-symptomatic stages of HD is still not entirely clear. Thus, our study makes a step forward in deciphering HD pathogenesis by attempting to understand pre-symptomatic alterations in HD; for this purpose we studied the implications of FL-mHTT on mitochondrial function and related oxidative stress in cortical and striatal isolated mitochondria from HD YAC128 transgenic mice.

In cortical mitochondria derived from pre-symptomatic (3 mo) YAC128 mice we observed an impairment in mitochondrial function. We described a decrease in both mitochondrial coupling and respiratory chain complexes II-IV activities, a decrease in mitochondrial Ca²⁺ handling, enhanced H₂O₂ production and apparent compensatory increase in GSH levels, but unchanged glutathione enzymes activities or antioxidant protein levels. In addition, in mitochondria of the same brain area from symptomatic (9-12 mo) YAC128 mice we showed unchanged OCR parameters, Ca²⁺ handling, ROS production, glutathione enzyme activities, despite a decrease in SOD2 and catalase protein levels at a later stage.

Surprisingly, in striatal mitochondria derived from pre-symptomatic YAC128 mice, enhanced mitochondrial function was described. We observed an increase in mitochondrial coupling and respiratory chain complexes I-IV activities, which was accompanied by enhanced H₂O₂ production, a decrease in glutathione peroxidase activity and in glutathione reductase protein levels; this occurred concomitantly with a decrease in mitochondrial Ca²⁺ handling after a second Ca²⁺ stimulus. Moreover, in symptomatic (9-12mo) YAC128 mouse striatal mitochondria, unchanged OCR parameters, Ca²⁺ handling, antioxidant enzyme activities or proteins were observed, whereas H₂O₂ production was largely increased, suggesting alternative oxidant events.

Overall, data evidence mitochondrial modifications in pre-symptomatic YAC128 mice, namely decreased mitochondrial function in the cortex and enhanced organelle activity in striatum. These results suggest that mitochondrial dysfunction in cortex might precede striatal neurodegeneration described in HD and that the response observed in 3 mo striatum might be a compensatory mechanism underlying mHTT-induced neuronal dysfunction.

Further studies are thus required, namely the measurement of mitochondrial membrane potential, since this is another important parameter to better understand the impact of mHTT on mitochondrial (dys)function. To clarify the apparent ambiguous evidences concerning the increase in ATP production observed in cortex-derived mitochondria, ATP synthase activity could be also measured. To complement the analysis of H_2O_2 levels, it would be interesting to determine aconitase activity, an important enzyme of the Krebs cycle that appears to be highly vulnerable to free radicals.

Altogether, we may suggest an early intervention aimed at slowing down HD progression, by focusing research efforts on developing therapies, such as bioenergetic agents or mitochondrial-targeted antioxidants that, if applied at early stages, may ameliorate mitochondrial function in cortex, which can influence the outcome in the striatum, namely through modified release of glutamate at cortico-striatal synapses known to cause striatal neurodegeneration by excitotoxicity.

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ATTACHMENTS

1 SUPPLEMENTARY METHODS

SUPPLEMENTARY TABLE 1 : MIX AND MEASURES CYCLE TIMES

Command	Nº cycles	Mix	Wait	Measurement
Calibration	3	1min	0.5min	3min
Port A	3	1min	0.5min	6min*
Port B	3	1min	0.5min	3min
Port C	3	1min	0.5min	3min
Port D	3	1min	0.5min	3min

*Measure times for State 3 respiration may be extended beyond 3min to observe the transition from State 3 to State 4 due to exhaustion of ADP in the microchamber (adapted from Rogers et al., 2011).

2 SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE 1: COUPLING AND ELECTRON FLOW EXPERIMENTS PERFORMED IN 3 MONTH-OLD YAC128 CORTICAL AND STRIATAL MITOCHONDRIA VERSUS WT AGE-MATCHED.

Coupling Experiment 3mo						
Brain area	Genotype	ADP	Oligomycin	FCCP	Antimycin A	
	WT	36.5 ± 4.7 n=7	64,8 ±12.6 n=7	36,9 ± 7.8 n=7	110.1 ± 12.7 n=7	
Cortex	YAC128	39.4 ± 2.7 n=7	80,5 ± 9.7 n=7	38.8 ± 7.6 n=7	79.9 ± 7.7 n=7 *	
	WT	31.9 ± 5.4 n=7	54.6 ± 15.1 n=7	30.5 ± 8.8 n=7	88 ± 13.9 n=7	
Striatum	YAC128	47.0 ± 5.8 n=7	93.5 ± 12.6 n=7	92.6 ± 10.3 n=7 **	165 ± 15.4 n=7 ****	
Electron Flow 3mo						
Brain area	Genotype	Rotenone	Succinate	Antimycin A	Ascorbate/TMPD	
	WT	10,1 ± 2,0 n=6	152,0 ± 10,4 n=6	146,5 ± 9,6 n=6	228,7 ± 11,2 n=6	
Cortex	YAC128	13,6 ± 2,1 n=5	114,1 ± 20,8 n=5	129,4 ± 21,6 n=5	176,5 ± 25,3 n=5	
	WT	17,4 ± 1.2 n=6	180,4 ± 16,5 n=6	168,7 ± 17,3 n=6	223,2 ± 16,4 n=6	
Striatum	YAC128	18,0 ± 1,9 n=6	221,2 ± 11,7 n=6	209,7 ± 12,7 n=6	273,8 ± 13,2 n=6	

Deltas obtained using data from SeaHorse XF24 Analyzer and show mean +/- SEM n=5-7. Statistical analysis: * p<0.05; ** p<0.01; **** p<0.001; **** p<0.001 by student's t-test

Coupling Experiment 9 mo						
Brain area	Genotype	ADP	Oligomycin	FCCP	Antimycin A	
Cortex _	WT	21,5 ± 6,8 n=3	44,2 ± 11,8 n=3	33,2 ± 4,4 n=3	59,4 ± 8,3 n=3	
	YAC128	36,1 ± 10,6 n=4	56,8 ± 14,9 n=4	41,4 ± 8,2 n=4	65,1 ± 11,9 n=4	
Striatum -	WT	35,2 ± 11,9 n=2	78,9 ± 14,5 n=2	40,5 ± 13,3 n=2	88,0 ± 24,5 n=2	
	YAC128	36,4 ± 9,1 n=4	83,9 ± 11,3 n=4	97,9 ± 20,3 n=4	140,5 ± 21,7 n=4	
Electron Flow 9mo						
Brain area	Genotype	Rotenone	Succinate	Antimycin A	Ascorbate/TMPD	
Cortex	WT	17,70 ± 1,706 n=2	66,45 ± 1,324 n=2	68,70 ± 1,374 n=2	69,80 ± 5,471 n=2	
	YAC128	49,22 ± 11,81 n=4	97,15 ± 19,34 n=4	92,51 ± 17,65 n=4	174,9 ± 32,55 n=4 *	
Striatum	WT	24,79 ± 4,126 n= 2	124,7 ±11,3 n= 2	124,5 ±11,1 n= 2	123,7 ±11,4 n= 2	
	YAC128	31,50 ± 4,990 n=4	90,5 ±9,6 n=4	88,8 ±9,3 n=4	157,9 ±22,4 n=4	

SUPPLEMENTARY TABLE 2: COUPLING AND ELECTRON FLOW EXPERIMENTS PERFORMED IN 9 MONTH-OLD YAC128 CORTICAL AND STRIATAL MITOCHONDRIA VERSUS WT AGE-MATCHED.

Deltas obtained using data from SeaHorse XF24 Analyzer and show mean +/- SEM n=2-4. Statistical analysis: * p<0.05; ** p<0.01; **** p<0.001; **** p<0.001 by student's t-test

SUPPLEMENTARY TABLE 3: COUPLING AND ELECTRON FLOW EXPERIMENTS PERFORMED IN 12 MONTH-OLD YAC128 CORTICAL AND STRIATAL MITOCHONDRIA VERSUS WT AGE-MATCHED

Coupling Experiment 12 mo						
Brain area	Genotype	ADP	Oligomycin	FCCP	Antimycin A	
Cortex	WT	42,3 ± 6,1 n=3	77,3 ± 3,7 n=3	43,8 ± 5,1 n=3	82,4 ± 5,1 n=3	
	YAC128	36,2 ± 7,9 n=4	63,7 ± 14,2 n=4	38,8 ± 12,5 n=4	74,9 ± 9,9 n=4	
Striatum -	WT	32,9 ± 6,6 n=4	68,6 ± 13,5 n=4	31,3 ± 3,7 n=4	74,3 ± 8,2 n=4	
	YAC128	39,9 ± 3,8 n=5	65,7 ± 6,4 n=5	36,9 ± 5,5 n=5	76,9 ± 4,9 n=5	
Electron Flow 12mo						
Brain area	Genotype	Rotenone	Succinate	Antimycin A	Ascorbate/TMPD	
Cortex	WT	10,5 ± 3,9 n=3	75,7 ± 22,7 n=3	77,7 ± 21,9 n=3	156,0 ± 48,4 n=3	
	YAC128	11,2 ± 3,9 n=3	61,4 ± 22,9 n=3	60,3 ± 23,1 n=3	107,1 ± 32,3 n=3	
Striatum -	WT	24,9 ±4,7 n=3	130,0 ±28,0 n=3	147,2 ±23,2 n=3	225,2 ±11,3 n=3	
	YAC128	43,1 ±16,4 n=3	180,6 ±57,4 n=3	109,7 ±18,5 n=3	164,0 ±40,7 n=3	

Deltas obtained using data from SeaHorse XF24 Analyzer and show mean +/- SEM n=2-4. Statistical analysis: * p<0.05; ** p<0.01; **** p<0.001; **** p<0.0001 by student's t-test



SUPPLEMENTARY FIGURE 1: EFFECT OF RU360 AND FCCP ON MITOCHONDRIA CA²⁺ HANDLING

A) Ca²⁺ green fluorescent following FCCP addition in cortical (i) and striatal (ii) mitochondria from 3 mo YAC128 and WT mice; B) Ca²⁺ green fluorescent with FCCP addition in cortical (i) and striatal (ii) mitochondria from 12 mo YAC128 and WT mice. C) Ca²⁺ green fluorescent after addition of RU360 (inhibitor of MCU) in cortical (i) and striatal (ii) mitochondria from 12 mo YAC128 and WT mice. Data represented in RFU are a representative independent experiment.



SUPPLEMENTARY FIGURE 2: REPRESENTATIVE TRACES OF MITOCHONDRIAL H₂O₂ PRODUCTION.

 H_2O_2 production in cortical **(A)** and striatal **(B)** mitochondria from 3 mo YAC128 and WT mice. Experiments were performed at mitochondria in reaction buffer containing 100 mM Sucrose, 100 mM KCl, 2 mM KH₂PO₄, 5mM HEPES, 10 μ M EGTA, and supplemented with 3 mM succinate in the absence or presence of the complex III inhibitor antimycin A (2 μ M). Data were obtained in RFU and are a representative independent experiment.



SUPPLEMENTARY FIGURE 3: ANALYSIS OF SOD2 ACETYLATION AND SOD1 PROTEIN LEVELS

A) Acetyl (K68) SOD2 levels in cortical and striatal mitochondria fraction from 3, 9 and 12mo YAC128 mice *versus* WT mice; **B)** SOD1 protein levels in cortical and striatal cytoplasmic fraction from 3 and 9 mo YAC128 mice *versus* WT mice. Protein levels were assessed in mitochondrial and cytoplasmic extracts by Western Blotting using Cx II (in **A**) and tubulin (in **B**) as loading controls. Data is presented as the mean ± SEM of 3-10 mice. Statistical significance: ^{tt} p<0.01 by Student's t-test.