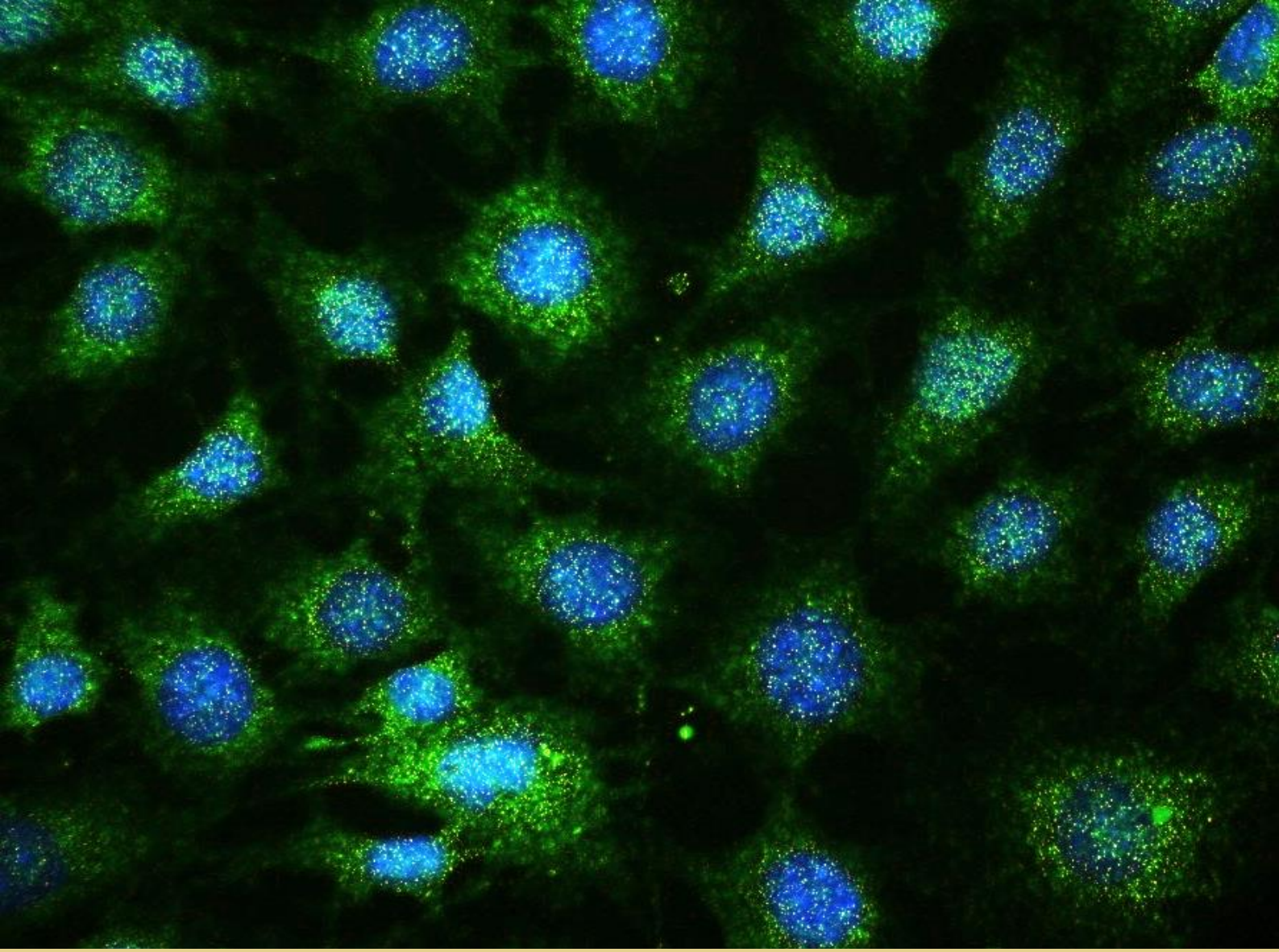

Márcio José do Coito Ribeiro

OXIDATIVE STRESS IN HUNTINGTON'S DISEASE KNOCK-IN STRIATAL CELLS

Tese de doutoramento em Biociências, especialidade em Biologia Celular e Molecular,
orientada por Professora Doutora Ana Cristina Carvalho Rego
e co-orientada por Professora Doutora Emília da Conceição Pedrosa Duarte
e apresentada ao Departamento de Ciências da Vida,
Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

Fevereiro de 2014

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Universidade de Coimbra

Márcio José do Coito Ribeiro

Oxidative Stress in Huntington's Disease Knock-in Striatal Cells

FCTUC FACULDADE DE CIÊNCIAS
E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Coimbra 2014

Dissertation submitted to the Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra for Degree of Doctor of Philosophy in Biosciences, specialization in Cellular and Molecular Biology

Dissertação apresentada ao Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do Grau de Doutor em Biociências, especialidade em Biologia Celular e Molecular

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The research described in the present thesis was performed at the Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal.

Márcio Ribeiro was funded by Fundação Bissaya Barreto before receiving a PhD fellowship from Fundação para a Ciência e Tecnologia (FCT), Portugal (fellowship: SFRH/BD/41285/2007). The research work was funded by European community fund FEDER through "Programa Operacional Temático Factores de Competitividade – COMPETE" and "Fundação para a Ciência e a Tecnologia" (FCT), Portugal, project references PEst-C/SAU/LA0001/2011, PTDC/SAU-FCF/66421/2006.

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Ribeiro, M., Rosenstock, T. R., Cunha-Oliveira, T., Ferreira, I. L., Oliveira, C. R. and Rego, A. C. (2012). Glutathione redox cycle dysregulation in Huntington's disease knock-in striatal cells. *Free Radical Biology & Medicine* **53**(10), 1857-67, 10.1016/j.freeradbiomed.2012.09.004.

Ribeiro, M., Silva, A. C., Rodrigues, J., Naia, L. and Rego, A. C. (2013). Oxidizing effects of exogenous stressors in Huntington's disease knock-in striatal cells--protective effect of cystamine and creatine. *Toxicological Sciences : an official journal of the Society of Toxicology* **136**(2), 487-99, 10.1093/toxsci/kft199.

Ribeiro, M.; Rosenstock, T. R.; Cunha-Oliveira, T.; Ferreira, I. L.; Oliveira, C. R.; Rego, A. C. Insulin and IGF-1 reduce mitochondrial-driven oxidative stress in Huntington's disease knock-in striatal cells. *Free Radical Biology & Medicine*. 2014 (Submitted).

Acknowledgments

My honest appreciation to Fundação Bissaya Barreto and to FCT for conceiving me this PhD fellowship grant SFRH/BD/41285/2007 (Supervisor: Dr Ana Cristina Rego, Ph.D.). Also, research work was funded by European community fund FEDER through "Programa Operacional Temático Factores de Competitividade – COMPETE" and "Fundação para a Ciência e a Tecnologia" (FCT), Portugal, project references PEst-C/SAU/LA0001/2011, PTDC/SAU-FCF/66421/2006 (PI: Dr Ana Cristina Rego, Ph.D.).

I would like to thank the Doctoral Programme in Biosciences, the Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, to the Center for Neuroscience and Cell Biology (CNC), and specially, to Professor Dr Catarina Isabel Neno Resende de Oliveira, coordinator of the scientific area of Neuroscience and Disease at CNC, for giving me the opportunity and honor to perform my Ph.D studies, in this institution, and for the advices and collaboration in the work that resulted in this thesis. I also would like to acknowledge Professor Dr Emília da Conceição Pedrosa Duarte for her acceptance as my co-supervisor in my PhD studies.

I would like to thank all the people who contributed in some way to the work described in this thesis. First and foremost, I would like to express my sincere gratitude to my supervisor Professor Dr Ana Cristina Rego, for accepting me in her group and to be my supervisor for the past years. Thank you for encouraging my research, for your patience, energy, motivation, enthusiasm, and immense knowledge you've shared with your students. I thank you for engaging me in new ideas, demanding a high quality of work in all my endeavors, for your assistance in writing papers and this thesis, and for allowing me to grow as a research scientist.

I would like to thank the various members and former members of the Mitochondrial Dysfunction and Signaling in Neurodegeneration group (PI: Dr Ana Cristina Rego) with whom I had the honor and opportunity to work with. They provided a friendly and cooperative atmosphere at work and also, useful feedback and insightful comments on my research work. Thank you "MDSN" group for making all these years much funnier and unforgettable.

To the CNC technicians, Isabel Nunes and Isabel Dantas for their advices, cell culture expertise and media supply.

Finally, I would like to acknowledge my friends and family who supported me during my time in Coimbra.

First, and foremost I would like to thank the best parents in the world, for their constant love, sacrifice, support and encouragement throughout my entire life.

At last, I would like to express my appreciation to my best friend, soul-mate and beloved fiancée. Thank you for all your love, patience, inspiration, encouragement and support, in every moment. I love you, Cláudia.

"Your beliefs become your thoughts

Your thoughts become your words

Your words become your actions

Your actions become your destiny"

Mahatma Ghandi

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CHAPTER 1

List of Abbreviations

$^{\bullet}$OH – Hydroxyl radical

$^{\bullet}$NO – Nitric oxide

β-TrCP – Beta-transducin repeat-containing protein

γ-GC – Gamma-glutamylcysteine

γ-glutamyl-AMC – Gamma-glutamyl-7-amino-4-methyl-coumarin

γ-GT – Gamma-glutamyl transpeptidase

ΔΨm – Mitochondrial transmembrane potential

λ-PP – Lambda protein phosphatase

$^{1}O_2$ – Singlet oxygen

2-OH-E^{+} – 2-Hydroxyethidium

3-HK – 3-Hydroxykynurenine

3-NP – 3-Nitropropionic acid

3-NT – 3-Nitrotyrosine

4-HNE – 4-Hydroxynonenal

4E-BP1 – Eukaryotic translation initiation factor 4E binding protein 1

6PDG – 6-Phosphogluconate dehydrogenase

6PG – 6-Phosphogluconate

6PGL – 6-Phospho-D-glucono-1,5-lactone

6PGLase – 6-Phosphogluconolactonase

8-OHdG – 8-Hydroxy-2'-deoxyguanosine

ABTS+ – 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

Ac-DEVD-AFC – N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin

ADP – Adenosine diphosphate

AIF – apoptosis-inducing factor

Akt – Protein kinase B

AMPA – Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR –AMPA receptor

AR – Androgen receptor gene

ARE – Antioxidant response element

Atg13 – Autophagy related 13

ATN1 – Atrophin-1 gene

ATP – Adenosine triphosphate

ATXN1/2/3/7 – Ataxin-1/2/3/7 gene

BAC – Bacterial artificial chromosome

BAD – B-Cell CLL/Lymphoma 2 (BCL2)-associated agonist of cell death

BDNF – Brain-derived neurotrophic factor protein

BRCA1 – Breast cancer 1, early onset

BTB – Bric-a-brac, tramtrack, broad domain

Ca^{2+} – Calcium

$CaCl_2$ – Calcium chloride

CACNA1A - Voltage-dependent P/Q-type calcium channel subunit alpha-1A gene

CAG – Cytosine, guanine, adenine

Cav-1 – Cholesterol-binding protein caveolin-1

CBP – CREB-binding protein

CDNB – 1-Chloro-2,4-dinitrobenzene

CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CK – Creatine kinase

CK2 – Casein kinase 2

CKB – Brain-specific cytosolic creatine kinase (or BB-CK)

CN – Caudate nucleus

CNS – Central nervous system

CoQ_{10} – Coenzyme Q_{10}

$CoQ_{10}H^{\bullet}$ – Semiquinone radical

CRE – Cyclic adenosine monophosphate (cAMP) response element

CREB – CRE binding protein

Cul1/3 – Cullin 1/3

DCF – Dichlorofluorescein

DEPC – Diethylpyrocarbonate

DHE – Dihydroethidium

DMEM – Dulbecco's Modified Eagle's Medium

DMF – Dimethyl fumarate

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DPP3 – Dipeptidyl-peptidase 3 protein

Drp1 – Dynamin related protein 1 (or dynamin 1-like (DMN1L))

DRPLA – Dentatorubral-pallidoluysian atrophy

DTT – DL-dithiothreitol

E^{+} – Ethidium

EAAC1 – Excitatory amino acid carrier 1

ECF – Enhanced ChemiFluorescence reagent

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol tetraacetic acid

Elk-1 – ELK1, member of ETS oncogene family

ENO1 – Non-neuronal enolase or enolase 1, (alpha)

ENO2 – Enolase 2 (gamma, neuronal)

Erk1/2 – Extracellular signal-regulated kinase 1/2

ETFQOR – Flavoprotein:quinone (Q) oxidoreductase

Exp1 – Exportin 1

F2-IsoPs – Prostaglandin F2-like compounds (F2-isoprostanes)

FAD – Flavin adenine dinucleotide

FBS – Fetal Bovine Serum

FCCP – Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

FDA – Food and Drug Administration

Fis1 – Mitochondrial fission 1

FMN – Flavin mononucleotide

FOXO – Forkhead boxO

FVB/N – Friend leukemia virus B strain

G6P – Glucose-6-phosphate

G6PD – Glucose-6-phosphate dehydrogenase

GAB1 – GRB2 associated binding protein-1

GABA – Gamma-aminobutyric acid

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GCL – Glutamate-cysteine ligase

GCLc – Glutamate-cysteine ligase catalytic subunit

GCLm – Glutamate-cysteine ligase modifier subunit

GFAP – Glial fibrillary acidic protein

GLUT – Glucose transporter(s)

GP – *Globus pallidus*

GPe – *Globus pallidus* external segments

GPi – *Globus pallidus* internal segments

GPx – Glutathione peroxidase

GRB2 – Growth factor receptor bound protein-2

GRed – Glutathione reductase

GS – Glutathione synthetase

GSH – Glutathione, reduced form

GSHee – Glutathione ethyl ester

GSK3-β – Glycogen synthase kinase 3 beta

GSSG – Glutathione, oxidized form

GST – Glutathione S-transferase

H_2DCFDA – 2',7'-Dichlorodihydrofluorescein diacetate

H_2O_2 – Hydrogen peroxide

HD – Huntington's disease

Hdh – Endogenous murine HD homologue gene

HE – Hydroethidium

HEAT – Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A and the lipid kinase TOR

HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HK – Hexokinase

$HO_2^{\bullet}$ – Hydroperoxyl radical

HO-1 – Heme oxygenase 1

HOBr – Hypobromous acid

HOCl – Hypochlorous acid

HPLC – High-performance liquid chromatography

HRP – Horseradish peroxidase

HSG – Huntington Study Group

Htt – Huntingtin protein

HTT – Huntingtin gene

IGF-1 – Insulin-like growth factor 1

IGF1R – IGF-1 receptor

IR – Insulin receptor

IRS – Insulin receptor substrate(s)

IT15 – Interesting transcript 15

JDP2 – c-Jun dimerization protein 2

JNK – c-Jun N-terminal kinase

K_2HPO_4 – Dipotassium phosphate

KCl – Potassium chloride

KCN – Potassium cyanide

Keap1 – Kelch-like ECH associated protein 1

KH_2PO_4 – Potassium dihydrogen phosphate

KPNA1 – Karyopherin alpha 1 (importin alpha 5)

KPNA6 – Importin alpha 7 (karyopherin alpha 6)

KPNB1 – Karyopherin (importin) beta 1

LA – Lipoic acid

LDH – Lactate dehydrogenase

Maf(s) – V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog(s)

MAP – Mitogen-activated protein

MAPK(s) – MAP kinase(s)

MCE – 2-mercaptoethanol

MDA – Malondialdehyde

MEK1/2 – MAP kinase kinases 1/2

MEKK – MAP kinase kinase kinase

MFF – Mitochondrial Fission Factor

Mfn1/2 – Mitofusin 1/2

$MgCl_2$ – Magnesium chloride

$MgSO_4$ – Magnesium sulfate

mHtt – Mutant huntingtin

Mid49 – Mitochondrial elongation factor 2

Mid51 – Mitochondrial elongation factor 1

MK-571 – 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid

MPT – Mitochondrial permeability transition

mRNA – Messenger ribonucleic acid

Mrp1 – Multidrug resistance-associated protein 1

mtDNA – Mitochondrial DNA

mTOR – Mammalian target of rapamycin

mTORC1/2 – mTOR complex 1/2

Na_2SO_4 – Sodium sulfate

Na_3VO_4 – Sodium orthovanadate

NAC – N-acetyl-L-cysteine

NaCl – Sodium chloride

NAD^+ – β-Nicotinamide adenine dinucleotide

NADH – β-Nicotinamide adenine dinucleotide, reduced

$NADP^+$ – β-Nicotinamide adenine dinucleotide 2′-phosphate

NADPH – β-Nicotinamide adenine dinucleotide 2′-phosphate, reduced

NaF – Sodium fluoride

NaH_2PO_4 – Monosodium phosphate

$NaHCO_3$ – Sodium bicarbonate

NaOH – Sodium hydroxide

NDA – 2,3-naphthalenedicarboxaldehyde

nDNA – Nuclear DNA

Neh – Nrf2-ECH homology domain

NEM – N-ethylmaleimide

NES – Nuclear export signal

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

NO_2^- – Nitrite anion

NOS – Nitric oxide synthase

NOX – NADPH oxidase

NPY – Neuropeptide Y

NQO1 – NAD(P)H:quinone oxidoreducase

NRF 1/2 – Nuclear respiratory factor 1/2

Nrf2 – Transcription factor nuclear factor (erythroid-derived 2)-like 2

$O_2^{\bullet-}$ – Superoxide anion

O_3 – Ozone

ONOO− – Peroxynitrite anion

ONOOH – Peroxynitrous acid

OPA – *Ortho*-phthaldialdehyde

Opa1 – Optic atrophy 1 (autosomal dominant)

OXPHOS – Mitochondrial respiratory chain oxidative phosphorylation

p21 – Cyclin-dependent kinase inhibitor 1A (Cip1, Waf1)

p38 – MAPK14

p62 – Selective autophagy substrate adaptor sequestosome1

p65 – A subunit of NF-κB transcription factor

PARP – Poly(ADP-ribose) polymerase

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PDH – Pyruvate dehydrogenase

PDPK1 – 3-phosphoinositide dependent protein kinase-1

PERK – Protein kinase RNA-like endoplasmic reticulum kinase

PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PH – Pleckstrin homology

PI-3K – Phosphatidylinositol 3-kinase

PIP2 – Phosphatidylinositol-4,5-bisphosphate

PIP3 – Phosphatidylinositol-3,4,5-trisphosphate

PKC – Protein kinase C

PMSF – Phenylmethanesulfonyl fluoride

PolyP – Polyproline(s)

PolyQ – Polyglutamine(s)

POZ – Pox virus and Zinc finger domain

Prdx – Peroxiredoxin

PTEN – Phosphatase and tensin homolog

PTMA – Prothymosin-alpha

PTPN11 – Protein tyrosine phosphatase, non-receptor type 11 (or SHP2)

PVDF – Polyvinylidene difluoride

Q – Glutamine(s)

QA – Quinolinic acid

R5P – Ribulose-5-phosphate

RAC3 – Receptor-associated coactivator 3

Raf-1 – V-Raf-1 murine leukemia viral oncogene homolog 1 (or c-Raf)

Ras – Rat sarcoma viral oncogene homolog (small GTPases (Ras superfamily))

Rbx1 – Ring box protein 1

RCS – Reactive chloride species

REST/NRSF – RE-1 silencing transcription factor/neuron-restrictive silencer factor

RFU – Relative fluorescence units

rhIGF-1 – Recombinant human IGF-1

RLU – Relative luminescence units

RNA – Ribonucleic acid

RNS – Reactive nitrogen species

$RO^{\bullet}$ – Alkoxyl radical

$RO_2^{\bullet}$ – Peroxyl radical

ROS – Reactive oxygen species

RPS6KB1 – Ribosomal protein S6 kinase, 70kDa, polypeptide 1

RSS – Reactive sulfur species

RT-PCR – Reverse-transcriptase polymerase chain reaction

SBMA – Spinal and bulbar muscular atrophy

SCA – Spinocerebellar ataxia

SDS – Sodium dodecyl sulphate

SDS-PAGE – SDS polyacrylamide gel electrophoresis

SH2 – Src Homology-2

SHC – Src Homology-2 (SH2)-containing protein

SIRT – Sirtuin

Skp1 – S-phase kinase-associated protein 1

SN – *Substantia nigra*

SNr – *Substantia nigra pars reticulata*

SOD – Superoxide dismutase

SOS – Son of sevenless

Sp1 – Specificity protein 1

SRXN1 – Sulfiredoxin-1

SS – Somatostatin

STS – Staurosporine

TAF4 – TAF4 RNA Polymerase II, TATA Box Binding Protein (TBP)-Associated Factor

***t*-BHP** – *tert*-Butyl hydroperoxide

***t*-BHQ** – *tert*-Butylhydroquinone

TBP – TATA box binding protein gene

TBP – TATA box binding protein

TCA – Tricarboxylic acid cycle

Tfam – Mitochondrial transcription factor A

TFB1M – Transcription Factor B1, Mitochondrial

TFB2M – Transcription Factor B2, Mitochondrial

TG2 – Transglutaminase 2

$TMRM^+$ – Tetramethylrhodamine methyl ester

TPP – Triphenylphosphonium

TPR – Translocated promoter region

Trx – Thioredoxin

TrxR – Thioredoxin reductase

ULK1 – Unc-51 like autophagy activating kinase 1

uMt-CK – Ubiquitous mitochondrial creatine kinase

USP15 – Ubiquitin specific peptidase 15

WST-1 – 2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt

WTX – APC membrane recruitment protein 1 (Wilms tumor on the X, AMER1)

Xc- – Cystine/glutamate antiporter

XO – Xanthine oxidase

YAC – Yeast artificial chromosome

"Every journey starts with a single step"

Confucius

Summary

Huntington's disease (HD) is a progressive neurodegenerative disorder characterized by motor and psychiatric disturbances and cognitive decline, largely affecting the striatum. HD is a polyglutamine expansion disorder caused by a CAG expansion in the *HTT* gene, leading to the expression of mutant huntingtin (mHtt). Themutant protein has been linked to several pathological mechanisms, including transcriptional deregulation, mitochondrial dysfunction and oxidative stress, which may result from impaired mitochondrial function and/or imbalanced levels of antioxidants. In this respect, several compounds used in HD clinical trials present antioxidant activity, including creatine and cystamine. Also, insulin-like growth factor 1 (IGF-1) was previously shown to protect HD cells, whereas insulin prevented neuronal oxidative stress. However, the role of IGF-1/Akt pathway in HD remains controversial.

The main objective of this thesis was to clarify the redox imbalance pathways following expression of full-length mHtt. We aimed to detail the changes in the antioxidant status, namely the glutathione redox system, and the efficacy of compounds with potential antioxidant activity used in clinical trials, as well as the role of insulin/IGF-1 pathway on oxidative stress in HD. Thus, we used striatal cells derived from HD knock-in mice expressing full-length mHtt with 111 glutamines (ST*Hdh*$^{Q111/Q111}$; mutant cells) *versus* wild-type striatal cells (ST*Hdh*$^{Q7/Q7}$).

In the first part of this work we detailed the changes in the glutathione antioxidant system by determining the activity and expression of proteins involved in the regulation of glutathione levels in HD striatal cells. Mutant cells showed increased reactive oxygen species (ROS) and caspase-3 activation. Interestingly, HD cells exhibited an increase in intracellular levels of both reduced and oxidized glutathione (GSH, GSSG), and enhanced activities of glutathione-related enzymes. Nevertheless, glutamate-cysteine ligase (GCL) and glutathione synthetase activities and levels of GCL catalytic subunit were decreased in mutant cells, suggesting decreased *de novo* synthesis of GSH. Enhanced intracellular total glutathione, despite decreased synthesis, could be explained by decreased glutathione export in mutant cells. Concordantly, we observed decreased mRNA expression levels and activity of the multidrug resistance-associated protein 1 (Mrp1). Data suggested that full-length mHtt affects the export of glutathione by decreasing the expression of Mrp1. Moreover, boosting GSH-related antioxidant defence mechanisms

induced by full-length mHtt was apparently insufficient to counterbalance increased ROS formation and emergent apoptotic features in HD striatal cells.

In the second part of the work we analysed the cellular antioxidant profile following hydrogen peroxide (H_2O_2) and staurosporine (STS) exposure, and further tested the protective effect of cystamine and creatine in striatal cells. Mutant cells displayed increased mitochondrial ROS production and decreased NADPH oxidase and xanthine oxidase (XO) activities, reflecting lower cytosolic superoxide anion generation, along with increased superoxide dismutases (SODs) and components of glutathione redox cycle. Exposure to H_2O_2 and STS enhanced ROS in mutant cells and largely increased XO activity. STS further boosted mitochondrial ROS and caspase-3 activity. Both stimuli slightly increased SOD1 activity, and decreased GRed, with a consequent rise in GSSG in mutant cells, whereas H_2O_2 only increased GPx activity. These results indicated that elevation of antioxidant levels accompanies mitochondrial-driven ROS generation in mutant striatal cells and that exposure to noxious stimuli induces a higher susceptibility to oxidative stress by increasing XO activity and lowering the antioxidant response. Additionally, creatine and cystamine increased mutant cells viability and prevented ROS formation in HD striatal cells subjected to H_2O_2 and STS. In the third part of this work we analysed the role of insulin/IGF-1 in HD striatal cells against ROS production and related antioxidant and signaling pathways. Insulin and IGF-1 decreased mitochondrial-driven ROS formation induced by mHtt, without changing SOD2 activity or glutathione levels. Insulin and IGF-1 promoted Akt and extracellular signal-regulated kinase (Erk) phosphorylation, respectively, and increased nuclear levels of phosphorylated nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which regulates the expression of detoxifying and antioxidant genes; however, this was not correlated with Nrf2 transcriptional activity or changes in mRNA levels of some Nrf2 target genes. Insulin and IGF-1 treatment also ameliorated mitochondrial function in HD cells. In the case of insulin, this occurred in a phosphatidylinositol 3-kinase (PI-3K)/Akt-dependent manner, concomitantly with reduced caspase-3 activation evoked by mHtt. Hence, insulin and IGF-1 improved mitochondrial function and reduced mitochondrial-driven ROS induced by mHtt, along with differential stimulation of Akt and Erk, in a process independent of Nrf2 transcriptional activity.

The present work defined the changes in antioxidant profile and cellular signaling following expression of full-length mHtt in mice striatal cells, demonstrating a higher susceptibility of these cells to stress and apoptotic cell death, thus revealing possible

targets for therapeutic intervention in HD. Importantly, treatment with cystamine and creatine rescued oxidative stress caused by stress stimuli, whereas activation of insulin or IGF-1 receptor-mediated kinase cascades reduced mitochondrial-driven oxidative stress through Nrf2-independent pathway in HD knock-in striatal cells, thus potentially delaying disease progression.

Keywords: Huntington's disease, mutant huntingtin, oxidative stress, glutathione redox system, insulin/IGF-1 signaling, reactive oxygen species, antioxidant response.

Resumo

A doença de Huntington (DH) é uma doença neurodegenerativa progressiva caracterizada por distúrbios motores e psíquicos e declínio cognitivo, afectando o estriado. A DH é umadoença de poliglutaminas causada por uma expansão de CAGs no gene *HTT*, conduzindo à expressão de huntingtina mutante (Httm). A proteína mutante tem sido associada a vários mecanismos patológicos, incluindo a desregulação da transcrição, disfunção mitocondrial e stresse oxidativo, que poderá resultar da desregulação da função mitocondrial e/ou do défice de antioxidantes. Neste contexto, o tratamento com compostos utilizados em ensaios clínicos da DH apresentaram actividade antioxidante. Por outro lado, o factor de crescimento semelhante à insulina tipo 1 (IGF-1) protegeu as células DH, enquanto a insulina preveniu o stresse oxidativo neuronal. Contudo, o papel da via IGF-1/Akt na DH permanence controverso.

O principal objectivo desta tese foi clarificar os mecanismos de (des)regulação redox após expressão da forma completa da Httm. Pretendemos avaliar as alterações de antioxidantes, nomeadamente o sistema redox da glutationa, assim como a eficácia de compostos usados em clínica (creatina e cistamina) com potencial actividade antioxidante, e o papel da insulina/IGF na regulação do stresse oxidativo na DH. Para tal, utilizámos células do estriado derivadas de murganhos knock-in para a DH, que expressam a Httm com 111 glutaminas (ST*Hdh*$^{Q111/Q111}$; células mutantes) *versus* células wild-type (ST*Hdh*$^{Q7/Q7}$).

Na primeira parte deste trabalho determinámos as alterações do sistema redox da glutationa através da análise da actividade e expressão de proteínas envolvidas na regulação dos níveis de glutationa em células estriatais DH. As células mutantes mostraram um aumento de espécies reactivas de oxigénio (ROS) e da actividade da caspase-3. Surpreendentemente, as células mutantes exibiram um aumento dos níveis intracelulares de glutationa reduzida e oxidada (GSH, GSSG), e um aumento das actividades de enzimas relacionadas com a glutationa. No entanto, as actividades da glutamato-cisteína ligase (GCL) e da glutationa sintetase e os níveis da subunidade catalítica da GCL diminuíram em células DH, sugerindo uma diminuição da síntese de novo da glutationa. Um aumento dos níveis totais intracelulares de glutationa, apesar da diminuição da síntese, foi explicado pela redução da exportação da glutationa em células mutantes. De facto, observou-se um decréscimo da expressão de mRNA e actividade da proteína-1 associada à resistência a múltiplas drogas (Mrp1). Estes resultados sugeriram

que a Httm afecta a exportação da glutationa através do decréscimo da expressão do Mrp1. Por outro lado, o aumento dos mecanismos de defesa antioxidante induzidos pela Httm não foi suficiente para contrabalançar a formação de ROS e as características apoptóticas em células estriatais de DH.

Na segunda parte do trabalho analisámos o perfil antioxidante celular após a exposição a péroxido de hidrogénio (H2O2) e estaurosporina (STS), e testámos o efeito protector da cistamina e creatina em células do estriado. As células mutantes mostraram um aumento da produção de ROS mitocondriais e uma diminuição das actividades da NADPH oxidase e da xantina oxidase (XO), reflectindo baixos níveis de anião superóxido citosólico, assim como um aumento da actividade das superóxido dismutases (SODs) e componentes do ciclo redox da glutationa. A exposição ao H2O2 e à STS aumentou os níveis de ROS em células mutantes e aumentou largamente a actividade da XO; a STS aumentou também a geração de ROS mitocondriais e a actividade da caspase-3. Ambos os estímulos aumentaram ligeiramente a actividade da SOD1 (Cu/Zn-SOD), e diminuíram a actividade da GRed, com um aumento consecutivo da GSSG em células mutantes, enquanto que o H2O2 apenas aumentou a actividade da GPx. Estes resultados indicaram que a geração de ROS mitocondriais é acompanhada pelo aumento de actividade do sistema antioxidante em células do estriado mutantes e que a exposição a estímulos nocivos induz um aumento da susceptibilidade ao stresse oxidativo através do aumento da actividade da XO e da diminuição da resposta antioxidante. Adicionalmente, a creatina e a cistamina aumentaram a viabilidade celular e preveniram a formação de ROS em células DH submetidas a H2O2 e STS.

Na terceira e última parte deste trabalho analisámos o papel da insulina/IGF-1 em células do estriado DH contra a produção de ROS e os mecanismos antioxidantes e de sinalização intracelular relacionados. A insulina e o IGF-1 diminuiram a formação de ROS derivados da mitocôndria induzidos pela Httm, sem alterarem a actividade da SOD2 ou os níveis de glutationa. A insulina e o IGF-1 promoveram a fosforilação do Akt e da cinase regulada por sinais extracelulares (Erk), respectivamente, e aumentaram os níveis nucleares do factor de transcrição nuclear Nrf2fosforilado, que regula a expressão de genes antioxidantes e de destoxificação celular; contudo, este resultado não foi relacionado com a actividade transcripcional do Nrf2 ou com alterações nos níveis de mRNA de genes alvo do Nrf2. O tratamento com insulina e IGF-1 também melhorou a função mitocondrial de células DH. No caso da insulina, este mecanismo ocorreu de forma dependente da via fosfoinositol 3-cinase (PI-3K)/Akt, em simultâneo com um

decréscimo da activação da caspase-3 induzida pela Httm. Assim, a insulina e o IGF-1 melhoraram a função mitocondrial e reduziram a formação de ROS mitocondriais induzidos pela Httm, e estimularam diferencialmente Akt e ErK, num processo independente da actividade transcripcional do Nrf2.

O trabalho apresentado nesta tese ajudou a clarificar a desregulação do perfil antioxidante e a sinalização celular que ocorre após a expressão da Httm em células estriatais. Para além disso, demonstrou-se uma maior susceptibilidade destas células ao stresse e à morte celular por apoptose, que poderá ser melhorada após o tratamento com citamina ou creatina, e após estimulação da cascata de cinases pela insulina ou IGF-1. Desta forma, este trabalho contribuiu para definir possíveis alvos de intervenção terapêutica que poderão reduzir a progressão da doença e melhorar os sintomas da DH.

Palavras-Chave: Doença de Huntington, huntingtina mutante, stresse oxidativo, sistema redox da glutationa, sinalização da insulina/IGF-1, espécies reactivas de oxigénio, resposta antioxidante.

Chapter 1 – Introduction

1.1 Huntington's disease

1.1.1 Huntington's disease: an overview and historical facts

> "*... in riding with my father on his professional rounds, I saw my first cases of 'that disorder.'... It made a most enduring impression upon my young mind ... an impression which was the very first impulse to my choosing chorea as my virgin contribution to medical lore. Driving with my father through a wooded road leading from Easthampton to Amagansett, we suddenly came upon two women, mother and daughter, both bowing, twisting, grimacing. I stared in wonderment, almost in fear. What could it mean? My father paused to speak with them, and we passed on. Then my Gamaliel-like instruction began; my medical education had its inception. From this point, my interest in the disease has never wholly ceased*." (Huntington, 1910).

> "*The hereditary chorea ... is confined to certain and fortunately a few families…hardly ever manifesting itself until adult or middle life... The tendency to insanity, and sometimes that form of insanity which leads to suicide, is marked... As the disease progresses the mind becomes more or less impaired... I have never known a recovery or even an amelioration of symptoms in this form of chorea; when once it begins it clings to the bitter end..."* (Huntington, 1872).

George Huntington (1850-1916) first encountered victims of hereditary chorea (a motor symptom characterized by abnormal and involuntary "dance-like" movementsand published in *Medical and Surgical Reporter of Philadelphia* (1872) at the age of 21 the main clinical aspects of the disease that had already been noted since the seventeen century by other physicians, including his father and grandfather. However, it was only

after the detailed and clear clinical descriptions given by George Huntington that the hereditary chorea was distinguished from other forms of chorea. After this description, other authors named the disease Huntington's chorea, and later it was identified as Huntington's disease (HD) because not all patients develop chorea, and due to the presence of cognitive and behavioral symptoms (Lanska, 2000).

In the decade from 1983 to 1993, HD was sequentially linked to a polymorphic DNA marker that maps to human chromosome 4 (Gusella et al., 1983), located in the p16 region at the short arm of chromosome 4 (Gusella et al., 1985; Landegent et al., 1986), and was included in the group of the nine inherited autosomal dominant (spinocerebellar ataxias (SCA) 1, 2, 3, 6, 7, 17, and dentatorubral-pallidoluysian atrophy (DRPLA)) or X-linked (spinal and bulbar muscular atrophy (SBMA)) polyglutamine (polyQ) neurodegenerative disorders, due to the discovery of an expanded trinucleotide CAG repeat (>36) in the exon 1 of the IT15 ("interesting transcript 15"), currently recognized as the huntingtin (*HTT*) or HD gene (Huntington's Disease Collaborative Research Group, 1993) (Table 1.1). These genetic disorders have in common the expansion of polyQ residues in different affected proteins. This modification alters the properties and activity of each one of the affected proteins and leads to a gain and/or a loss of function, as well as the accumulation of protein aggregates in the cytoplasm and/or in the nucleus. The number of CAG repeats is dynamic and can expand or contract from generation to generation; in the majority of polyQ diseases high CAG expansion result from paternal transmission (anticipation) (Pasternak, 2005), whereas most maternal transmissions show no change or a decrease in repeat number. In general, the severity of disease symptoms correlates with the number of CAG repeats in the affected protein, whereas the age of onset has an inverse correlation with the number of CAG repeats. Although the affected protein in the disease is ubiquitously expressed, specific brain regions are affected (Havel et al., 2009) as shown in Table 1.1.

Table 1.1 – Summary of the polyQ disorders genetic features, affected protein and brain regions.

PolyQ disorder	Chromosomal locus	Mutated gene	Expanded exon	Pathological CAG repeat number	Affected protein	Normal subcellular localization	Putative function	Affected brain regions
HD[1]	4p16.3	*HTT*	1	>36	Huntingtin	Cytoplasm	Anti-apoptotic, signaling, transport, transcription	Striatum and cortex
SBMA[2]	Xq11-12	*AR*	1	>35	Androgen receptor	Nuclear and cytoplasmic	Transcription	Motor neurons
SCA1[3]	6p22.3	*ATXN1*	8	>40	Ataxin-1	Nuclear and cytoplasmic	Transcription	Purkinje cells in the cerebellum; brainstem nuclei and cerebellar dentate nuclei

[1] Driver-Dunckley and Caviness, 2007; Sharon et al., 2010; Warby et al., 2011.
[2] Finsterer, 2009; La Spada, 1999; 2011; Rhodes et al., 2009; Rocchi and Pennuto, 2013.
[3] Matilla-Dueñas et al., 2008; Orr, 2012; Subramony and Ashizawa, 1998; 2011; Whaley et al., 2011.

SCA2[4]	12q24.1	*ATXN2*	1	>31	Ataxin-2	Cytoplasmic	RNA metabolism	Cerebellar Purkinje cells
SCA3[5]	14q32.12	*ATXN3*	10	>52	Ataxin-3	Nuclear and cytoplasmic	De-ubiquitylating activity	Cerebellum, brainstem, and substantia nigra
SCA6[6]	19p13.2	*CACNA1A*	47	20 to 33	Voltage-dependent P/Q-type calcium channel subunit alpha-1A	Membrane associated	P/Q-type alpha1A Ca^{2+} channel subunit	Cerebellar Purkinje cells

[4] Kasumu and Bezprozvanny, 2012; Laffita-Mesa et al., 2012; Lastres-Becker, 2008; Pulst, 1998; 2013.
[5] Bettencourt and Lima, 2011; Colomer Gould, 2012; Costa and Paulson, 2012; Paulson, 1998; 2012.
[6] Bunn et al., 2012; Craig et al., 2004; Fujioka et al., 2013; Gomez, 1998; 2013; Unno et al., 2012.

SCA7[7]	3p14.1	*ATXN7*	3	>36	Ataxin-7	Nuclear and cytoplasmic	Transcription	Cerebellar Purkinje cells, brainstem, spinal cord
SCA17[8]	6q27	*TBP*	3	> 45 CAG/CAA	TATA Box Binding Protein	Nuclear	Transcription	Cerebellar Purkinje cells, Cortex, Striatum
DRPLA[9]	12p13.31	*ATN1*	5	48 to 93	Atrophin-1	Nuclear and cytoplasmic	Transcription	Dentatorubral and pallidoluysian systems

[7] Garden, 1998; 2012; Garden and La Spada, 2008; Horton et al., 2013.
[8] Gao et al., 2008; Toyoshima et al., 2005; 2012.
[9] Tsuji, 1999; 2010; 2012.

1.1.2 Huntington's disease genetics and prevalence

HD is the most prevalent polyQ disorder with a worldwide prevalence of 5-10 cases per 100,000 persons (Table 1.2) but varies geographically as a result of ethnicity, local migration and past immigration patterns, and *HTT* gene haplotypes with no differences of prevalence between men and women. The identity of the genetic change responsible for HD was first described in individuals living in the Lake Maracaibo region of Venezuela, which is believed to have the highest prevalence of HD in the world where HD affects up to 700 per 100,000 persons (Table 1.2). In fact, the rate of occurrence is highest in individuals of Western European descent, and is less frequently in Japan, China, and Finland, and among African descents. A epidemiological study of the prevalence of HD in the UK between 1990 and 2010 found that the average prevalence for the UK was 12.3 per 100,000, whereas the frequency of HD in Japan has been estimated between 0.1 and 0.38 per 100,000 (Evans et al., 2013; Pringsheim et al., 2012; Warby et al., 2011) (Table 1.2).

HD is inherited in a dominant fashion and a single HD-causing allele is sufficient to cause the disease. Unaffected alleles have CAG repeat size ranging from 10 to 35. With a median size of 18, the most common alleles in all populations contain repeats of 15-20 CAGs (Warby et al., 2009). HD-causing alleles are classified as: 1) Reduced-penetrance HD-causing alleles with 36-39 CAG repeats. An individual with an allele in this range is at risk for HD but may not develop symptoms (Langbehn et al., 2004). 2) Full-penetrance HD-causing alleles with 40 or more CAG repeats. Alleles of this size are associated with development of HD with great certainty (Warby et al., 1998; 2010). Individuals with adult-onset HD usually have a CAG expansion from 40 to 55. In that range, 50-70% of age of symptom onset appears to be explained by the length of the polyQ stretch, whereas the remainder is determined by other modifying genes and environmental factors (Wexler et al. 2004). For longer polyQ stretches, a greater proportion of age of symptom onset is explained by the length of the polyQ stretch. Like other polyQ disorders, there is an inverse correlation between the length of CAG repeats and the age of onset. Thus, the longer the CAG stretch, the earlier symptoms typically appear. HD juvenile onset cases have CAG expansions greater than 60 that are often inherited from the father (paternal transmission) (Warby et al., 2011).

1.1.3 Huntington's disease symptoms

HD symptoms onset usually occurs between 30-50 years of age (Table 1.2). Although each person with HD is clinically unique, chorea is the most prevalent and manifested symptom in HD. Chorea is derived from the Greek word meaning "dance", and is characterized by abnormal and involuntary rapid "dance-like" movements of hands or feet with an extension to other parts of the body as the disease progresses. Generally, involuntary movements of face, fingers, feet or thorax and saccades, or abnormal fast movements of the eyes, are an early manifestation of the disease (Berardelli et al., 1999; Lasker et al., 1987; Penney et al., 1990). The treatment for chorea normally requires dopamine-depleting drugs, such as tetrabenazine, the only drug so far approved by the Food and Drug Administration (FDA) for the treatment of HD. Other antichoreic agents include haloperidol, a dopamine antagonist and antipsychotic drug (Killoran and Biglan, 2012). Slowness of movements (bradykinesia), rigidity and the involuntary contraction of muscles (dystonia) progressively appear and normally dictate the final stage of the disease, in which the patient will become severely rigid, grossly akinetic and dysphagic. Also, ataxia and abnormal postural are common in advance stages of HD (Berardelli et al., 1999; Penney et al., 1990; Louis et al., 2000); abnormal facial expression and difficulties in chewing, swallowing and speaking (Walker, 2007), weight loss and malnutrition (in advanced stages of HD) (Aziz et al., 2008) and sleep disturbances (Gagnon et al., 2008) are also frequent symptoms.

Emotional disturbances, including depression, dysphoria, agitation, irritability, anxiety, apathy, and delusional behavior are common in HD patients (Caine and Shoulson, 1983; Paulsen et al., 2001), which progressively aggravate with the course of disease. Psychosis and paranoia are also manifested in HD patients (Josiassen et al., 1983). Some behavioral symptoms may occur before the diagnosis, which can include irritability, depression, and obsessive-compulsive behaviour (Duff et al., 2007; Kirkwood et al., 2002). Between 30-50% of HD patients develop depressive symptoms in the course of disease and can lead to personality changes in early stages. Moreover, the incidence of suicide is high in HD patients (Sorensen and Fenger, 1992).

Early manifestations of the disease includes deficits in cognition, which progressively worse and are related with progressive caudate nucleus (CN) atrophy (Aylward, 2000; 2007; Montoya et al., 2006). In the early stages, HD patients often experience impaired problem-solving abilities, visuospatial skills difficulties, attention

deficit, difficulties in planning, and cognitive inflexibility, which can lead to a decline in performance at work. Although memory in general is relatively preserved, deficits in episodic, procedural and working memory can manifest in the course of disease, leading to subcortical dementia syndrome (Ho et al., 2003; Montoya et al., 2006; Paulsen et al., 1995; Rohrer et al., 1999).

The juvenile form of HD is a distinct clinical variant of HD, also called the "Westphal variant" of HD and accounts for 5-10% of HD cases. As described earlier, it affects individuals before age 20 and occurs when the length of CAG repeats is greater than 60. The disease progression is faster and the symptoms are more severe in juvenile cases. Bradykinesia without chorea, rigidity and spasticity, dystonia, epileptic seizures and deficits in cognition are the most manifested symptoms (Telenius et al., 1993; Warby et al., 1998; 2010).

The death of the HD patients occurs 15-20 years after the symptoms onset and may result from complications such as heart failure, physical injuries provoked by falls, inanition, dysphagia, or aspiration pneumonia (Walker, 2007).

Table 1.2 – Summary of the polyQ disorders prevalence and clinical features.

PolyQ disorder	Estimated worldwide mean prevalence	Highest prevalence	Lowest prevalence	Mean age of symptoms onset (range in years)	Average duration (range in years)	Major Clinical Features
HD[10]	5-7.5 per 100,000	700 per 100,000 (Maracaibo region of Venezuela)	0.1-0.38 per 100,000 (Japan)	30-50	15-20	Motor, cognitive decline, psychiatric triad
SBMA[11]	1-2 per 100,000	15 per 100,000 (Vaasa region in Western Finland)	-	20-50	Normal life expectancy but wheelchair dependent 20-30 years after onset	Slowly progressive weakness, atrophy, and fasciculations of bulbar, facial and limb muscles associated with mild androgen insensitivity
SCA1[12]	1-2 per 100,000	-	-	30-50	10-28	Cerebellar ataxia, dysarthria, and eventual deterioration of bulbar functions

[10] Driver-Dunckley and Caviness, 2007; Sharon et al., 2010; Warby et al., 2011.
[11] Finsterer, 2009; La Spada, 1999; 2011; Rhodes et al., 2009; Rocchi and Pennuto, 2013.
[12] Matilla-Dueñas et al., 2008; Orr, 2012; Subramony and Ashizawa, 2011; Whaley et al., 2011.

SCA2[13]	1-2 per 100,000	43 per 100,000 (Holguin region in Cuba)	-	30-50	10-15	Progressive cerebellar syndrome characterized by ataxic gait, cerebellar dysarthria, dysmetria and dysdiadochokinesia.
SCA3[14]	1-2 per 100,000	1 per 239 (Flores island, Azores)	-	20-50	7-29	Ataxia, brainstem dysfunction, dystonia, parkinsonism
SCA6[15]	>1 per 100,000	5.21 per 100,000 (UK)	-	43-52	>25	Slowly progressive cerebellar ataxia, dysarthria, and nystagmus

[13] Kasumu and Bezprozvanny, 2012; Laffita-Mesa et al., 2012; Lastres-Becker, 2008; Pulst, 1998; 2013.
[14] Bettencourt and Lima, 2011; Colomer Gould, 2012; Costa and Paulson, 2012; Paulson, 1998; 2012.
[15] Bunn et al., 2012; Craig et al., 2004; Fujioka et al., 2013; Gomez, 1998; 2013; Unno et al., 2012.

SCA7[16]	>1 per 100,000	-	-	3rd-4th decade (0.5-60)	20 (1-45)	Progressive cerebellar ataxia, including dysarthria and dysphagia, and cone-rod and retinal dystrophy with progressive central visual loss resulting in blindness in affected adults
SCA17[17]	>100 families	0.16 per 100,000 (North-East England)	0.47 per 1,000,000 (Japan)	34.6 (3-75)	19 (10-28)	Ataxia, chorea, brainstem dysfunction, dystonia, dementia
DRPLA[18]	>1 per 100,000	0.48 per 100,000 (Japan)	-	28.8 (1-60)	13	Ataxia, chorea, seizures, dementia, myoclonus

[16] Garden, 1998; 2012; Garden and La Spada, 2008; Horton et al., 2013.
[17] Gao et al., 2008; Toyoshima et al., 2005; 2012.
[18] Tsuji, 1999; 2010; 2012.

1.1.4 Huntington's disease protein and neuropathological features

Althought HD pathology has been observed in peripheral tissues (Björkqvist et al., 2008, van der Burg et al., 2009), like other polyQ disorders, HD is predominantly a central nervous system (CNS) disorder, characterized by cell loss and atrophy (Sathasivam et al., 1999), classified in five grades (0–4) designated in ascending order of severity (Vonsattel et al., 1985).

Within the brain, atrophy of the striatum (the largest component of the basal ganglia system) is the most prominent (Vonsattel and DiFiglia, 1998), which primarily include a selective degeneration of medium spiny projection neurons (which represent about 96% of striatal neurons, and receive synaptic input primarily from the cortex) in the CN and putamen, while aspiny interneurons (which represent around 2% of the striatal neurons) of the striatum are generally spared (Cowan and Raymond, 2006; Ferrante et al., 1985; 1987a,b). Counts of neurons in the CN revealed that 50% are lost in grade 1 and that 95% are lost in grade 4 (Vonsattel et al., 1985). However, progressive degeneration within medium spiny projection neurons in the CN and putamen appears to be different. Early in the course of HD, striato-*globus pallidus* (GP) internal segments (GPi) medium spiny projection neurons (which express substance P/gamma-aminobutyric acid (GABA) and projects to and inhibit GPi resulting in thalamus desinhibition and consequent excitation of upper motor neurons in the motor areas of the cortex, which increase movement (direct pathway)) are relatively spared, whereas striato-GP external segments (GPe) (which express enkephalins and projects to and inhibit GPe resulting in subthalamic nucleus inhibition and consequent less inhibition of *substantia nigra* (SN) *pars reticulata* (SNr) and GPi which inhibits thalamus resulting in the inhibition of upper motor neurons; thus decreasing movement (indirect pathway)) and striato-SNr (which express substance P/GABA and projects to and inhibit SNr (direct pathway)) medium spiny projection neurons degenerate (Reiner et al., 1988; Richfield et al., 1995a,b); loss of striato-GPe neurons was also demonstrated in presymptomatic HD (Albin et al., 1992). The early and relatively selective loss of striato-GPe and striato-SNr neurons was suggested to be a plausible explanation for the chorea and oculomotor abnormalities that are prominent clinical features of early HD (Albin, 1995). However, in later stages of adult HD, both populations of striatal projection neurons are affected, with concomitant loss of markers of the direct pathway (substance P/GABA-containing neurons), including dopamine D1 receptors and substance P (Reiner et al., 1988; Richfield et al., 1991). Also,

in juvenile HD cases, degeneration of both direct and indirect pathway striatal neurons was observed (Albin et al., 1990). Thus, the degeneration of both the direct and indirect pathways was suggested to be functional correlated with bradykinetic rigid phenotype observed in late stage and juvenile HD (Albin et al., 1990). Also, within the spared aspiny interneurons in HD there appears to be a differential level of vulnerability. Aspiny interneurons projection arbors are restricted to the striatum, and comprises: a) large cholinergic aspiny interneurons; b) medium somatostatin (SS)/neuropeptide Y (NPY)/diaphorase or nitric oxide synthase (NOS)-positive aspiny interneurons; and c) medium GABAergic-containing/parvalbumin-positive aspiny interneurons). Striatal SS/NPY/NOS neurons are relatively spared in HD (Ferrante et al., 1985; Dawbarn et al., 1985). Although markers of striatal cholinergic function decline in HD, striatal cholinergic aspiny interneurons are preserved, indicating functional impairment but not degeneration of striatal cholinergic aspiny interneurons (Hirsch et al., 1989). Moreover, it was suggested that striatal GABA/parvalbumin-containing interneurons are preserved early in the course of HD, but degenerate with disease progression (Albin, 1995; Harrington and Kowall, 1991).

On the other hand, non-striatal brain regions can also be affected, particularly in latter stages of the disease, and can include the SN, hippocampus, and mostly various regions of the cortex (Van Raamsdonk et al., 2005), whereas pyramidal neurons of deeper cortical layers appear to be more affected in HD, which can be correlated with dementia and personality changes in HD patients (Vonsattel, 2008).

The reason why there is selective brain degeneration in HD is not known. However, it might be due to the role and importance of the protein that is affected in HD might have in the brain. The *HTT* gene encodes a polymorphic stretch of glutamines (Q) within the N-terminal of a high molecular weight protein, with approximately 3144 amino acids (~348 kDa), known as huntingtin (Htt) (Hoogeveen et al., 1993; Huntington's Disease Collaborative Research Group, 1993) (Figure 1.1). Htt polyQ tract begins at the 18 amino acid and is followed by a proline rich sequence (polyP) composed by 38 amino acids, which is thought to be important in Htt solubility (Steffan et al., 2004) (Figure 1.1). The first 17 amino acids of Htt are important for nuclear shuttling since they interact with TPR (translocated promoter region), a protein of the nuclear pore that actively exports proteins from the nucleus. When the first 17 amino acids were eliminated, Htt nuclear accumulation was observed (Cornett et al., 2005). Htt also contains multiple regions of so-called HEAT (**H**untingtin, **E**longation factor 3, a subunit of protein phosphatase 2**A**

and the lipid kinase **T**OR) repeats, a sequence of ~40 amino acids named after the first four proteins in which it was discovered (Andrade and Bork, 1995; Neuwald and Hirano, 2000). Although the exact function of HEAT repeats are currently unclear, studies have suggested that these domains play a role in a variety of interactions between proteins, including transportation in the cytoplasm and nucleus, microtubule dynamics and chromosome segregation (Neuwald and Hirano, 2000).

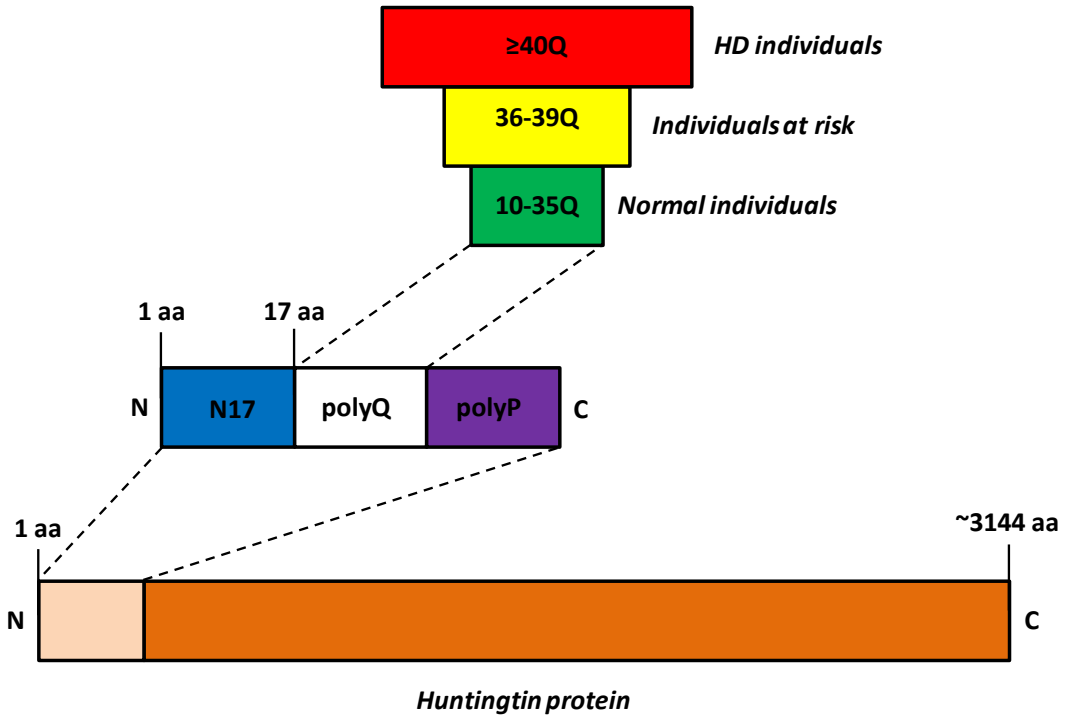


Figure 1.1 – Huntingtin protein structure. The CAG expansion which occurs in the exon-1 of *HTT* gene is translated into an expansion of glutamines (Q) in a region of the N-terminal Htt protein, which is composed by ~3144 aminoacids (aa). The polyQ region is preceded by 17 aminoacids (N17) and followed by a polyproline (polyP) rich region. The polyQ region comprised by 10 to 35Q is present in normal individuals. Between 36 to 39Q, individuals may not develop symptoms but are at risk to develop HD. Above 39Q, individuals develop HD symptoms, which are more severe with increased Q number.

Htt mRNA and protein are ubiquitously expressed throughout the development and in the adult, in a variety of cells and peripheral tissues, and homogenously throughout the brain where it has its higher expression, with a predominance of neuronal over glial expression (Landwehrmeyer et al., 1995; Li et al., 1993; Sharp et al., 1995; Strong et al., 1993). In the brain, Htt mRNA was detected in both grey and white matter (lowest expression levels), and the highest expression levels of Htt were found in the cortex (with differential expression between cortical layers), hippocampus, SN and cerebellum,

followed by the striatum (Landwehrmeyer et al., 1995; Li et al., 1993; Strong et al., 1993), with no differences in the distribution and levels of Htt mRNA between symptomatic HD patients and control individuals, except in the striatum where the intensity of labeling was significantly reduced (Landwehrmeyer et al., 1995). However, presymptomatic HD brains revealed a striatal expression similar to controls and surviving striatal neurons in more advanced HD had an expression of Htt mRNA within normal limits. Thus, HD brain selective degeneration does not seem to result from altered Htt mRNA expression (Landwehrmeyer et al., 1995).

Wild-type Htt protein within neurons can be found in the cytoplasm, neurites and synapses, and associates with various organelles and structures, such as microtubules, plasma membrane, endosomal and endoplasmic compartments, clathrin-coated vesicles and mitochondria (DiFiglia et al., 1995; Gutekunst et al., 1995; Kegel et al., 2005; Trottier et al., 1995). Although mostly located in the cytoplasm, Htt is also detected in the nucleus (Hoogeveen et al., 1993; Kegel et al., 2002). Due to its subcellular localization Htt interacts with numerous proteins involved in gene expression, intracellular transport, signaling and metabolism (Borrell-Pages et al., 2006a,b; Harjes and Wanker, 2003; Li and Li, 2004a,b). However, the normal function of Htt is not completely known. On the other hand, it was shown that wild-type Htt is involved in embryonic development, since homozygous *HTT* locus knockout mice are lethally affected at early embryonic development stages (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). On the other hand, the presence of one fully functional allele (at least 50% Htt expression) is compatible with life in humans and HD is not caused by a simple loss of function of the *HTT* gene (Cattaneo et al., 2005). In fact, patients with Wolf-Hirshhorn syndrome, having a partial deletion of chromosome 4 that comprises the CAG triplet repeats region and therefore have only one copy of the *HTT* gene, are born and do not develop HD (Gusella et al., 1985). Also, conditional inactivation of wild-type Htt, to avoid early embryonic lethality of homozygous knockout mice, in which Htt levels were reduced to below 50% of normal presented defects in the epiblast (the structure that will give rise to the neural tube), which led to reduced neurogenesis and profound malformations of the cortex and striatum, thus indicating that Htt plays a role in neurogenesis (Dragatsis et al., 2000; White et al., 1997). Other evidences suggest that wild-type Htt has an anti-apoptotic function in a gene and protein-dependent manner (Leavitt et al., 2006). Wild-type Htt acts downstream mitochondrial cytochrome c release (a protein at the mitochondrial intermembrane space that when released from mitochondria can bind to caspases to

activate the cell death process), preventing the activation of both caspase-9 (Rigamonti et al., 2001) and -3 (Rigamonti et al., 2000). Moreover, wild-type Htt physically interacts with active caspase-3 and inhibits its activity (Zhang et al., 2006). Furthermore, Htt appears to act as an activator or enhancer of gene transcription. Particularly, Htt was shown to regulate the gene transcription of brain-derived neurotrophic factor protein (BDNF), a neurotrophin essential for striatal neuronal survival and for the activity of cortico-striatal synapses, by binding and trapping in the cytoplasm (inhibit) the BDNF transcriptional repressor REST/NRSF (RE-1 silencing transcription factor/neuron-restrictive silencer factor) (Gauthier et al., 2004; Zuccato et al., 2001; 2003; 2007).

Expanded polyQ Htt (mutant Htt; mHtt) appears to possess at least certain properties of wild-type Htt. In fact, mHtt can rescue the embryonic lethal phenotype seen in Htt-null knockout mice (Hodgson et al., 1996; Dragatsis et al., 1998). However, it is still not clear if neuronal cytotoxicity in HD is due to a loss or a novel gain of Htt function or both. The proteolytic cleavage of mHtt into N-terminal fragments containing the polyQ stretch and their subsequent translocation to the nucleus, and formation of intranuclear aggregates (DiFiglia et al., 1997; 2002; Goldberg et al., 1996), is a hallmark of the disease detected in *post-mortem* HD human brains. The aggregates can be found before the onset of the first symptoms (DiFiglia et al., 1997; Weiss et al., 2008), and the rate of aggregate formation was shown to correlate with the length of the polyQ repeat (Legleiter et al., 2010). However, the toxicity of mHtt nuclear inclusions remains controversial, since their formation is correlated with disease progression, but is not linked with neuronal degeneration. In fact, some studies have shown that mHtt inclusions are protective against mHtt-induced toxicity in cultured cells (Arrasate et al., 2004; Davies et al., 1997; Saudou et al., 1998). Exposure of mHtt-transfected striatal neurons to conditions in which nuclear localization of mHtt was blocked and consequently, suppressing its ability to form intranuclear inclusions, resulted in increased cell death, suggesting that the formation of intranuclear inclusions might reflect a cellular mechanism to protect against mHtt-induced cell death (Saudou et al., 1998). On the other hand, the decrease of proteolytic cleavage of mHtt reduced its toxicity and slowed disease progression (Gafni et al., 2004; Wellington and Hayden, 2000). In addition, expression of smaller N-terminal mHtt fragments resulted in increased toxicity in cultured cells (Hackam et al., 1998) and transgenic animals (Davies et al., 1997; Schilling et al., 1999; Yu et al., 2003), when compared to the full-length mHtt expression with the same polyQ

expansion. This result suggested that the susceptibility to neuronal death is greater with decreased protein length and increased polyQ size.

Therefore, several HD models have been generated to mimic the neuropathological features that occur in Humans. These models express only a fragment or a full-length mHtt to study the cellular and molecular mechanisms of mHtt-induce neurodegeneration.

1.1.4.1 **Huntington's disease models**

1.1.4.1.1 Knock-in models

Knock-in HD models were generated by targeting an expanded polyQ repeat and/or adjacent human mHtt exon 1 sequences (including the polyP region) to replace the corresponding sequences in the endogenous murine HD homologue gene (*Hdh*), thus expressing mHtt from the endogenous *Hdh* locus in a similar manner to the expression in HD patients. Therefore, *Hdh* knock-in mice are commonly considered as one of the most precise genetic HD mouse models (Gusella and Macdonald, 2006). When compared to N-terminal mHtt fragments expressing models, *Hdh* knock-in mice display slow progression and moderately mild phenotypes, and their lifespan is usually normal. These mouse models are important to evaluate early pathological processes induced by mHtt in humans. Originally, knock-in models were disappointing because the first mice generated with an extended stretch of 50 or 80 CAG repeats into the endogenous mouse *Hdh* gene (HdhQ50; HdhQ80) presented no behavioural phenotypes or abnormalities, or mHtt aggregates (Shelbourne et al., 1999; White et al., 1997). Therefore, other knock-in models that represented better the human pathology were developed.

1.1.4.1.1.1 HdhQ111

The HdhQ111 model is a knock-in mouse model of HD with an insertion of a chimeric murine *Hdh*/human mHtt exon 1 into the endogenous *Hdh* locus; the human mHtt portion includes 111 CAG repeats and a polyP region (Wheeler et al., 1999). The behavioral phenotypes of HdhQ111 are very mild and slowly progressive. Rotarod, clasping or open field abnormalities were not detected in heterozygous or homozygous HdhQ111 mice until 17 months of age, and gait abnormalities were detected only at 24 months of age (Wheeler et al., 2002). HdhQ111 homozygous mice show selective and

progressive accumulation of nuclear mHtt at 2.5 months of age (Wheeler et al., 2000), nuclear inclusion formation at 10 months of age, and reactive gliosis in the striatum at 24 months of age (Wheeler et al., 2000, 2002).

1.1.4.1.1.2 CAG140

The CAG140 HD knock-in mice model was developed with a replacement of the endogenous murine *Hdh* exon 1 with a chimeric mouse and human exon 1 with 140 CAG repeats. CAG140 HD mice display early hyperactivity at 1 month of age, followed by hypoactivity at 4 months of age, gait abnormality at 12 months of age, with nuclear mHtt microaggregates in the striatum and cortex, and nuclear inclusions in the striatum at 4 months of age and in the cortex at 6 months. However, nuclear microaggregates were also observed at 6 months of age in the cerebellum, a relatively spared region in human HD brain, but presented no cell loss or brain atrophy (Menalled et al., 2003).

1.1.4.1.1.3 Hdh(CAG)150

Hdh(CAG)150 HD knock-in mice was developed by a replacement of the short CAG repeat in the murine *Hdh* exon 1 with a stretch of 150 CAG repeats (Lin et al., 2001). Homozygous Hdh(CAG)150 mice revealed several slowly progressive motor abnormalities (Heng et al., 2007; Woodman et al., 2007). Homozygous HdhCAG(150) mice display progressive rotarod deficits at 18 months of age and mHtt aggregates in the striatum and hippocampus at 6 months and widespread in the brain at 10 months of age (Tallaksen-Greene et al., 2005). Striatal neuronal loss is observed at 100 week-old (Heng et al., 2007).

1.1.4.1.2 Transgenic models

In transgenic mouse models, full-length or a fragment of the mutant *HTT* gene is inserted randomly into the mouse genome, leading to the expression of a mutant protein in addition to the endogenous Htt. Thus, transgenic mouse models of HD express N-terminal mHtt fragments or full-length human *HTT* gene with an expanded polyQ tract.

1.1.4.1.2.1 N-terminal huntingtin transgenic models

The first transgenic mice models of HD include the insertion of a fragment of the human *HTT* gene. R6/2 HD mice contain a mutant N-terminal segment of the exon 1 of

the human *HTT* gene with 144 CAG repeats (Mangiarini et al., 1996). These mice display choreiform-like movements, inclusion formation at 4-5 weeks, followed by an early death around 12-14 weeks, but with minimal neuronal death when compared to human HD patients (Hersch and Ferrante, 2004; Li et al., 2005; Mangiarini et al., 1996). Another transgenic model developed is the R6/1 HD mice that express a truncated *HTT* gene with around 115 CAG repeats, and exhibit a more progressive pathology and lower expression of the mutant transgene, a marked decline in rotarod performance develops at 13-20 weeks, correlating with the numbers of striatal neurons exhibiting intranuclear inclusions of mHtt, and with death occurring within 4-5 months of age (Davies et al., 1997; Hansson et al., 2001a,b). The N171-82Q mouse model of HD contains a longer N-terminal fragment of mHtt (exon 1 and exon 2) with 82 CAG repeats. The lifespan of the N171-82Q HD is ~17-20 weeks with HD-like symptoms beginning at 10-12 weeks of age. In these mice, neuropathological features are more similar to human HD, so that neurodegeneration is more prominent and seems more selective for the striatum (Duan et al., 2003; Hersch and Ferrante, 2004; Schilling et al., 1999).

1.1.4.1.2.2 Full-lenght huntingtin transgenic models

Two genomic transgenic models expressing full-length mHtt from the human genomic locus on a yeast artificial chromosome (YAC) (Hodgson et al., 1999; Slow et al., 2003; Van Raamsdonk et al., 2007a,b) or on a bacterial artificial chromosome (BAC) (Gray et al., 2008) were generated. A series of YAC transgenic models of HD expressing full-length human mHtt with 18, 46, 72, and 128 CAG repeats (i.e., YAC18, YAC46, YAC72, and YAC128) were generated after microinjection of YAC DNA construct into the friend leukemia virus B strain (FVB/N) pronuclei and maintained on the inbred mouse FVB/N background strain; in contrast to the C57BL/6 background strain common to most HD mouse models, the FVB/N strain shows higher neuronal loss when exposed to excitotoxic stress after injection of kainic acid or quinolinic acid (QA) (Hodgson et al., 1999; Slow et al., 2003; Van Raamsdonk et al., 2007a,b). YAC128 mice are the latest of the series and exhibit by far the most robust phenotypes among all the YAC models; therefore, YAC128 is used as a preclinical model in HD (Slow et al., 2003). At the protein level, the YAC128 line expresses human mHtt at about 75% of the level of the endogenous murine *Hdh* (Slow et al., 2003). YAC128 mice exhibit hyperactivity at 2 months of age and hypoactivity at 8–12 months of age. They also exhibit rotarod deficits at 4 months of age, which become more prominent at 6 months of age (Graham et al.,

2006; Van Raamsdonk et al., 2005a,b). mHtt nuclear localization is detected in the striatum at 1-2 months and in the cortex and hippocampus at 3 months of age. Nuclear inclusions are detected only at 18 months in the striatum. As in humans, selective atrophy in the striatum and cortex, but not in cerebellum is detected (Van Raamsdonk et al., 2005a,b). BACHD is a more recent generated transgenic mouse model of HD maintained in the FVB/N background strain. BACHD mice exhibit mild rotarod deficits at 2 months, with progressive deficits and hypoactivity at 6 months of age. At 12 months of age, cortical and striatal atrophy is detected, but no early nuclear mHtt localization is detected (Gray et al., 2008; Menalled et al., 2009).

1.1.4.1.3 Toxic lesion models

Taking into account the reduced activity of mitochondrial respiratory complex II (succinate dehydrogenase) in the CN and putamen of symptomatic HD patients, and the report that accidental ingestion of the mitochondrial toxin 3-nitropropionic acid (3-NP, an irreversible complex II inhibitor) produced preferential degeneration of the putamen and CN associated with severe neurological symptoms in humans, pharmacologic inhibitors of mitochondrial complex II (3-NP and malonate, the latter a reversible inhibitor) were tested in a tentative to reproduce HD features. They have been found to induce striatal damage and motor phenotypes in animals, closely resembling the symptoms seen in HD patients (Brouillet et al., 2005; Kumar et al., 2010). The main advantage of 3-NP is that HD symptoms develop spontaneously after its systemic administration (Reynolds et al., 1998). This model has been extended to non-human primates in which chronic systemic administration of 3-NP mimics behavioural, histological and neurochemical features of HD (Brouillet et al., 1995; Brouillet and Hantraye, 1995). Malonate was shown to cause motor impairments and neuronal pathology resembling HD after intrastriatal administration (as malonate does not cross the blood-brain barrier) in rodents. Similar to 3-NP, malonate produces age-dependent striatal lesions that can be attenuated by *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonists. Further indirect evidence contributes to malonate-induced neurodegeneration (Kumar et al., 2010).

There is increased evidence of excitotoxicity in HD, a pathological process that occurs due to excessive stimulation of ionotropic glutamate receptors such as the NMDA, in particular, and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR) by the excitatory neurotransmitter glutamate leading to neuronal

damaged and death (Dong et al., 2009). Indeed, the excitotoxin QA-induced striatal lesions closely resembling those of HD, as they result in marked depletion of both GABA and substance P, selectively sparing SS/NPY neurons (Beal et al., 1986). Thus, QA was proposed as a model to replicate the neurochemical characteristics of HD. QA is an extensively studied endogenous metabolite of the tryptophan degradation pathway, also known as the kynurenine pathway. QA is a NMDA receptor agonist, preferentially acting on discrete populations of these receptors containing GluN2A and GluN2B subunits. QA exerts excessive excitation of NMDA receptors and recruits enhanced cytoplasmic Ca^{2+} concentrations, mitochondrial dysfunction, decreased ATP levels, cytochrome c release, selective loss of GABAergic and cholinergic neurons, and oxidative stress. The intrastriatal injection of QA reproduces striatal lesions observed in HD by targeting a subset of medium spiny neurons - the GABAergic and substance P medium spiny neurons. The QA model has been successfully tested in primates, exhibiting similar neuropathological lesions (Beal et al., 1986; Ferrante et al., 1993; Lugo-Huitrón et al., 2013; Pérez-De La Cruz et al., 2012; Schwarcz and Kohler, 1983).

1.1.4.1.4 Invertebrate models

In a tentative to overcome the limitations of human genetic studies, models ranging from yeast, the fruit fly *Drosophila melanogaster*, and the nematode *Caenorhabditis elegans* to mammals and human cell culture systems, have been used to study specific functional aspects of the genes/proteins identified in neurodegenerative diseases.

Drug development is an expensive and time-consuming process which has to be proofed by clinical trials. In this respect, genetic studies performed during the past decade in invertebrate models such as Drosophila and C. elegans provided new strategies and rationales for the development of neuroprotective drugs. The “low-cost” and the rapid drug screening that Drosophila and C. elegans provide, have allowed the pre-selection of candidate drugs for neuroprotection, and enabled the identification and study of key modulators of conserved biological processes, in advance of their discovery and study in more complex mammalian. Also, they are good model systems to study *in vivo* how postmitotic cells may respond to the stress produced by disease proteins during developmental and adult life, and represent an attractive intermediate by combining sufficient complexity to allow investigation of both cellular and behavioral phenotypes

with simplicity that facilitates rapid, high-throughput testing of hypotheses. In fact, under normal conditions, C. elegans development to adult stage is completed in approximately 3 days with a lifespan of approximately 2 weeks, thus allowing experiments to be designed and carried out quite rapidly compared with other animal models. Also, Drosophila has a short life span ranging from 40 to 120 days depending on diet and stress, and it shows complex behaviour, including learning and memory, driven by a sophisticated brain and nervous system. At least 50% of fly genes display homology to human genes, and among human diseased genes ~75% have a Drosophila ortholog, including *HTT*. However, despite a high level of genetic conservation for numerous essential pathways involved in development and/or cell maintenance, nematodes and flies are physiologically distantly related to humans. Notwithstanding the limitations, expression of polyQ-containing proteins was shown to be neurotoxic in both Drosophila retinal neurons and C. elegans chemosensory or mechanosensory neurons (Brignull et al., 2006; Hirth, 2010; Neri, 2011).

1.1.4.1.5 In Vitro Models

Several cell lines have been used to model the pathological features of HD, such as the non-neuronal human HeLa cells, the human embryonic (HEK293T) and monkey kidney fibroblast cell lines (COS-7), as well as the Neuro2a (N2a) neuroblastoma (mouse) and neuron-like PC12 (rat) cells, ST14A (derived from embryonic day 14 rat striatal primordia by retroviral transduction of the temperature-sensitive SV40 large T antigen), SK-N-SH and SH-SY5Y (human neuroblastoma), rat and mouse primary striatal neurons, ST*Hdh*$^{Q111/ Q111}$ striatal cells, and HD cybrids (Cisbani and Cicchetti, 2012).

These cell lines have been widely used and provide a cost and time-effective model to investigate pathways associated with HD. In this section we will focus and resume some described features of ST*Hdh*$^{Q111/ Q111}$ striatal cells.

1.1.4.1.5.1 ST*Hdh*$^{Q111/ Q111}$ striatal cells

The long polyQ segments in the precise genetic models of juvenile HD, HdhQ92 and HdhQ111 knock-in mice, altered mHtt physical properties, producing HD-like *in vivo* brain pathology specifically in the striatum; this included nuclear localization of mHtt predominantly in medium spiny neurons, and subsequent formation of N-terminal inclusions and insoluble aggregate (Wheeler et al., 2000). Thus, immortalized wild-type

or homozygous mutant progenitor striatal cells that express endogenous normal/wild-type Htt (Q7) or mHtt with 111 Q (Q111) derived from E14 striatal primordia of wild-type or HdhQ111 knock-in mice, respectively, were developed. The cells represent early phases of HD pathogenesis because they show no visible Htt aggregates, no toxicity occurs under non-stimulated conditions, and also because they are derived from the embryonic phase of the Hdh knock-in mice (Trettel et al., 2000). Moreover, ST*Hdh*^{Q111} cells are selectively vulnerable to 3-NP. The 3-NP treatment caused significantly greater cell death in mutant striatal cells, compared with wild-type cells (Ruan et al., 2004). Also, mitochondrial respiration and ATP production were significantly reduced in ST*Hdh*^{Q111} cells (Milakovic and Johnson, 2005). Furthermore, in a recent study, ST*Hdh*$^{Q111/Q111}$ cells displayed more fragmented mitochondria compared to ST*Hdh*$^{Q7/Q7}$, correlating with decreased dynamin related protein 1 (Drp1) and optic atrophy 1 (autosomal dominant) (Opa1) protein levels, key regulators of mitochondrial fission and fusion, respectively. In fact, ST*Hdh*$^{Q111/Q111}$ cells presented significantly decreased mitochondrial fusion and hydrogen peroxide (H_2O_2)-induced increased swollen mitochondria, which correlated with increased mitochondrial oxidized state (Jin et al., 2013). Indeed, mtDNA damage was associated with reduced mitochondrial bioenergetics in ST*Hdh*$^{Q111/Q111}$ cells (Siddiqui et al., 2012). ST*Hdh*$^{Q111/Q111}$ cells also showed higher basal levels of mitochondrial-generated ROS and mitochondrial DNA (mtDNA) lesions and a lower spare respiratory capacity (Siddiqui et al., 2012). Therefore, ST*Hdh*$^{Q111/Q111}$ striatal cells represent a highly accurate model of HD, providing a reliable tool for the study of mHtt-induced cytotoxicity in the striatum in the early phases of the disease.

1.2 Mechanisms of cytotoxicity induced by mutant huntingtin

The expanded polyQ stretch in the protein Htt results in a conformational change leading to the deregulation of several cellular processes, such as transcription, mitochondrial function and energy homeostasis, reactive oxygen species (ROS) production, and/or modified intracellular signaling pathways, which can be originated from and/or enhance apoptotic cell death (Figure 1.2) as discussed in the next sections.

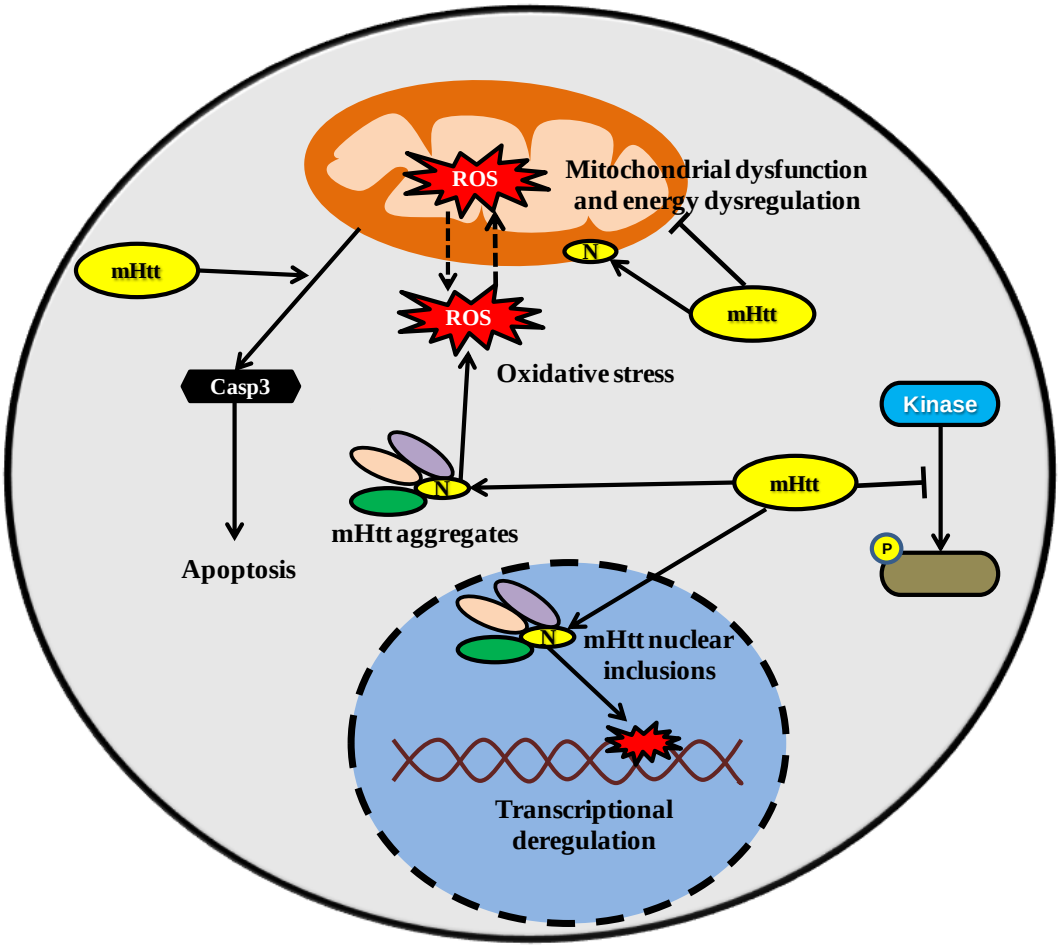


Figure 1.2 – Mutant huntingtin-induced deregulation of several cellular processes. Mutant huntingtin (mHtt) has been described to induce apoptotic cell death by activating caspases cascade, namely caspase-3 (Casp3). The formation of cytosolic aggregates and nuclear inclusions of mHtt fragments composed by the N-terminal Htt (N) recruits other proteins leading to their inactivation, many of these proteins are transcription factors which results in transcriptional deregulation. mHtt fragments also have been linked to increased reactive oxygen species (ROS) production and to interact with mitochondrial components resulting in mitochondrial dysfunction and energy deregulation. Signaling pathways have been also described to be deregulated in HD. Indeed mHtt appears to reduce kinases activity leading to reduced target proteins phosphorylation.

1.2.1 Mutant huntingtin induces apoptotic cell death

Several proteases were shown to cleave Htt *in vitro* and *in vivo*, and the corresponding cleavage products have been found in the brain of patients and murine models (Mende-Mueller et al., 2001). These proteases include caspase-1, -2, -3 and -6 (Goldberg et al., 1996; Graham et al., 2006; Hermel et al., 2004; Wellington et al., 1998; 2000; 2002), calpains (Bizat et al., 2003; Gafni and Ellerby, 2002; Goffredo et al., 2002) and aspartic proteases (Lunkes et al., 2002). Both normal and mHtt are substrates for caspase-3 activity, suggesting that the proteolytic cleavage is a physiologic event in the cell. However, the abnormal polyQ tract of truncated mHtt changes the conformation of the native structural protein, which renders it the preferred enzymatic substrate, in respect to normal Htt, and consequently induces the formation of insoluble aggregates (Davies et al., 1997; Scherzinger et al., 1997). mHtt fragments within the striatum of HD brains clearly differ from those of control brains (Mende-Mueller et al., 2001) and HD mice expressing mHtt resistant to cleavage by caspase-6 but not caspase-3 maintained normal neuronal function and did not develop striatal neurodegeneration, and protected against neurotoxicity induced by NMDA, QA, and staurosporine (STS), suggesting that the proteolytic cleavage of mHtt by caspase-6 is necessary to induce neurodegeneration (Graham et al., 2006). Moreover, mHtt has been shown to induce the activation of the intrinsic apoptotic pathway. Expression and Mitochondrial cytochrome c release were found to be increased in HD striatal neurons (Kiechle et al., 2002; Wellington et al., 1998) and in excitotoxic lesion models of HD (Antonawich et al., 2002; Bizat et al., 2003; Vis et al., 2001), which may be associated with increased activation of caspase-1, -2, -3, -6, -7, -8 and -9 verified in HD mouse models and HD human brain (Hermel et al., 2004; Kiechle et al., 2002; Maglione et al., 2006; Ona et al., 1999; Sanchez et al., 1999; Wellington et al., 1998). In this respect, mHtt was also found to bind and enhanced the pro-apoptotic factor p53 activation, which concomitantly increased caspase-6 activation in skeletal muscle, and in the brain from HD patients and mouse models (Bae et al., 2005; Ehrnhoefer et al., 2013; Steffan et al., 2000). Interestingly, p53 activation was found to regulate Htt expression at transcriptional level, both *in vitro* and *in vivo*, in the striatum and cortex of HD mouse brain, suggesting that a p53 stress response could be a modulator of the process of HD (Feng et al., 2006), whereas p53 deficient HdhQ140 HD mice resulted in a reduction of mHtt expression in brain and testis, and a significant increase in

nuclear aggregate formation in the striatum. The authors suggested a functional rescue of at least several aspects of the HD phenotype by a deficiency in p53 (Ryan et al., 2006).

1.2.2 Mutant huntingtin leads to transcriptional deregulation

Another consequence of mHtt expression is transcriptional deregulation. Nuclear mHtt aggregates interfere with normal transcriptional (Davies et al., 1997; DiFiglia et al., 1997). Several transcription factors have been reported to interact with mHtt. The mutated form of Htt was shown to interact with both the polyQ (Nucifora et al., 2001) and the acetyltransferase domains (Steffan et al., 2000; 2001) of CBP ((cyclic adenosine monophosphate (cAMP) response element (CRE) binding protein (CREB))-binding protein), and with TAF4 (TAF4 RNA Polymerase II, TATA Box Binding Protein (TBP)-Associated Factor); this factor, together with TBP, which also interacts with mHtt, is fundamental for RNA polymerase II activity (Dunah et al., 2002; Schaffar et al., 2004; Shimohata, 2002). The interaction of TBP with mHtt leads to the sequestration of TBP into mHtt aggregates, preventing the binding of TBP to DNA promoters (Friedman et al., 2008; Huang et al., 1998). CRE-mediated transcription is regulated by TAF4, which is part of the basal transcriptional machinery and can abnormally interact with mHtt, rendering the transcriptional complex ineffective (Dunah et al., 2002). mHtt can also alter CRE-mediated transcription through inhibition of CBP transcriptional activities. CBP plays a role in histone acetylation by acting as an acetyltransferase, opening the chromatin structure and exposing the DNA to transcription factors such as TAF4, thus enhancing CRE-mediated transcription. In the presence of mHtt, the interaction between the mutant protein and CBP is enhanced, leading to histone hypoacetylation and inhibition of CBP-mediated transcription (Cong et al., 2005; Steffan et al., 2000). Also, in the presence of mHtt, CBP becomes abnormally ubiquitinated resulting in its higher degradation and in reduced transcriptional expression of CREB-controlled genes (Jiang et al., 2003). Concordantly, deletion of CREB in the brain causes selective neurodegeneration in the hippocampus and striatum (Mantamadiotis et al., 2002).

Specificity protein 1 (Sp1) transcription factor involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling, was also shown to interact with the soluble form of mHtt, which inhibited Sp1 binding to DNA and suppressed its transcriptional activity in *post-mortem* brain tissues of both presymptomatic and affected

HD patients, and in the brain of R6/2 HD mice, in a polyQ-dependent manner, whereas overexpression of Sp1 and the coactivator TAF4 reduced the cellular toxicity, the neuritic extension defects, and the transcriptional inhibition caused by intranuclear mHtt (Dunah et al., 2002; Li et al., 2002; Zhai et al., 2005). Interestingly, genes whose mRNA levels were decreased in HD had abnormal Sp1-DNA binding, whereas genes with unchanged mRNA levels had normal levels of Sp1 association, suggesting that mHtt dissociates Sp1 from target promoters, inhibiting transcription of specific genes (Chen-Plotkin et al., 2006). More recently, Sp1 was found to regulate human *HTT* gene expression. The expression of Sp1 enhanced *HTT* gene transcription and the inhibition of Sp1-mediated transcriptional activation reduced *HTT* gene expression, suggesting that the dysregulation of Sp1-mediated Htt transcription, combining with mHtt detrimental effect on other Sp1-mediated downstream gene function, may contribute to the pathogenesis of HD (Wang et al., 2012).

1.2.3 Mutant huntingtin promotes mitochondrial dysfunction and dysregulation of energy metabolism

1.2.3.1 Glucose transport, uptake and metabolism in HD

The normal function of the brain requires high amounts of energy which is provided mainly through the glucose metabolism within cells (Siegel et al., 1999). When glucose metabolism is reduced, several dysfunctions in the brain may occur. The first evidence that energy metabolism has an important role in HD pathogenesis came from the observation that HD patients exhibit prominent weight loss, despite sustained caloric intake (O'Brien et al., 1990). Indeed, glucose hypometabolism appears to be present in HD and was demonstrated to begin in the asymptomatic phase of the disease, and in degenerating regions of HD, striatum and cortex (Andrews and Brooks, 1998; Kuwert et al., 1990). However, more recently, a study suggested that hypothalamus, but not the basal ganglia, is the brain region responsible for metabolic impairments in HD, since the selective hypothalamic expression of a short fragment of mHtt in BACHD mice was sufficient to recapitulate the glucose metabolic disturbances that occur in HD patients, whereas the selective hypothalamic inactivation of the expanded *HTT* gene prevented the development of the metabolic phenotype (Hult et al., 2011). An essential step for glucose consumption by cells is its transportation across plasma membrane, which is facilitated

via several glucose transporters (GLUT). Thus, a dysregulation in these proteins may reflect a lower cellular capacity to use glucose. Although no differences were observed at earlier HD stages (grade 1), in advanced stages (grade 3) GLUT1 and -3 protein levels were decreased by three- and four-fold, respectively, in the CN of *post-mortem* HD patients (Gamberino and Brennan, 1994), suggesting reduced glucose transport by striatal cells. In fact, a significant decrease in glucose uptake in the cortex and striatum of both pre-symptomatic and symptomatic HD patients was verified (Antonini et al., 1996; Ciarmiello et al., 2006). Also, GLUT1 and -4 gene expression were downregulated in COS-7 and SK-N-SH cells expressing N-terminal (exon-1) mHtt with 74Q, and in the muscle of 11- and 15-week-old R6/2 HD mice, respectively, whereas GLUT1 overexpression or treatment with glucose decreased mHtt aggregation and cell death (Kita et al., 2002; Ravikumar et al., 2003; Strand et al., 2005), indicating a protective role of GLUT1 against mHtt-induced degeneration by increasing intracellular glucose concentrations.

When enters the cell, glucose is irreversibly phosphorylated by hexokinase (HK) in an ATP-dependent process, resulting in glucose-6-phosphate (G6P) that serves as a substrate for both the pentose phosphate pathway and glycolysis (Berg et al., 2006). On contrary to the reported decreases in glucose transport and cellular uptake, HK was shown to be actively increased in erythrocytes and fibroblasts from HD patients (Cooper et al., 1998; Zanella et al., 1980), in posterior brain (the posterior part of the striatum, hippocampus, thalamus, parietal and occipital cortices) homogenates of advanced symptomatic 20-week-old N171-82Q HD mice and 3-NP-treated mice (Oláh et al., 2008), suggesting increased glucose phosphorylation and enhanced metabolism in HD. The increase in HK activity might reflect increased G6P levels, which can be used by the pentose phosphate pathway. In this pathway, β-nicotinamide adenine dinucleotide 2′-phosphate, reduced (NADPH) is the major cofactor produced by the action of glucose-6-phosphate dehydrogenase (G6PD) and 6-phophogluconate dehydrogenase (6PGD) and is a required cofactor in the regeneration of reduced glutathione (GSH) by glutathione reductase (GRed), and for the reductive reactions of lipid biosynthesis (Heales and Bolanos, 2002). Indeed, G6PD activity was reported to be increased in erythrocytes from HD patients, correlating with increased HK activity (Zanella et al., 1980). However, in HD cybrids, G6PD activity was significantly decreased (Ferreira et al., 2011). Thus, it might be possible that different tissues or cells in HD have contrary glucose metabolic rate or it might be dependent on the stage of the disease. In fact, in the striatum at 4-week-

old R6/2 HD mice, the expression levels of the non-neuronal enolase (ENO1 or enolase 1, (alpha), a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate), were significantly increased, whereas in the striatum of symptomatic (10 week-old) R6/2 HD mice, enolase activity was significantly decreased, which correlated with increased ENO1 and enolase 2 (gamma, neuronal; ENO2) protein oxidation (Perluigi et al., 2005), suggesting a decreased glycolytic rate due to increased protein oxidation in the striatum along HD progression. Also in the striatum of symptomatic (10-week-old) R6/2 HD mice, pharmacological inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, a glycolytic enzyme that catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate) induced decreased protein levels of glutamate transporters and enhanced glutamate neurotoxicity (Estrada-Sánchez et al., 2010), indicating a higher dependence of glycolysis to induce neuroprotection. Moreover, GAPDH has been described to bind to mHtt polyQ repeats (Burke et al., 1996), and to co-localize with mHtt aggregates in a N-terminal polyQ-region dependent manner (Wu et al., 2007), leading to GAPDH inactivation, which may cause a deficit in cerebral energy metabolism (Cooper et al., 1998). Indeed, mHtt interaction with GAPDH altered the subcellular localization of GAPDH, increasing its nuclear localization and decreasing its glycolytic activity in both fibroblasts from HD patients and in neurons from a transgenic HD mouse model expressing full-length mHtt carrying 89Qs (Mazzola and Sirover, 2001, 2002; Senatorov et al., 2003). Interestingly, GADPH was also shown to induce mHtt nuclear translocation involving a ternary complex of mHtt, GAPDH and Siah1, an ubiquitin-E3-ligase thus enhancing cytotoxicity (Bae et al., 2006); conversely GAPDH knockdown in SK-N-SH neuroblastoma cells expressing mHtt (exon 1) with 103Qs reduced mHtt aggregation by 45-50% (Lazarev et al., 2013), indicating an aggregation-dependent GAPDH process in HD. Importantly, GAPDH was recently shown to supply energy, independently of mitochondria, for the both anterograde and retrograde fast axonal transport, in a wild-type Htt-dependent mechanism (Zala et al., 2013), which might indicate that the fast axonal transport is compromised by the expression of mHtt and/or loss of wild-type Htt activity in HD.

Pyruvate, the glycolytic final product, can be converted into lactate by the reversible action of lactate dehydrogenase (LDH) or consumed by pyruvate dehydrogenase (PDH) to produce NADH and acetyl-coenzyme A. The latter is used by the tricarboxylic acid cycle (TCA, Krebs or citric acid cycle) for production of NADH that is required for oxidative phosphorylation to produce high levels of ATP (Nelson and

Cox, 2004). In neurons, LDH isoforms are adapted to favor lactate to pyruvate conversion, whereas in astrocytes LDH primarily functions to convert pyruvate into lactate, which is then released to the extracellular space where it is taken up by neurons and converted to pyruvate. Thus, apart from direct glucose utilization, lactate brain levels are essential for neuronal activation (O'Brien et al., 2007; Turner and Adamson, 2011). In the basal ganglia, striatum and in the cortex of presymptomatic, early manifest and advanced stages HD patients, lactate levels and lactate/pyruvate ratio were significantly elevated (Jenkins et al., 1993; 1998; Koroshetz et al., 1997; Reynolds et al., 2005). Increased lactate/pyruvate ratio was also found in cybrid lines from HD patients (Ferreira et al., 2011). However, in a juvenile HD case, an 8-year-old boy with 85 CAG repeats did not present significant lactate changes in the basal ganglia (Schapiro et al., 2004), whereas a decrease in lactate levels was detected in the cerebrospinal fluid of symptomatic adult-onset HD patients (Garseth et al., 2000), which appears to indicate that lactate alterations in HD are a region/tissue and/or polyQ length dependent, and also can change with disease progression. In fact, decreased lactate levels were detected in cortex, cerebellum and brainstem of 4 week-old (presymptomatic) and 8-week-old (early symptomatic) R6/2 HD mice, and in the muscle of 8-week-old R6/2 HD mice (Tsang et al., 2006). On the other hand, in 12 week-old R6/2 HD mice (symptomatic), lactate levels were increased in striatum, cortex, cerebellum and in brainstem and decreased in muscle and in urine (Fox et al., 2007; Tsang et al., 2006). Also, a region-selective increase in striatal lactate was detected in 3-NP-treated primates and rats (Dautry et al., 1999; Matthews et al., 1998). Thus, neuronal consume of lactate might be affected leading to reduced pyruvate levels. In fact, administration of pyruvate in rodents significantly reduced QA-mediated striatal neuronal degeneration and oxidative damage, whereas lactate, was ineffective against oxidative damage and was only partially effective in reducing lesions and neuronal degeneration (Ryu et al., 2004), indicating that striatal pyruvate production in HD could be diminished or its consume by PDH to produce NADH and acetyl-coenzyme A is necessary to offer neuroprotection in HD. In fact, PDH complex activities were reduced in CN and putamen of HD patients (Sorbi et al., 1983), and decreased PDH activity in basal ganglia was significantly augmented with increasing duration of illness, possibly due to a progressive loss of neurons in HD CN (Butterworth et al., 1985). Decreased PDH activity along with decreased mitochondrial β-nicotinamide adenine dinucleotide, reduced (NADH)/β-nicotinamide adenine dinucleotide (NAD)(t) ratio were also observed in HD cybrids, which was correlated with a decrease in PDH

E1alpha subunit protein levels (Ferreira et al., 2011). Also, in R6/2 HD mice PDH expression levels decreased with age (Perluigi et al., 2005). Therefore, the supply of cofactors for the TCA cycle might be compromised in HD. In fact, both the TCA enzymes citrate synthase and aconitase activities were decreased in CN, putamen and cortex of HD patients (Tabrizi et al., 1999; Sorolla et al., 2008), and in the striatum of 12-week-old R6/2 HD mice (Tabrizi et al., 2000).

Thus, the energy production by glycolysis might be affected in HD, which can contribute to decreased TCA enzymes activities and consequently less NADH available for the oxidative phosphorylations to produce ATP. However, other intracellular systems can be an alternative for the energy production, such as the phosphocreatine/creatine system.

1.2.3.2 **Phosphocreatine/creatine system and ATP in HD**

The phosphocreatine/creatine system can generate ATP 10 times faster than mitochondrial oxidative phosphorylation and 40 times faster than glycolysis (Wallimann et al., 1992). Creatine is a substrate of cytosolic and mitochondrial creatine kinases (CK), which catalyzes the reversible phosphorylation of creatine, using ATP as a phosphate donor to form phosphocreatine and ADP as a by-product. This reaction can be reversible, as CK can convert phosphocreatine and ADP into creatine plus ATP (Woznicki and Walker, 1979), controlling thereby ATP concentrations through the transfer of high-energy phosphate from phosphocreatine to ADP (Wyss and Kaddurah-Daouk, 2000). The brain expresses high levels of CKs due to the requirement to consume elevated energy levels (Wallimann, 2007). Brain-specific cytosolic (BB-CK or CKB) and ubiquitous mitochondrial ($_{u}$Mt-CK) are the two isoforms of CK expressed in brain tissues and are an easy target of oxidative damage (Zhang et al., 2011). Indeed, oxidation and decreased mRNA and protein levels and activity of CKB and $_{u}$Mt-CK were detected in the striatum (Crocker et al., 2006; Kim et al., 2010; Perluigi et al., 2005; Sorolla et al., 2008; 2010; Zhang et al., 2011) and blood (Kim et al., 2010) of HD patients, and in the striatum of R6/2, N171-82Q, and HdhQ111 HD mouse models, in 3-NP-treated rats, and in HD ST*Hdh*^{Q109} and ST14A 109Q striatal cells (Lagoa et al., 2009; Lin et al., 2013; Zhang et al., 2011), which might be associated with reduced striatal ATP and creatine levels (which correlated with motor and cognitive performance and with the CAG repeat length) verified in presymptomatic and symptomatic adult-onset HD patients, in juvenile HD

cases (Alcauter-Solórzano et al., 2010; Clarke et al., 1998; Reynolds et al., 2005; 2008; Sánchez-Pernaute et al., 1999; Schapiro et al., 2004; van den Bogaard et al., 2011), in ST*Hdh*$^{Q111/Q111}$ striatal cells (Gines et al., 2003; Milakovic and Johnson, 2005), and in 3-NP-treated rodents and primates (Dautry et al., 1999; Matthews et al., 1998; Mochel et al., 2010). Moreover, mHtt suppressed the activity of the promoter of the CKB gene, which contributed to decreased CKB expression in HD, whereas the overexpression of CKB rescued ATP depletion, aggregate formation, impaired proteasome activity and shortened neurites induced by mHtt (Lin et al., 2013).

1.2.3.3 **Mitochondrial abnormalities in HD**

Under normal conditions, the ATP produced by the mitochondrial respiratory chain oxidative phosphorylation (OXPHOS) is responsible, for more than 80% of the ATP produced in cells (Papa et al., 2012). However, altered mitochondrial function can lead to a disruption of energy balance and consequent neuronal degeneration, which can be due to increased mtDNA damage. In fact, in HD patients, decreased activities of mitochondrial complexes I, II/III, and IV, were previously detected in striatum CN and putamen (Benchoua et al., 2006; Brennan et al., 1985; Browne et al., 1997; Butterworth et al., 1985; Gu et al., 1996; Tabrizi et al., 1999), in muscle (Arenas et al., 1998), and platelets (Parker et al., 1990; Silva et al., 2013), which can be associated with increased mtDNA deletions that were detected in leukocytes, lymphocytes and cortex from HD patients (Banoei et al., 2007; Chen et al., 2007; Horton et al., 1995; Liu et al., 2008), correlating with CAG repeat length (Liu et al., 2008). Moreover, in symptomatic R6/2 and N171-82Q HD mouse models, a significant reduction in striatal and cortical activities of mitochondrial complexes I, II and IV, and increased striatal mtDNA deletions and damage which increased with age, were also observed (Acevedo-Torres et al., 2009; Aidt et al., 2013; Benchoua et al., 2006; Chaturvedi et al., 2010; Kim et al., 2011; Oláh et al., 2008; Tabrizi et al., 2000). Decreased activities of mitochondrial complexes I, IV and V, and decreased mtDNA copy number and transcripts and accumulation of mtDNA deletions, were further verified in ST*Hdh*$^{Q111/Q111}$ striatal cells (Napoli et al., 2013). Indeed, striatal cells showed higher basal levels of mitochondrial-generated ROS, mtDNA lesions and a lower spare respiratory capacity compared to wild-type cells (Siddiqui et al., 2012). Considering the role of ROS in mtDNA damage, treatment with a synthetic mitochondria-specific antioxidant, XJB-5-131, improved mitochondrial function in

isolated organelle fractions from HdhQ150 knock-in mice, and restored mtDNA copy number *in vivo* to levels similar to those of the controls (Xun et al., 2012). These results suggest that oxidative stress-induces mitochondrial abnormalities that are potentiated upon expression of mHtt.

The replication and transcription of the mitochondrial genome can be effectuated by mitochondrial transcription factors and regulators of mitochondrial biogenesis; thus maintaining mitochondrial function and energy metabolism, and cell survival (Marín-García, 2013). Such proteins include the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a transcriptional co-activator and a master regulator of mitochondrial biogenesis (Puigserver and Spiegelman, 2003). PGC-1α up-regulates the expression and forms heteromeric complexes with the nuclear respiratory factors (NRF)-1 and -2 (Lin et al., 2005; Wu et al., 1999), which in turn regulate the expression of many nuclear-encoded mitochondrial genes, such as cytochrome c, and the mitochondrial transcription factors A (Tfam) and B (TFB1M, and TFB2M). Importantly, Tfam acts on the promoters within the D-loop region of mtDNA and regulates the replication and transcription of the mitochondrial genome (Gleyzer et al., 2005; Handschin and Spiegelman, 2006; Kelly and Scarpulla, 2004; Scarpulla, 2008a,b). Moreover, NRF-1 and -2 were shown to regulate all ten nuclear-encoded subunits of complex IV in neurons (Dhar et al., 2008), and several nuclear-encoded subunits of complexes I, II, III and V (Marín-García, 2013). The findings that PCG-1α knockout mice revealed neurological abnormalities, such as dystonia and clasping and the fact that they were lean and presented the most pronounced degeneration in the striatum (Lin et al., 2004) suggested that PGC-1α could represent a target in HD. In fact, PGC-1α expression and activity were reported to be impaired in cell models and transgenic mouse models of HD and in brain and muscle tissues from HD patients (Chaturvedi et al., 2009; Cui et al., 2006; McGill and Beal, 2006; Weydt et al., 2006), whereas overexpression in *in vitro* models of HD and in and N171-82Q HD mice or lentiviral delivery of PGC-1α to the striatum of R6/2 HD mice, prevented mHtt protein aggregation, restored mitochondrial function, reduced mitochondrial toxicity, completely prevented striatal atrophy at the site of PGC-1α injection, rescued HD neurological phenotypes, and neurodegeneration in part by attenuating oxidative stress (Cui et al., 2006; Weydt et al., 2006; Tsunemi et al., 2012). In a study performed in over 400 German HD patients, polymorphisms in NRF-1, Tfam, and PGC-1α showed significant correlation with the age at onset (Taherzadeh-Fard et al., 2009; 2011). Also, reductions in Tfam and PGC-1α observed in HD *post-mortem*

brain tissue correlated with reductions in the number of mitochondria (Kim et al., 2010). mHtt was also reported to bind to the PGC-1α promoter, causing decreased transcription of its mitochondrial target genes (e.g. Tfam and cytochrome c) and impaired mitochondrial function (Cui et al., 2006; McGill and Beal, 2006).

Mitochondrial bioenergetics seems strongly dependent on mitochondrial morphology and changes in morphology seem to impact on bioenergetic state. Mitochondrial shape is largely determined by a balance between fission and fusion events and this equilibrium maintains steady state mitochondrial morphology, mtDNA and metabolic integration, bioenergetic functionality and organelle number (Griparic and van der Bliek, 2001). Mitochondrial fission is an essential event for the accurate rearrangement of mtDNA during cell division and also for the transport of mitochondria to new cells during mitosis and meiosis (Hales, 2004; Szabadkai et al., 2004). Mitochondrial fission is regulated and maintained by two GTPase genes: mitochondrial fission 1 (Fis1) and cytosolic dynamin family member, Drp1. Fis1 is primarily localized on the outer mitochondrial membrane and participates in mitochondrial division (Chen and Chan, 2005). Drp1 is cytosolic recruited by mitochondrial elongation factors 1 (Mid51) and 2 (Mid49), and Mitochondrial Fission Factor (MFF) to mitochondria (Elgass et al., 2013), often at sites where mitochondria make contact with the endoplasmic reticulum (Friedman et al., 2011) to form spirals around mitochondria that constrict to sever both inner and outer membranes (Elgass et al., 2013). In contrast, mitochondrial fusion is a two-step process, in which the outer and inner membranes fuse by separate events. It is accepted that this event occurs as a way to recover the integrity and function of damaged/depolarized membranes, being essential to maintain a homogenous organelle population and ensuring inter-complementation of mtDNA (Twig et al., 2008a,b). The outer membrane fusion is controlled by two large membrane GTPase proteins, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), whereas inner membrane fusion is controlled by OPA1 (Chen and Chan, 2010). In HD, increased mitochondrial fission and decreased fusion has been described. Increased expression of Drp1 and Fis1 and reduced expression of Mfn's and OPA1 was verified in cellular models of HD, and in HD *post-mortem* brain tissues. Interestingly, mHtt was shown to bind and increase Drp1 enzymatic activity and thus mitochondrial fission (Haun et al., 2013; Kim et al., 2010; Shirendeb et al., 2011; 2012; Song et al., 2011; Wang et al., 2009). Moreover, S-nitrosylation of Drp1, resulting in increased Drp1 activity, was shown to mediate mHtt-induced mitochondrial fragmentation, resulting in dendritic spine loss in both human HD brains and animal

models of HD (Haun et al., 2013). Furthermore, mitochondrial fission was described to participate in mHtt-induced cell death in HD (Liot et al., 2009). Mitochondrial fragmentation, increased vacuolization, disrupted cristae, swollen and also giant mitochondria and increased susceptibility towards apoptotic stimuli were also observed in in skin fibroblasts and muscle of HD patients, and in HD transgenic mice and cellular models (Bayram-Weston et al., 2012; Costa et al., 2010; Squitieri et al., 2010). In progenitor and differentiated neuron-like ST*Hdh*$^{Q111/Q111}$ cells, changes in mitochondrial morphology and reduced mitochondrial import of Cys-rich proteins were also observed (Napoli et al., 2013). Changes in mitochondrial dynamics and morphology in HD appear to be related with the previous observation that mHtt aggregates and/or N-terminal fragments of mHtt localize on brain mitochondria of transgenic and knock-in HD models, and in human neuroblastoma and ST*Hdh*$^{Q111/Q111}$ cells, whereas mHtt-induced mitochondrial permeability transition (MPT) pore opening contributing for the occurrence of intrinsic apoptotic cell death, and impaired vesicular and mitochondrial fast axonal trafficking in mammalian neurons *in vitro* and in whole animals *in vivo*, which occurred early in development prior to the onset of measurable neurological or mitochondrial abnormalities (Chang et al., 2006; Choo et al., 2004; Orr et al., 2008; Panov et al., 2002; Petrasch-Parwez et al., 2007; Trushina et al., 2004).

Taken together, these data clearly show that energy metabolism dysregulation and mitochondrial dysfunction is present in HD, which could be related to increased oxidative stress that will be discussed in the next chapter.

1.2.4 Mutant huntingtin induces oxidative stress

Oxidative stress classical definition is the imbalance of pro-oxidants and antioxidants in cells, which can result from: 1) an increase in oxidant formation; and/or 2) a decrease in antioxidant protection; and/or 3) a failure in the repair of oxidative lesions. When the pro-oxidant/antioxidant equilibrium is lost, oxidative stress takes place, altering and damaging many intracellular molecules, including DNA, RNA, lipids and proteins (Veskoukis et al., 2012). However, the contemporary definition of oxidative stress has been refined to account for two different mechanistic outcomes, macromolecular damage, and disruption of thiol redox circuits, involving aberrant cell signaling and dysfunctional redox control (Jones, 2006; 2008).

1.2.4.1 Mutant huntingtin causes increased generation of reactive species and oxidative damage

Damage in cells is induced by free radicals, which are molecules containing one or more unpaired electrons in their atomic or molecular orbitals (Halliwell and Gutteridge, 1999). Reactive species comprise both free radical and non-free radical intermediates and can be classified into four groups based on the main atom involved: i) ROS, ii) reactive nitrogen species (RNS), iii) reactive sulfur species (RSS) and iv) reactive chloride species (RCS) (Sosa et al., 2013). Oxygen-derived reactive species are the major secondary products formed in cells of aerobic organisms and characterize the most important class of reactive compounds generated in living systems (Raha and Robinson, 2001). Depending on the stability of the molecule, ROS half-lives vary from a few nanoseconds to hours, and include superoxide anion ($O_2^{\bullet-}$), H_2O_2, hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($RO_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), hypochlorous acid (HOCl), hypobromous acid (HOBr), singlet oxygen ($^1O_2$) and ozone (O_3) (Dalle-Donne et al., 2005; Sosa et al., 2013). H_2O_2 is not a free radical, although it can freely diffuse across biological membranes and may cause severe damage to essential macromolecules. In the presence of transition metals, such as iron or copper, H_2O_2 can be converted to highly reactive $^{\bullet}OH$, which is recognized as one of the most potent oxidants in nature (Fridovich, 1995). The most abundant RNS is nitric oxide ($^{\bullet}NO$), which is able to react with certain ROS; $^{\bullet}NO$ easily reacts with $O_2^{\bullet-}$ to form peroxynitrite anion ($ONOO^-$), which is an oxidant and nitrating molecule, that can cross cell membranes and damage intracellular components. $^{\bullet}NO$ is also converted into peroxynitrous acid (ONOOH) and into $^{\bullet}OH$ and nitrite anion (NO_2^-) (Sosa et al., 2013). Several enzymatic systems contribute to intracellular ROS production, including NADPH oxidase (NOX) (Krause, 2004), cytochrome P450-dependent oxygenases (Bernhardt, 1996), and xanthine oxidase (XO) (Harrison, 2002). Non-enzymatic sources of ROS occur mainly in mitochondria, believed to be major intracellular source of ROS. During respiration, electrons released from the mitochondrial electron transport chain incompletely reduce O_2 to form $O_2^{\bullet-}$ as by-product. The site of electron leakage from the electron transport chain to form $O_2^{\bullet-}$ is generally considered to be a semiquinone radical ($CoQ_{10}H^{\bullet}$) or reduced flavin (FMN and FAD), at coenzyme Q_{10} (CoQ_{10})-binding-sites of complexes III and I, respectively (Brand, 2010; Murphy, 2009; Poyton et al., 2009; Turrens, 2003). Although

mitochondrial complexes I and III are the most studied and believed to be the major mitochondrial source of ROS, other mitochondrial enzyme complexes have also been reported or demonstrated to produce ROS. Examples include the dihydrolipoamide dehydrogenase-containing FAD-linked pyruvate and alpha-ketoglutarate dehydrogenase complexes (Starkov et al., 2004), as well as the flavoenzymes alpha-glycerophosphate dehydrogenase (Tretter et al., 2007), and the electron-transferring flavoprotein:quinone (Q) oxidoreductase (ETFQOR) of fatty acid β-oxidation (St-Pierre et al., 2002).

Although ROS have some biological positive effects by performing an essential physiological role in cell signaling, excessive generation and/or long-term exposure to high amounts of ROS can lead to profound toxic effects, by reacting with vital cell components and alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, and DNA damage ultimately resulting in apoptotic or necrotic cell death (Fialkow et al., 2007; Koppula et al., 2012). The nervous system is particularly vulnerable to ROS. In fact, the brain contains high amounts of polyunsaturated fatty acids that are highly susceptible to lipid peroxidation, presents high oxygen consumption for energy production and possesses a deficit in the antioxidant systems comparatively to other organs (Mariani et al., 2005). The striatum, the area that is largely affected in HD, is highly susceptible to dysfunction of mitochondrial oxidative phosphorylation (Pickrell et al., 2011), and it is known that acute poisoning with mitochondrial toxins (cyanide, sodium azide, and 3-NP) and also mitochondrial defects of genetic origins (e.g., mutation or deletion of mtDNA or nuclear DNA (nDNA)) can lead to striatal degeneration (Beal, 1992; Brouillet et al., 1999). Moreover, the striatum of young rats has been shown to display decreased antioxidant capacity compared with the cortex or other brain areas (Balu et al., 2005). Furthermore, the brain expresses high levels of NOX and XO, which makes the brain more prone to increased ROS production. Activated forms of NOX, which require interaction between cytosolic and membrane-bound subunits, and which primary function is to catalyze the transfer of electrons across the plasma membrane from NADPH to molecular oxygen via their "Nox" catalytic subunit, generating $O_2^{\bullet-}$ and H_2O_2, are among the major sources of ROS in the CNS. Increasing evidence suggests that NOX has important roles in neurodegenerative diseases. NOX was shown to mediate striatal neuronal injury after transient global cerebral ischemia (Gao et al., 2012; Koppula et al., 2012; Serrano et al., 2003; Yoshioka et al., 2011). In HD, NOX activity was found to be elevated in human

HD *post-mortem* cortex and striatum and highest in striatum of presymptomatic individuals. Synaptosome fractions obtained from cortex and striatum of HD(140Q/140Q) mice also showed increased NOX activity at 3 months of age, which was potentiated with increased age, compared to controls, correlating with increased ROS and neurite swelling. Concordantly, NOX inhibition significantly reduced ROS formation, neurite swelling and neuronal cell death. Also, mHtt colocalized at plasma membrane lipid rafts with gp91-phox, a catalytic subunit for the NOX2 isoform, and HD(140Q/140Q) mice bred to gp91-phox knock-out mice presented decreased NOX activity and normal ROS levels, as well as improved survival (Valencia et al., 2013). Moreover, NOX was involved in striatal damage evoked by QA in adult rats (Maldonado et al., 2010). XO is an enzyme involved in the purine/adenosine metabolism. While the major role of XO is the conversion of hypoxanthine and xanthine to uric acid, an interconvertible form, xanthine dehydrogenase, also exists and is responsible for conversion of NAD^+ to NADH. The action of these enzymes produces $O_2^{\bullet -}$ and H_2O_2 (Zhang et al., 1998), while uric acid is a potent reducing agent (electron donor) and antioxidant. In blood plasma, over half of the antioxidant capacity derives from uric acid (Maxwell et al., 1997). In the brain of patients with depression or with schizophrenia were found significant increases in XO activity in the thalamus and putamen, and significant decreases in XO activity in the occipital cortex and thalamus, respectively, with a significant correlation between treatment with chlorpromazine (a dopamine antagonist of the typical antipsychotic class of medications for the treatment of schizophrenia) and increased XO activity in the putamen and the temporal cortex (Michel et al., 2010; 2011). In this respect, XO was demonstrated to actively diminish dopamine transporter function in rat striatum (Fleckenstein et al., 1997). Also, an age-related increase in XO activity, which significant correlated with increased lipid peroxidation, was found in the cerebral cortex of adult female mice (Vida et al., 2011). In HD human *post-mortem* cerebral cortex, uric acid levels were found to be reduced (Beal et al., 1992), which suggest that XO activity might be decreased and also decreased antioxidant capacity due to low levels of uric acid. On the other hand, treatment with allopurinol or oxypurinol, inhibitors of XO activity, blocked peroxide accumulation and cell death caused by 3-hydroxykynurenine (3-HK) (a tryptophan metabolite whose level in the brain is markedly elevated in HD) in primary neuronal cultures prepared from rat striatum, suggesting that XO is involved in 3-HK-induced neurotoxicity in HD (Okuda et al., 1996), and that cytosolic ROS production might be enhanced in HD striatum.

Nevertheless, mitochondrial ROS formation, including $O_2^{\bullet-}$, was also verified in fibroblasts from HD patients (Wang et al., 2013). Increased ROS formation was also observed in HD cybrids and potentiated with treatment with 3-NP or to STS, an inducer of apoptosis (Ferreira et al., 2010). Increased ROS, including $O_2^{\bullet-}$ formation, were found in the brain striatum and cortex of symptomatic R6/1, R6/2, and N171-82Q HD mouse models (Ellrichmann et al., 2011; Pérez-Severiano et al., 2004; Sadagurski et al., 2011; Stack et al., 2010; Tabrizi et al., 2000), and in YAC128 HD mice embryonic fibroblasts (Wang et al., 2013). Furthermore, H_2O_2 treatment potentiated mHtt-induced increased ROS levels and cell death, and induced aggregation of mHtt N-terminal fragments, which in turn directly caused ROS production in a polyQ length-dependent manner, preceding cell death and impairing proteasomal function in neuronal and non-neuronal cellular models (Goswami et al., 2006; Hands et al., 2011; Wyttenbach et al., 2002).

Since molecular products from oxidative/nitrosative stress are generally more stable than oxidants and nitrosants themselves, ROS/RNS measurements often involve determining levels of their oxidation target products (Griffiths et al., 2002). Several biomarkers are normally used to detect oxidations in proteins, lipids and DNA.

Oxidative damage to nucleotides causes modification of pyrimidine and purine bases and is commonly measured by in the detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) adducts (Evans et al., 2004; Kasai, 2002). Particularly, mtDNA is highly susceptible to ROS-induced damage because it is located in close proximity to the production site of ROS, mtDNA repair mechanisms are limited and lacks histone protection. As a result, deletions in mtDNA accumulate, ultimately leading to a decline in mitochondrial function and concomitant enhanced ROS production (Lin and Beal, 2006). Significant increases in the levels of nuclear and mitochondrial DNA-8-OHdG adducts has been shown in the CN, cortex, serum, leukocytes, and in plasma of HD patients (Browne et al., 1997; Chen et al., 2007; Polidori et al., 1999; Shirendeb et al., 2011; Hersch et al., 2006; Túnez et al., 2011). Also, in HD mouse models, DNA oxidation, including mtDNA, was significantly increased in forebrain tissue, cortex, striatum, urine and plasma of R6/2 (Acevedo-Torres et al., 2009; Bogdanov et al., 2001; Tabrizi et al., 2000) with striatum being more vulnerable to damage than cortex (Acevedo-Torres et al., 2009), in striatal tissues of N171-82Q (Stack et al., 2010), and in embryonic fibroblasts from YAC128 (Wang et al., 2013). Moreover, systemic and intrastriatal injections of 3-

NP or QA in rodents induced striatal increases in DNA oxidation (Acevedo-Torres et al., 2009; Maldonado et al., 2012).

Reaction of free radicals with unsaturated lipids may trigger lipid peroxidation chain reaction, resulting in the oxidative breakdown of cellular membranes. Usually lipid peroxidation is detected using various biomarkers, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), acrolein and isoprostanes (Dalle-Donne et al., 2006; Mariani et al., 2005; Morrow et al., 1999). MDA is a physiologic ketoaldehyde produced by peroxidative decomposition of unsaturated lipids as a byproduct of arachidonic acid metabolism (Dalle-Donne et al., 2006). Acrolein is the simplest unsaturated aldehyde and is formed *in vivo* by the metal-catalyzed oxidation of polyunsaturated fatty acids, including arachidonic acid (Uchida et al., 1998). 4-HNE is a major and toxic aldehyde generated by free radical attack on omega-6 polyunsaturated fatty acids (arachidonic, linoleic, and linolenic acids) (Uchida, 2003) and is considered a second toxic messenger of oxygen free radicals (Esterbauer et al., 1991; Dalle-Donne et al., 2006). Prostaglandin F2-like compounds (F2-isoprostanes, F2-IsoPs), are formed *in vitro* and *in vivo* (Morrow et al., 1990) by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid (Comporti et al., 2008). Due to F2-IsoPs release into the circulation and are less reactive that other lipid peroxidation products, they can be detected more easily in plasma and urine, and thus are considered the most reliable markers of oxidative stress (Comporti et al., 2008; Morrow et al., 1996). MDA, 4-HNE, and F2-IsoPs levels were found to be elevated in the striatum, cortex, cerebrospinal fluid, and in blood of HD patients (Browne et al., 1999; Chen et al., 2007; Lee et al., 2011; Montine et al., 1999; Stoy et al., 2005), with increased MDA levels correlating significantly with disease severity (Chen et al., 2007). Also, in HD mouse models, increased lipid peroxidation was detected in the striatum, cortex and whole brain of symptomatic R6/2, N171-82Q, and CAG140 (Lee et al., 2011; Pérez-Severiano et al., 2000; Sadagurski et al., 2011; Tabrizi et al., 2000), co-localizing with mHtt inclusions (Lee et al., 2011). Furthermore, systemic and intrastriatal administration of QA in rats or exposure to 3-NP, QA or 3-NP plus QA in striatal and cortical mice or rat synaptosomes and slices, also induced an increase in striatal lipid peroxidation (Colle et al., 2012; Herrera-Mundo and Sitges, 2013; Maldonado et al., 2012; Túnez et al., 2006). Also, increased cellular immunoreactivity for 4-HNE was observed in a cell line model of HD regulated by doxycycline, the Tet-mtHtt-Q103-EGFP cells, which was enhanced following exposure to H_2O_2 (Lee et al., 2011).

Oxidative changes in essential proteins may result in functional loss of proteins. Irreversible oxidation in proteins are usually detected by determining the levels of protein carbonyl groups and 3-nitrotyrosine (3-NT) and reversible protein oxidations can be measured by determining cysteine modification products, which includes sulfenic acid (S-sulfenation), s-nitrosothiols (S-nitrosylation), s-glutathione (S-glutathionylation) and protein disulfides (Cai and Yan, 2013) formation. Protein carbonyl groups (aldehydes and ketones) can be formed by oxidation of several amino acid residues, including arginine, histidine, lysine, proline, threonine and cysteine (Berlett and Stadtman, 1997; Cai and Yan, 2013). 3-NT is a product of nitration of protein tyrosine residues by $ONOO^-$ (Maruyama et al., 1996), thus representing a biomarker for $ONOO^-$. Furthermore, the severe impairment in mitochondrial TCA-cycle enzyme aconitase reported in HD brain, has been attributed to Fe–S clusters within the protein, which make it a particularly vulnerable target for free radical-mediated oxidative damage (Tabrizi et al., 1999). Indeed, loss of aconitase activity and increased 3-NT levels were reported in the striatum and cortex of HD patients (Browne et al., 1999; Sorolla et al., 2008; Tabrizi et al., 1999). Moreover, aldolase C, glial fibrillary acidic protein (GFAP), tubulin, ENO2, and CKB were found to be the targets of oxidative modification in both striatum and cortex from HD patients (Sorolla et al., 2008). More recently, it was shown that the oxidation of mitochondrial enzymes resulted in decreased catalytic activity in striatal samples of HD patients, providing a link to the bioenergetic deficits observed in HD (Sorolla et al., 2010). Oxidation of the mHtt protein was also reported in cellular models. Cysteine oxidation within N-terminal of mHtt promoted oligomerization and delayed the clearance of soluble protein (Fox et al., 2011). Oxidation of a methionine residue at position 8 of exon 1 mHtt was also shown to occur *in vivo* and *in vitro* after mHtt aggregation, creating additional interactions among mHtt aggregates and altering overall morphologies of the aggregates (Mitomi et al., 2012). Also, in HD mouse models, increased 3-NT, protein carbonyl groups, and decreased aconitase activity were detected in whole brain, striatum, and cortex of R6/2 and N171-82Q (Sadagurski et al., 2011; Stack et al., 2010; Tabrizi et al., 2000; Zourlidou et al., 2007). Moreover, a proteomic analysis in the brain of R6/2 mice revealed increased oxidation in several proteins, namely CK, aconitase, ENO1 and -2, heat shock protein (Hsp) 90 and the voltage-dependent anion channel 1 (Perluigi et al., 2005).

1.2.4.2 Mutant huntingtin promotes dysregulation of antioxidant systems

To maintain a physiological redox homeostasis, cells are provided of a vast variety of endogenous antioxidant proteins that can be produced and regulated via transcription. The promoter antioxidant response element (ARE) is a cis-acting regulatory element that regulates the antioxidant protein gene transcription of more than 200 cytoprotective genes. The activation of ARE-induced gene transcription is induced through the binding of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Itoh et al., 2003; 2004; Kobayashi and Yamamoto, 2006; Zhang et al., 2013). Under normal (unstressed) conditions, Nrf2 half life (~20 min) is regulated by several proteins (Table 1.3 and 1.4). The most known endogenous inhibitor is Kelch-like ECH associated protein 1 (Keap1), which in association with cullin 3 (Cul3)-Ring box protein 1 (Rbx1) ubiquitin ligase complex binds and maintains Nrf2 in cytoplasm to be ubiquitinated and degraded by proteasome. However, under conditions of increased ROS formation, Nrf2 dissociates from Keap1 prolonging Nrf2 half-life (100-200 min) (Figure 1.3). Oxidative stress-induced dephosphorylation of Keap1 at Tyr141 and oxidation of cysteine residues within Keap1 domain that binds to the Cul3/Rbx1 ubiquitin ligase complex imposes a conformational change that weakens its activity as an E3 ligase adaptor, cause the formation of disulfide bonds between cysteines of two Keap1 peptides, and also cause the switching of Cul3-dependent ubiquitination from Nrf2 to Keap1 leading to its degradation and stabilization of Nrf2 (Dinkova-Kostova et al., 2002; Jain et al., 2008; Zhang et al., 2004; Zhang and Hannink, 2003; Fourquet et al., 2010). However, modifications of cysteine thiol groups of Keap1 are important, but are not sufficient for activation of Nrf2. The phosphorylation, acetylation, and the conservation of Nrf2 cysteine residues (with the exception of Cys183 oxidation which decreases the nuclear export signal (NES) of Nrf2, resulting in increased Nrf2 retention in the nucleus), are necessary for Nrf2 activation, nuclear translocation, and ARE activation (He and Ma, 2009; Li et al., 2006; Niture et al., 2014) (Table 1.3 and 1.4). After phosphorylation, Nrf2 is imported to the nucleus, where it is acetylated by CBP and forms heterodimers with the receptor-associated coactivator 3 (RAC3)/AIB-1/steroid receptor coactivator-3, c-Jun, c-Jun dimerization protein 2 (JDP2), and small V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog(s) (Mafs) (MafK, MafG or MafF) proteins for high-affinity binding, leading to increased promoter specific DNA binding to ARE, and consequent enhancement of Nrf2-ARE transcription

of antioxidant genes (Kim et al., 2013; Sun et al., 2009; Tanigawa et al., 2013; Zhang et al., 2013) (Table 1.5). In the late response (post-induction of Nrf2) to oxidative stress, Keap1 is deubiquitinated, leading to enhanced Keap1-Cul3-Rbx1 complex formation, stability and enzymatic activity, and nuclear importation, whereas Nrf2 is deacetylated and dephosphorylated, disrupting its binding to ARE, binds to Keap1-Cul3-Rbx1 complex and both Nrf2 and Keap1 are phosphorylated at Tyr568 and Tyr85, respectively, increasing Nrf2 and Keap1-Cul3-Rbx1 complex interaction with exportin 1 (Exp1), leading to Nrf2-Keap1-Cul3-Rbx1 nuclear exportation and consequently Nrf2 degradation, thereby providing a negative feedback mechanism to switch off Nrf2 activation and restore normal cellular homeostasis (Jain and Jaiswal, 2006; Kawai et al., 2011; Kaspar et al., 2012; Sun et al., 2007; Villeneuve et al., 2013) (Figure 1.4).

Table 1.3 – Nrf2 cellular endogenous inhibitors.

Endogenous inhibitors	Interaction	Function	In HD
Keap1[19]	Nrf2 N-terminus Neh2 domain[20] interacts with the C-terminus Kelch-DC domain of the Keap1, which in turn through its N-terminal BTB/POZ domain[21], binds to and function as an adaptor for the Cul3[22]-E3 ubiquitin ligase complex with Rbx1[23,24]	Nrf2 ubiquitination and consequent proteasomal degradation	Increased Keap1 mRNA but decreased Keap1 protein levels in ST*Hdh*$^{Q111/Q111}$ cells[25]
β-TrCP[26]	Both N-terminus and C-terminus portions of Nrf2 Neh6 domain interacts with β-TrCP, which acts as a substrate receptor for the Skp1[27]-Cul1-Rbx1 ubiquitin ligase complex[28]	Nrf2 ubiquitination and consequent proteasomal degradation	β-TrCP was shown to interact with both wild-type and mHtt in primary cultures of striatal neurons from wild-type mice or HdhQ111/Q111 knock-in mice and in HEK293 cells containing Htt fragments, decreasing mHtt-induced neuronal cell death[29]
GSK3-β[30]	Nrf2 Neh6 N-terminus portion is phosphorylated by GSK3-β, resulting in a phosphodegron[31] for the binding of β-TrCP[32]	Nrf2 ubiquitination and consequent proteasomal degradation	GSK3-β was found to be actively increased in the striatum and cerebral cortex from N171-82Q and YAC128 HD mouse models around the onset of behavioral deficits, whereas pharmacological

[19] Kelch-like ECH associated protein 1 (Keap1)
[20] Nrf2-ECH homology 2 (Neh2) domain
[21] Bric-a-brac, tramtrack, broad (BTB)/pox virus and Zinc finger (POZ) domain
[22] Cullin 3 (Cul3)
[23] Ring box protein 1 (Rbx1)
[24] Kaspar and Jaiswal, 2010
[25] Jin et al., 2013
[26] Beta-transducin repeat-containing protein (β-TrCP)
[27] S-phase kinase-associated protein 1 (Skp1)
[28] Chowdhry et al., 2013; Rada et al., 2011; 2012
[29] Godin et al., 2010
[30] Glycogen synthase kinase 3 β (GSK3-β)
[31] A phosphodegron is defined as one or a series of phosphorylated residues on the substrate that directly interact with a protein–protein interaction domain in an E3 Ubiquitin-ligase (e.g. an F-box protein), thereby linking the substrate to the conjugation machinery (Ang and Harper, 2005)
[32] Chowdhry et al., 2013; Rada et al., 2011; 2012

			inhibition GSK3-β activity attenuated symptoms, and prolonged survival[33]
USP15[34]	Interacts with Keap1[35]	Specifically deubiquitinates Keap1 leading to enhanced Keap1-Cul3-Rbx1 complex formation, stability and enzymatic activity, and consequent Nrf2 ubiquitination and degradation	mRNA and protein levels of USP15 were decreased in N171-82Q HD mice[36]
Cav-1[37]	Nrf2 Cav-1-binding domain (amino acids 281-289) interacts with the scaffolding domain of Cav-1 (amino acids 82-101)[38]	Recruits Nrf2 to caveolae plasma membranes, blocking its nuclear translocation and supressing its transcriptional activity	Cav-1 protein was shown to be increased ST*Hdh*$^{Q111/Q111}$ cells and to interact with mHtt, resulting in an increase of intracellular cholesterol levels in the CN of *post-mortem* HD patient's brains (Vonsattel grade 4), in the striatum of HdhQ111/Q111 mice, and in ST*Hdh*$^{Q111/Q111}$ cells[39]
PTMA[40]	The nuclear protein PTMA binds to the double glycine repeat (DGR)/Kelch domain of Keap1, in the late response (post-induction of Nrf2) to oxidative stress[41]	Transports Keap1 in complex with Cul3 and Rbx1 into the nucleus, presumably to regulate nuclear level of Nrf2 and rapidly switch off the activation of Nrf2 downstream gene expression	PTMA was shown to interact with mHtt, enhancing mHtt aggregates but preventing mHtt-induced cytotoxicity and cell death in both non-neuronal and neuronal cell models expressing N-terminal mHtt fragments[42]
SIRT1[43]	Interacts with Nrf2[44]	Deacetylates Nrf2 decreasing its transcriptional activity and enhancing its cytoplasmic localization	SIRT1 was shown to interact with mHtt, resulting in loss of SIRT1 deacetylase activity, hyperacetylation of SIRT1-target proteins, and decreased neuroprotection. However, a phase II clinical trial is

[33] Chiu et al., 2011; Valencia et al., 2010
[34] Ubiquitin specific peptidase 15 (USP15)
[35] Villeneuve et al., 2013
[36] Menzies et al., 2010
[37] Cholesterol-binding protein caveolin-1 (Cav-1)
[38] Li et al., 2012; Volonte et al., 2013
[39] del Toro et al., 2010; Trushina et al., 2006
[40] Prothymosin-alpha (PTMA)
[41] Karapetian et al., 2005; Niture and Jaiswal, 2009; Sun et al., 2007
[42] Dong et al., 2012
[43] Sirtuin 1 (SIRT1)
[44] Kawai et al., 2011

			being conducted to treat HD with the highly specific Sirt1 inhibitor, EX-527[45]
KPNA6[46]	KPNA6 directly interacts with the Kelch domain of Keap1[47]	Facilitates Keap1 nuclear import, attenuates Nrf2 signaling, and accelerates Nrf2 clearance from the nucleus during the post-induction phase, therefore promoting cytosolic restoration of Nrf2	-
p65[48]	Physically interacts *in vivo* and *in vitro* with Keap1[49]	Induces Keap1 nuclear translocation, inhibiting Nrf2-dependent transcription, and enhancing Nrf2 ubiquitination	Decreased expression and activity of p65 was verified in the striatum of HD patients and R6/2 HD mice, and in ST*Hdh*$^{Q111/Q111}$ cells[50]
λ-PP[51]	Interacts with Nrf2[52]	Dephosphorylates Nrf2	-
Src subfamily of tyrosine kinases Fyn, Src, Yes and Fgr kinases	Interacts with Nrf2[53]	Phosphorylates Nrf2 at Tyr568, resulting in Nrf2 nuclear export and degradation, thereby providing a negative feedback mechanism to switch off Nrf2 activation and restore normal cellular homeostasis	-

[45] Arrowsmith et al., 2012; Jeong et al, 2011; Jiang et al, 2012
[46] Importin alpha 7 (karyopherin alpha 6; KPNA6)
[47] Sun et al., 2011
[48] p65, a subunit of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factor
[49] Yu et al., 2011
[50] Ghose et al., 2011; Laprairie et al., 2014
[51] Lambda protein phosphatase (λ-PP)
[52] Apopa et al., 2008
[53] Jain and Jaiswal, 2006; Niture et al., 2011

Table 1.4 – Nrf2 cellular endogenous activators.

Endogenous activators	Interaction	Function	In HD
PI-3K/Akt[54]	No direct interaction between PI-3K or Akt and Nrf2 was reported until now	Activation of PI-3K/Akt results in increased Nrf2 nuclear translocation and transactivation, which could be due to its direct phosphorylation by Akt or Akt phosphorylation and inhibition of GSK3-β, thus preventing Nrf2 phosphorylation which results in the binding of β-TrCP and consequent Nrf2 degradation[55]	(See targeting insulin/IGF-1/PI-3K/Akt/Erk pathway section)
PKC[56]	PKC interacts with the Neh2 domain of Nrf2 (which Keap1 binds)[57]	Phosphorylates Nrf2 at Ser40, leading to Keap1-Nrf2 dissociation and Nrf2 nuclear translocation in response to oxidative stress	PKC isoforms (conventional PKC-α and -β) mRNA and protein levels were shown to be significantly reduced in both striatum and cortex of symptomatic R6/2 HD mice, and in the putamen of HD patients. Also, mHtt was shown to associate with three PKC isoforms (conventional PKC-α, novel PKC-δ, and atypical PKC-ζ), whereas novel PKC-δ specifically accumulated and associated with mHtt intranuclear aggregates in brain tissues from symptomatic R6/1 and R6/2 HD mouse models[58]
MAPKs (JNK1 and 2, Erk2, p38, MEKK3 and 4)[59]	Interacts with Nrf2[60]	Phosphorylates Nrf2 at serines 215, 408, and 577, resulting only in a moderate contribution for Nrf2 nuclear translocation and transactivity	Activation of JNK and p38 have been demonstrated in several HD models and were associated with enhanced neurotoxicity, whereas increased Erk activity has been largely associated with HD neuroprotection[61]
CK2[62]	Interacts with Nrf2 Neh4 and Neh5 transcription activation	Mediates two steps of phosphorylation of Nrf2 within its TA domains Neh4 and Neh5, resulting in two forms of Nrf2 migrating with differing MW at 98 kDa (Nrf2-98), which is	CK2 phosphorylates mHtt at serines 13 and 16, resulting in decreased toxicity and cell death, and altered mHtt subcellular localization[64]

[54] Phosphatidylinositol 3-kinase (PI-3K)/Protein kinase B (Akt)
[55] Chowdhry et al., 2013; Lee et al., 2001; Nakaso et al., 2003; Wang et al., 2008
[56] Protein kinase C (PKC)
[57] Huang et al., 2002
[58] Harris et al., 2001; Hashimoto et al., 1992; Rajput et al., 2011; Zemskov et al., 2003a,b
[59] Mitogen-activated protein kinases (MAPKs); c-Jun N-terminal kinase (JNK); extracellular signal-regulated kinase (Erk) 2; mitogen-activated protein kinase 14 (p38); mitogen-activated protein kinase kinase kinase (MEKK) 3 and 4
[60] Sun et al., 2009; Xu et al., 2006
[61] Apostol et al., 2006; Gianfriddo et al., 2004; Perrin et al., 2009
[62] Casein kinase 2 (CK2)

	(TA) domains, and also interacts with Keap1[63]	transcriptional active, and 118 kDa (Nrf2-118), which do not bind to ARE, and are more susceptible for degradation. Also, CK2 phosphorylates Keap1 at Thr55, leading to dissociation of the Keap1-Cul3-Rbx1 complex from Nrf2, and consequent activation of Nrf2	
PERK[65]	Interacts with Nrf2[66]	Phosphorylates Nrf2 during endoplasmic reticulum stress response, promoting its dissociation from Keap1, nuclear translocation, and transactivation	PERK inhibition or overexpression was shown to not affect mHtt aggregation[67]
CBP[68]	When in the nucleus, Neh4 and Neh5 TA domains of Nrf2 bind individually and cooperatively to CBP[69]	After binding to Nrf2 Neh4 and Neh5 TA domains of Nrf2, CBP acetylates the Neh1 DNA-binding domain of Nrf2, leading to increased Nrf2 promoter-specific DNA binding to ARE	CBP was shown to interact with mHtt, resulting in inhibition of CBP acetyltransferase and transcriptional activity, altered CBP normal localization, CBP recruitment to mHtt aggregates, enhanced CBP ubiquitination and degradation, and increased cytotoxicity and cell death[70]
KPNA1/ KPNB1[71]	Interacts with Nrf2[72]	KPNA1 and KPNB1 forms a heterodimer nuclear import system which facilitates Nrf2 nuclear import	-
BRCA1[73]	Physically interacts with Nrf2[74]	Promotes Nrf2 stability and transactivation	The levels of non-phosphorylated BRCA1 were significantly decreased whereas the phosphorylated (DNA damage responsive form of BRCA1) was significantly increased in ST*Hdh*$^{Q111/Q111}$ cells and in the striatum of symptomatic R6/2 HD mice, indicating increased DNA damage in HD[75].
p21[76]	Physically interacts with Nrf2[77]	Competes with Keap1 for Nrf2 binding, and disrupts Keap1-dependent Nrf2 ubiquitination, inducing Nrf2 transcriptional	mHtt exon1 protein repressed the transcription of p21[78].

[64] Atwal et al., 2011; Fan et al., 2008
[63] Apopa et al., 2008; Niture and Jaiswal, 2010; Pi et al., 2007
[65] Protein kinase RNA-like endoplasmic reticulum kinase (PERK)
[66] Cullinan et al., 2003
[67] Lee et al., 2012
[68] CREB-binding protein (CBP)
[69] Katoh et al., 2001; Sun et al., 2009
[70] Choi et al., 2012; Jiang et al., 2003; 2006; Steffan et al., 2000; 2001; Nucifora et al., 2001
[71] Karyopherin alpha 1 (importin alpha 5) (KPNA1) and karyopherin (importin) beta 1 (KPNB1)
[72] Theodore et al., 2008
[73] Breast cancer 1, early onset (BRCA1)
[74] Gorrini et al., 2013
[75] Jeon et al., 2012
[76] Cyclin-dependent kinase inhibitor 1A (p21, Cip1, Waf1)
[77] Chen et al., 2009

		activity	
p62[79]	Physically interacts with the C-terminus Kelch-DC domain of Keap1[80]	Competes with the interaction between Nrf2 and Keap1, resulting in Nrf2 stabilization and transactivation, and Keap1 degradation via autophagy	p62 was shown to interact with mHtt and to co-localize with mHtt nuclear inclusions in the striatum and frontal cortex of HD patients (Vonsattel grades 3, 3-4 and 4), and in the whole brain, in the striatum and hippocampus of symptomatic R6/1 and R6/2 HD mouse models. Also, increased p62 protein levels were found in the striatum of HD patients, and R6/2 and YAC128 HD mouse models, whereas in in the striatum, cortex and hippocampus of early symptomatic 12 week-old R6/1 and in ST*Hdh*$^{Q111/Q111}$ cells, decreased p62 protein levels were verified[81]
WTX[82]	Binds to Keap1[83]	Inhibits Nrf2 ubiquitination and degradation	-
DPP3[84]	Binds to Keap1[85]	Prevents Keap1 association with Nrf2, thus inhibiting Nrf2 ubiquitination and driving Nrf2-dependent transcription	-

[78] Steffan et al., 2000
[79] Selective autophagy substrate adaptor sequestosome1 (p62)
[80] Bae et al., 2013; Komatsu et al., 2010; Copple et al., 2010
[81] Jin et al., 2013; Lee et al., 2012; Nagaoka et al., 2004; Rué et al., 2013
[82] APC membrane recruitment protein 1 (Wilms tumor on the X; WTX, AMER1)
[83] Camp et al., 2012
[84] Dipeptidyl-peptidase 3 (DPP3) protein
[85] Hast et al., 2013

Table 1.5 – Nrf2/ARE target antioxidant genes.

System	Target genes	Function
Superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) decomposition	Superoxide dismutase (SOD) 1-3[86]	Catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and molecular oxygen
	Catalase[87]	Catalyzes the conversion of H_2O_2 into water and molecular oxygen
Glutathione	Glutathione reductase (GRed)[88]	Catalyzes the NADPH-dependent reduction of disulfide-oxidized glutathione (GSSG) to thiol-reduced glutathione (GSH) [GSSG + NADPH + $H^+ \rightarrow$ 2GSH + $NADP^+$]
	Glutathione peroxidase (GPx) 1-4[89]	Converts 2GSH and H_2O_2 into GSSG and H_2O
	Glutathione S-transferase (GST)[90]	Catalyzes the conjugation of various electrophiles, reactive alkenals, and numerous other xenobiotic to GSH
	Glutamate-cysteine ligase (GCL) catalytic (GCLc) and modifier (GCLm) subunits[91]	Catalyzes the first step of the synthesis of GSH, involving the ligation of glutamate to L-cysteine, in an ATP-dependent manner, producing gamma-glutamylcysteine (γ-GC)
	Glutathione synthetase (GS)[92]	Catalyzes the second step of GSH synthesis, involving the conversion of γ-GC and glycine, in an ATP-dependent manner, into GSH
	Multidrug resistance-associated protein 1 (Mrp1)[93]	Catalyzes the exportation of glutathione-S conjugates and glutathione itself to the extracellular millieu in an ATP-dependent manner

[86] Cho et al., 2002; Huang et al., 2014; Park and Rho, 2002; Zhang et al., 2013
[87] Zhu et al., 2005
[88] Shih et al., 2003; Thimmulappa et al., 2002; Zhu et al., 2005
[89] Banning et al., 2005; Cho et al., 2002; Thimmulappa et al., 2002
[90] Cho et al., 2002; Shih et al., 2003; Chanas et al., 2002
[91] Jeyapaul and Jaiswal, 2000; Moinova and Mulcahy, 1999; Shih et al., 2003; Wild et al., 1999
[92] Shih et al., 2003
[93] Hayashi et al., 2003; Ji et al., 2013; Maher et al., 2007; Shih et al., 2003

	Gamma-glutamyl transpeptidase (γ-GT)[94]	Catalyzes the cleavage of the gamma-glutamyl bond of GSH and the transfer of gamma-glutamyl to water or to some amino acids and peptides
	Excitatory amino acid carrier 1 (EAAC1)[95]	Mediates the cysteine uptake in cells
	Cystine/glutamate antiporter (Xc-)[96]	Mediates the oxidized form of cysteine (cystine) uptake in cells
NADPH-producing enzymes	Glucose-6-phosphate dehydrogenase (G6PD)[97]	Catalyzes the conversion of D-glucose 6-phosphate and $NADP^+$ into 6-phospho-D-glucono-1,5-lactone (6PGL) and NADPH
	6-Phosphogluconate dehydrogenase (6PGD)[98]	Catalyzes the conversion of 6-phosphogluconate and $NADP^+$ into ribulose 5-phosphate and NADPH
	Malic enzyme[99]	Catalyzes the conversion of (S)-malate and $NADP^+$, into pyruvate, CO_2, and NADPH.
Thioredoxin	Thioredoxin (Trx)[100]	Catalyzes the reduction of oxidized proteins by exchanging cysteine thiol and protein disulfides
	Thioredoxin reductase (TrxR)[101]	Catalyzes the reduction of oxidized Trxs by consuming NADPH
	Peroxiredoxin (Prdx)[102]	Catalyzes the reduction of peroxides by using Trxs
	Sulfiredoxin-1 (SRXN1)[103]	Catalyzes the protein deglutathionylation and the reduction of Prdx-cysteine

[94] Zhang et al., 2006
[95] Escartin et al., 2011
[96] Shih et al., 2003
[97] Thimmulappa et al., 2002
[98] Thimmulappa et al., 2002
[99] Thimmulappa et al., 2002
[100] Im et al., 2012; Kim et al., 2003; Nakaso et al., 2003
[101] Chen et al., 2005; Suvorova et al., 2009; Tanito et al., 2007
[102] Chowdhury et al., 2009; Kim et al., 2007; Erttmann et al., 2011

		sulfinic acid
Detoxification	NAD(P)H:quinone oxidoreducase (NQO1)[104]	Catalyzes the two-electron reduction of endogenous quinones, using NADH or NADPH as reducing cofactors, and producing the redox-stable hydroquinone
	Heme oxygenase 1 (HO-1)[105]	Catalyzes the degradation of toxic heme into the antioxidant biliverdin, free iron, and carbon monoxide

[103] Bae et al., 2009; Soriano et al., 2008
[104] Thimmulappa et al., 2002
[105] Alam et al., 1999; 2003; Thimmulappa et al., 2002

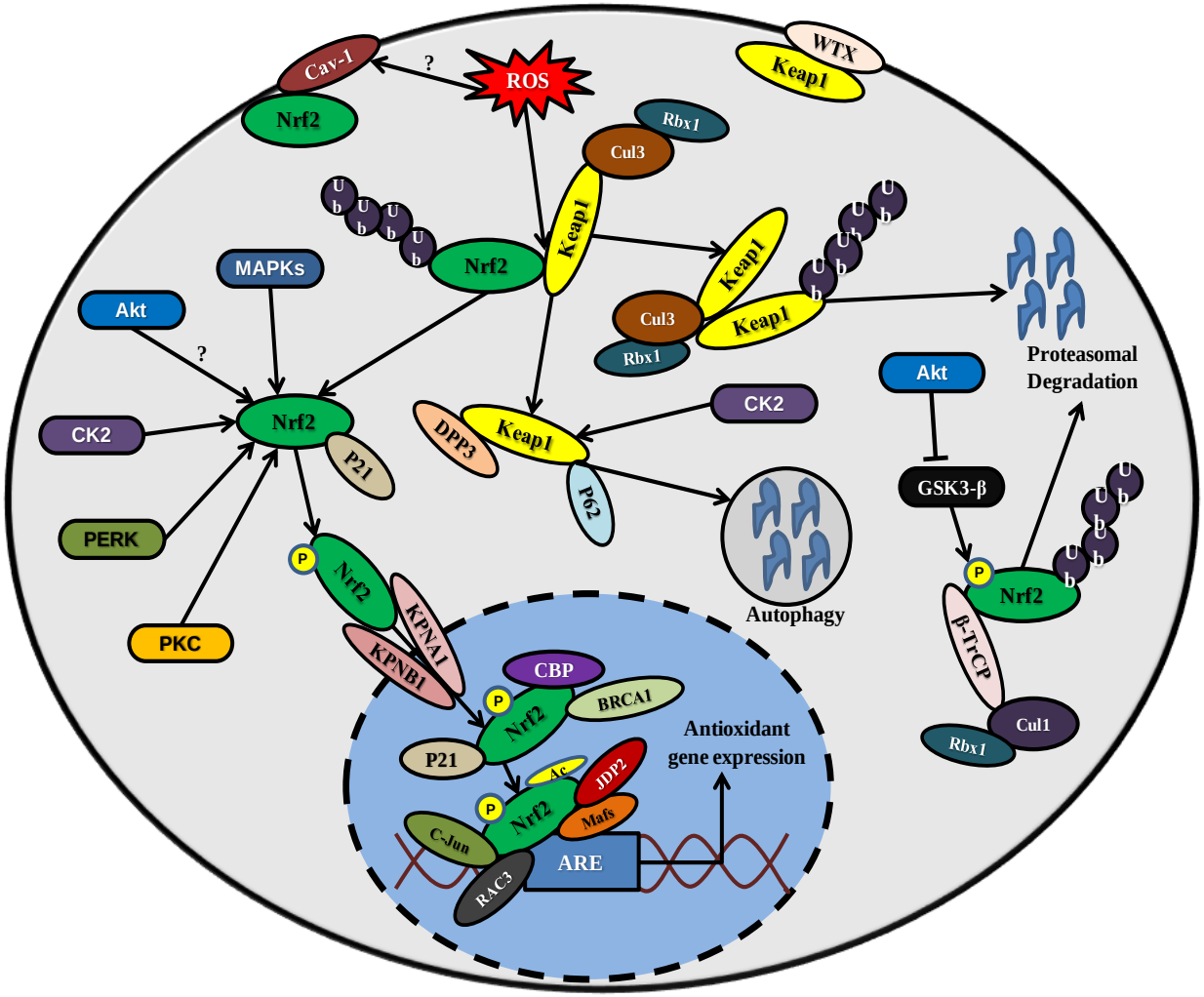

Cav-1
?
ROS
WTX
Keap1
Nrf2
Rbx1
Cul3
Keap1
Ub
Nrf2
MAPKs
Akt
?
Keap1
Cul3
Rbx1
Keap1
Ub
Proteasomal
Degradation
Akt
CK2
Nrf2
DPP3
Keap1
CK2
P21
P62
GSK3-β
Autophagy
PERK
P
Nrf2
KPNA1
KPNB1
β-TrCP
P
Nrf2
Ub
PKC
CBP
BRCA1
P
Nrf2
Antioxidant
gene expression
Cul1
Rbx1
P21
Ac
JDP2
P
Nrf2
Mafs
C-Jun
ARE
RAC3

Figure 1.3 – Nrf2 pathway regulation upon oxidative stress conditions. Under normal conditions, Kelch-like ECH associated protein 1 (Keap1) is linked to the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to promote its ubiquitination and degradation through the Keap1 binding to cullin 3 (Cul3)-ring box protein 1 (Rbx1) ubiquitin (Ub) ligase complex. Also, glycogen synthase kinase 3 beta (GSK3-β) phosphorylates Nrf2 to promote beta-transducin repeat-containing protein (β-TrCP) to act as a receptor substrate for the S-phase kinase-associated protein 1 (Skp1)-cullin 1 (Cul1)-Rbx1 ubiquitin ligase complex, leading to Nrf2 ubiquitination and degradation. Caveolin-1 (Cav-1) also recruits Nrf2 to caveolae membranes inhibiting its activation. However, under conditions of increased reactive oxygen species (ROS) formation, Keap1 cysteine residues are oxidized resulting in a displacement from Nrf2 and formation of Keap1 dimers which results in Keap1 ubiquitination by the Cul3-Rbx1 ubiquitin ligase complex. Also Keap1 phosphorylation by casein kinase 2 (CK2), its association to the APC membrane recruitment protein 1 (Wilms tumor on the X; WTX, AMER1) or to dipeptidyl-peptidase 3 (DPP3) protein prevents Keap1 binding to Nrf2. Furthermore, Keap1 association with the selective autophagy substrate adaptor sequestosome1 (p62), results in Keap1 degradation via autophagy. Moreover, cyclin-dependent kinase inhibitor 1A (p21, Cip1, Waf1) competes with Keap1 for Nrf2 binding, and disrupts Keap1-dependent Nrf2 ubiquitination. Then several kinases (e.g. mitogen-activated protein (MAP) kinases (MAPKs), casein kinase 2 (CK2), protein kinase RNA-like endoplasmic reticulum kinase (PERK), protein kinase C (PKC)) are able to phosphorylate Nrf2, resulting in its translocation to the nucleus. Also, Akt (or protein kinase B) phosphorylates and inhibits GSK3-β leading to decreased Nrf2 ubiquitination by β-TrCP-Cul1-Rbx1 complex. After Nrf2 phosphorylation, Nrf2 is imported to the nucleus via the heterodimer nuclear import system composed by karyopherin alpha 1 (importin alpha 5) (KPNA1) and karyopherin (importin) beta 1 (KPNB1). Inside the nucleus, Nrf2 is acetylated by the CREB-binding protein (CBP) and associates with breast cancer 1, early onset (BRCA1) and p21 resulting in the enhancement of Nrf2 binding to the antioxidant response element (ARE) where it forms heterodimers with the receptor-associated coactivator 3 (RAC3)/AIB-1/steroid receptor coactivator-3, c-Jun, c-Jun dimerization protein 2 (JDP2), and small Mafs (MafK, MafG or MafF) proteins for high-affinity binding, leading to increased promoter specific DNA binding to ARE, and consequent enhancement of Nrf2-ARE transcription of antioxidant genes.

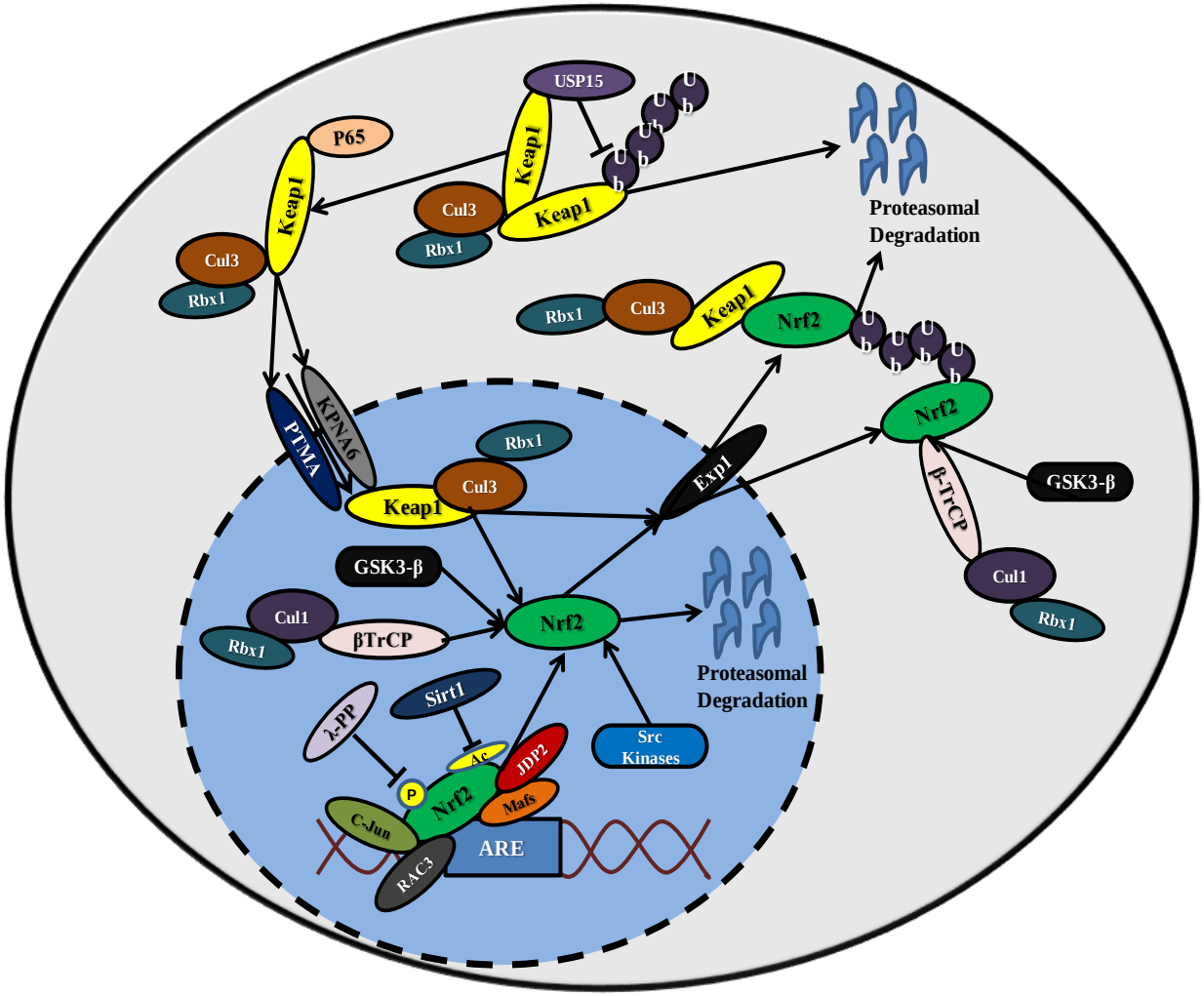

USP15
Ub
P65
Keap1
Cul3
Rbx1
Proteasomal
Degradation
Nrf2
KPNA6
PTMA
Exp1
GSK3-β
Cul1
βTrCP
Sirt1
Ac
JDP2
Mafs
C-Jun
RAC3
ARE
Src
Kinases
P

Figure 1.4 – Nrf2 pathway regulation in the late response (post-induction of Nrf2) to oxidative stress. After oxidative stress conditions, Keap1 is deubiquitinated by ubiquitin specific peptidase 15 (USP15) and associates with p65, a subunit of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factor, which leads to nuclear import of Keap1 in complex with Cul3-Rbx1 through prothymosin-alpha (PTMA) and/or importin alpha 7 (karyopherin alpha 6; KPNA6). In the nucleus, Nrf2 is dephosphorylated by lambda protein phosphatase (λ-PP) and deacetylated by sirtuin 1 (SIRT1), leading to its dissociation from the antioxidant response element (ARE) and consequent association with Keap1-Cul3-Rbx1 or GSK3-β/β-TrCP-Cul1-Rbx1 ubiquitin (Ub) complexes, resulting in Nrf2 degradation or nuclear export via exportin 1 (Exp1) and consequent degradation in the cytoplasm.

Therefore, one way that cells regulate its endogenous antioxidant capacity is through activation of Nrf2. In HD, the protection against ROS production may be affected. In fact, in the plasma and erythrocytes of HD patients, a reduction in the antioxidant systems correlated with disease stage (Túnez et al., 2011). The basal levels of striatal Nrf2 and ARE activity were significantly reduced in N171-82Q HD mice (Chaturvedi et al., 2010), and in ST*Hdh*$^{Q111/Q111}$ cells (Jin et al., 2013), respectively, whereas Nrf2 overexpression reduced the number of fragmented mitochondria in striatal ST*Hdh*$^{Q111/Q111}$ cells (Jin et al., 2013). In this respect, Nrf2-deficient cells and neurons derived from Nrf2 knockout mice were shown to be significantly more vulnerable to malonate or 3-NP (Calkins et al., 2005; Shih et al., 2005). Importantly, intrastriatal transplantation of Nrf2-overexpressing astrocytes before treatment with malonate or 3-NP had a strong neuroprotective effect (Calkins et al., 2005), revealing the importance of Nrf2 in the context of mitochondrial dysfunction associated with HD. On the other hand, mHtt was described to activate Nrf2-responsive genes in a PC12 model of HD (van Roon-Mom et al., 2008). Also, although no changes in Nrf2 protein levels were detected in the striatum of pre-, early-, and symptomatic R6/1 HD mice, Nrf2 protein levels were significantly increased in cortex at late stages of the disease, when compared to wild-type mice (Rue et al., 2013). Nrf2-target antioxidant proteins were also been reported to be modified in HD. Although, no differences were found in total superoxide dismutase (SOD), SOD1 (Cu/Zn-SOD) and 2 (Mn-SOD) and in glutathione peroxidase (GPx) activities, significantly lower specific activities for catalase were found in skin fibroblasts from HD patients (del Hoyo et al., 2006). In fact, reduced activities of catalase (Zanella et al., 1980), SOD1, and GPx (Chen et al., 2007) were also detected in HD patient's erythrocytes. Moreover, GSH levels were found to be decreased in plasma (Klepac et al., 2007) and oxidized GSH (GSSG) levels were also decreased in the CN of HD patients (Sian et al., 1994). Conversely, proteomic analysis of HD *post-mortem* striatal and cortical brain samples revealed increased activities of SOD2 and catalase, and an induction of GPx1 and 6 and peroxiredoxin (Prdx) 1, 2 and 6 (Sorolla et al., 2008). Moreover, increased GRed and G6PD activities were found in erythrocytes from HD patients, compared to control individuals (Zanella et al., 1980). Also, a significant increase in gamma-glutamyl transpeptidase (γ-GT) activity in human *post-mortem* HD CN was observed and the levels of γ-GT significantly increased with increasing duration of illness (Butterworth et al., 1985). In addition, heme oxygenase 1 (HO-1) was elevated

in human HD striatum and cortex as compared with age-matched control brain individuals (Browne et al., 1999). Also, in animal models of HD, deregulated antioxidant capacity has been described. A significant increase in total SOD and SOD1 activities was observed in the striatum of young (19 week-old) R6/1 HD mice; however, a decrease in total SODs activities was observed in the striatum of this HD animal model at the age of 35 weeks, when mice exhibited a severe phenotype (Santamaria et al., 2001). Conversely, significant increases in mRNA levels of GPx1, catalase and SOD1 were observed in ST*Hdh*$^{Q111/Q111}$ knock-in HD striatal cells (Lim et al., 2008). Although GPx transcription was unaltered, increased GRed, SOD2, and glutamate-cysteine ligase (GCL) catalytic subunit (GCLc) transcription was verified in the basal ganglia of 8-week-old R6/2 HD mice, whereas GSH levels were increased in the forebrain of 5-6 and 11-12 week-old R6/2 HD mice (Fox et al., 2004). Also, GPx activity was unchanged in the striatum of R6/1 HD mice from 11 to 35 weeks of age (Perez-Severiano et al., 2004). In contrast, 8 and 12 week-old R6/2 HD mice showed increased GSH levels in the striatum (Tkac et al., 2007). Although the striatum and cortex of ~12 week-old R6/2 mice did not present any changes in cellular GSH, mitochondrial GSH was significantly increased compared to wild-type mice (Choo et al., 2005); nevertheless, GCL was reported to be actively decreased in both striatum and cortex of R6/2 HD mice, not explaining the increase in GSH levels (Choo et al., 2005).

1.3 Neuroprotective targets in Huntington's disease

Presently there is no effective protective treatment or cure for HD. Therefore, several approaches have been tested to prevent or delay disease progression. In this section some targets for neuroprotection in HD are described.

1.3.1 Targeting Nrf2 and use of antioxidant therapies

As delineated before in this thesis, a central role for the induction of the cellular antioxidant defense is carried out by the transcription factor Nrf2. Oxidative stress was reported as a cytotoxic mechanism in several HD models and patients, and Nrf2 pathway seems to be altered in HD as well as the antioxidants that are dependent of Nrf2. Thus, the discovery of novel drugs that increase Nrf2 activation and consequently induce the expression of antioxidant defenses or directly scavenge ROS might be relevant for delaying HD progression. Here we describe some known Nrf2 inducers as well as antioxidants that have shown to be protective in HD.

Sulforaphane is a natural dietary isothiocyanate and an electrophile that can react with protein thiols to form thionoacyl adducts and is believed to affect the Cys residues in Keap1 protein, therefore releasing Nrf2 from Keap1 (Keum, 2011). Treatment with sulforaphane enhanced mHtt degradation, reduced mHtt cytotoxicity (Liu et al., 2014), and increased ARE activity in ST*Hdh*$^{Q111/Q111}$ cells (Jin et al., 2013). *tert*-Butylhydroquinone (*t*-BHQ) is a metabolite of the widely used food antioxidant butylated hydroxyanisole, approved for human use by FDA. *t*-BHQ possesses an oxidizable 1,4 diphenolic structure that confers its potent ability to dissociate Keap1/Nrf2 complex (Van Ommen et al., 1992), and appears to have a Nrf2-specific action *in vivo* since dietary supplementation of *t*-BHQ attenuated 3-NP-induced toxicity in Nrf2$^{+/-}$ but not in Nrf2$^{-/-}$ mice (Shih et al., 2005). Importantly, *t*-BHQ significantly increased nuclear Nrf2 protein levels and glutathione S-transferase (GST) activity, and prevented increased lipid peroxidation in rat striatal slices treated with QA or 3-NP (Tasset et al., 2010). Dimethyl fumarate (DMF) is an orally bioavailable fumaric acid ester that is metabolized to methyl hydrogen fumarate (Kees, 2013). DMF was reported to increase neuronal Nrf2 and promote recovery in R6/2 and YAC128 HD mouse models (Ellrichmann et al., 2011). Oral administration of triterpenoids upregulated Nrf2/ARE induced genes, decreased

oxidative stress, improved motor performance, rescued striatal atrophy and increased longevity in N171-82Q mice (Stack et al., 2010). Moreover, synthetic triterpenoids were shown to activate Nrf2 and rendered protection against 3NP-mediated increased DNA and protein oxidation, lipid peroxidation, and disrupted glutathione homeostasis (Yang et al., 2009). Lipoic acid (LA) is an essential cofactor for several enzyme complexes and is present in mitochondria as a cofactor for PDH and alpha-ketoglutarate dehydrogenase complexes. It is an effective antioxidant and has been used to treat diseases associated with impaired energy metabolism (Johri and Beal, 2012). LA can also increase Nrf2-dependent transcriptional activity by forming lipoyl-cysteinyl mixed disulfides on Keap1 (Dinkova-Kostova et al., 2002), and thus decreasing Keap1 binding to Nrf2. In both the R6/2 and N171-82QHD mouse models, dietary supplementation with LA resulted in significant extension of survival and delayed weight loss in N171-82Q transgenic mice (Andreassen et al., 2001).

Ascorbic acid is a potent antioxidant which oxidizes readily to dehydroxyascorbic acid in the presence of ROS (Johri and Beal, 2012). Despite symptomatic R6/1 HD mice did not show any changes in ascorbate levels in the striatum, cortex and cerebellum (Petersén et al., 2001), deficits in ascorbate release in the striatum began in the presymptomatic phase of R6/2 HD mice (Rebec et al., 2002), which was attributed to dysfunctional corticostriatal pathway (Dorner et al., 2009), and in symptomatic CAG140 knock-in HD mice (Dorner et al., 2007). Moreover, regular systemic administration of ascorbate in R6/2 HD mice beginning at symptom onset restored the behavior-related release of ascorbate in striatum and attenuated the neurological motor signs of HD (Rebec et al., 2003; 2006). N-acetyl-L-cysteine (NAC) is a known GSH precursor, and it has been widely used to increase GSH levels in several models of oxidative damage. Treatment with GSH ethyl ester (GSHee), a cell permeable form of GSH, or NAC significantly protected against mHtt-mediated cell death (Wyttenbach et al., 2002), disruption in mitochondrial transmembrane potential ($\Delta\Psi m$) and the increase in ROS formation, $^{\bullet}NO$ levels and protein oxidation (Firdaus et al., 2006), in COS-7 or SK-N-SH cells expressing mHtt exon1 with 74Q or 103Q, respectively, and completely prevented 3-NP-mediated decrease in $\Delta\Psi m$ in ST*Hdh*$^{Q111/Q111}$ striatal cells (Mao et al., 2006). Indeed, it was described that expanded polyQ directly inhibit mitochondrial function by inducing oxidative stress, which was prevented with NAC treatment in isolated

mitochondria (Fukui and Moraes, 2007). Taken together, these results demonstrate that antioxidant therapy is beneficial to counteract mHtt-induced cytotoxicity.

1.3.2 Targeting insulin/IGF-1/PI-3K/Akt/Erk pathway

Insulin, insulin-like growth factor (IGF)-1 and -2 belong to the insulin-like peptide hormone superfamily. Insulin is mainly produced by beta-pancreatic cells, which acts to decrease blood glucose levels by stimulating glucose uptake after binding and activating the insulin receptor (IR). Insulin also promotes the transport of amino acids from the bloodstream into cells, increasing the rate of amino acid incorporation into proteins, and stimulates lipid synthesis from carbohydrates, decreases fatty acid release from tissues and leads to a net increase in total body lipid stores (Ghasemi et al., 2013a,b). Insulin present in adult CNS is mainly derived from beta-pancreatic cells, which can get across the blood-brain barrier by a carrier-mediated, saturable, regulatable, and temperature-sensitive active transporter system (Banks, 2004; Burns et al., 2007). However, insulin and also IGF-1 can be produced in the brain, where they appear to be involved in the regulation of brain metabolism, neuronal differentiation and neuromodulation, after activating the IR and IGF-1 receptor (IGF1R) which are both expressed in neurons (Cardona-Gomez et al., 2001; Havrankova et al., 1978a,b; Hoyer, 2004; Rotwein et al., 1988). Due to structural and functional homology, insulin and IGF-1 can bind to (and activate) both IR and IGF1R, with insulin binding to the IR with higher affinity (< 1 nM) than IGF-1 (100–500-fold lower affinity), whereas IGF1R preferentially binds IGF-1 (< 1nM) as compared to insulin (100–500-fold lower affinity) (Rechler et al., 1980). However, the IGF1R receptor and the IR can also form functional hybrids that have similar affinities for IGF-1 and insulin, which is indicative of cooperation between these two receptors (Fernandez and Torres-Aleman, 2012).

The IR and IGF1R are both a membrane-bound glycoprotein formed by two subunits held together by disulfide bridges, an extracellular alpha-subunit and a transmembrane beta-subunit with intracellular tyrosine kinase activity (Kuemmerle, 2003; Shelton et al., 2004; Torres Aleman, 2005). Insulin, IGF-1 or IGF-2 binds to the extracellular alpha subunit of the receptor, resulting in conformational change, which enables ATP to bind to the intracellular component of the receptor's beta subunit. ATP binding in turn triggers phosphorylation of the beta subunit, conferring tyrosine kinase activity. This facilitates the recruitment of SHC (Src Homology-2 (SH2)-containing

protein), and insulin receptor substrate(s) (IRS), which are then tyrosine phosphorylated. Following activation, IRS binds and activates phosphatidylinositol 3-kinase (PI-3K) through its SH2 domains (Benarroch, 2012; Tseng et al., 2002; Wilcox, 2005), whereas SHC in turn interact with the adapter protein growth factor receptor bound protein-2 (GRB2), recruiting the SOS (son of sevenless) to the plasma membrane for activation of the membrane-associated guanine nucleotide-binding protein Ras (which also requires stimulation of the protein tyrosine phosphatase, non-receptor type 11 (PTPN11; or SHP2), through its interaction with receptor substrates such as GAB1 (GRB2 associated binding protein-1) or IRS1/2), which in turn stimulates a serine kinase cascade through the activation of Ser/Thr kinase V-Raf-1 murine leukemia viral oncogene homolog 1 (Raf-1 or c-Raf), MEK1/2 (MAP kinase kinases 1/2) and extracellular signal-regulated kinases (Erk) 1 and -2 (Heinrich et al., 2006; Hu et al., 2001; Polivka and Janku, 2013; Yart et al., 2001). After activation, PI-3K catalyzes the conversion of PIP2 (phosphatidylinositol-4,5-bisphosphate) to PIP3 (phosphatidylinositol-3,4,5-trisphosphate), which is reversed by PTEN (phosphatase and tensin homolog). Elevated PIP3 binds to the PH (pleckstrin homology) domain of Akt (protein kinase B) and PDPK1 (3-phosphoinositide dependent protein kinase-1) which in turn phosphorylates residue Thr308 on Akt, increasing cell glucose uptake by promoting the translocation of GLUT's to the membrane (Mora et al., 2005; Polivka and Janku, 2013). However, Akt can also be phosphorylated at Ser473 by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (Dowling et al., 2010). Phosphorylation of these two residues (Thr308 and Ser473) are necessary for full activation of Akt kinase activity (Alessi et al., 1997a,b; Feng et al., 2004); however, it was previously described that Akt phosphorylation on Thr308, but not Ser473, better correlates with Akt protein kinase activity (Vincent et al., 2011).

Akt and Erk1/2 are considered to be crucial elements in a multiplicity of pathways to promote cell survival and prevent cell damage (Chong et al., 2005; de la Monte et al., 2012; Numakawa et al., 2013). Erk1/2 promotes cell survival through preventing activation of the pro-apoptotic protein BAD (B-Cell CLL/Lymphoma 2 (BCL2)-associated agonist of cell death), and through its translocation to the nucleus where it catalyzes the phosphorylation of transcription factors such as Elk-1 (ELK1, member of ETS oncogene family), initiating a transcriptional program that leads to cellular proliferation or differentiation (Cohen-Armon, 2007; Jin et al., 2004). Activation of PI-3K/Akt pathway leads to activation or inactivation by phosphorylation of several

downstream targets, including the activation of mTORC1 (which in turn has a number of downstream biological effects including activation of mRNA translation via the phosphorylation of downstream targets (eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and ribosomal protein S6 kinase, 70kDa, polypeptide 1 (RPS6KB1)), and suppression of autophagy through the phosphorylation and inhibition of autophagy related 13 (Atg13) and unc-51 like autophagy activating kinase 1 (ULK1)), and the inactivation of proteins involved in apoptotic pathways, such as BAD, caspase-9, glycogen synthase kinase 3 beta (GSK3-β), and the members of the forkhead boxO (FOXO) class of transcription factors -1 and -3, (Chong et al., 2012; Dowling et al., 2010; Kim et al., 2001; Wu et al., 2011). Akt is also known to phosphorylate CREB at Ser133, promoting the recruitment of CBP, and the complex formed by CREB and its co-activator CBP is competent to initiate gene transcription (Du and Montminy, 1998). Moreover, it was previously described that Akt can accumulate in mitochondria in its active state, inducing neuroprotection (Bijur and Jope, 2003; Mookherjee et al., 2007). Akt translocation to mitochondria was associated with increased expression and activity of complex I. Also, within mitochondria, Akt phosphorylated alpha and beta subunits of ATP synthase, leading to its activation, and phosphorylated and inactivated GSK3-β leading to activation of PDH (Li et al., 2013).

In HD, higher plasma IGF-1 was associated with cognitive decline in HD patients (Saleh et al., 2010), and genetic enhancement of IRS-2 levels in the brains of R6/2 HD mice significantly reduced life span and increased neuronal oxidative stress and mitochondrial dysfunction (Sadagurski et al., 2011). In contrast, insulin or IGF-1-induced IRS-2 activation caused the elimination of mHtt exon1 aggregates by macroautophagy (Yamamoto et al., 2006), and reduced IGF-1 mRNA levels were detected in *post-mortem* striatum and in fibroblasts of HD patients, and in ST*Hdh*$^{Q111/Q111}$ cells (Pouladi et al., 2010), as well as an age-dependent reduction in insulin mRNA, decreased PI-3K protein levels, diminished expression of key regulators of insulin gene transcription, decreased blood insulin and plasma IGF-1 levels, and impaired glucose tolerance in R6/2 mice (Andreassen et al., 2002; Duarte et al., 2011; Rajput et al., 2011). Moreover, peripheral or intranasal administration of IGF-1 enhanced blood insulin levels in R6/2 mice (Duarte et al., 2011), and enhanced IGF-1 cortical levels and improved motor activity and both peripheral and central metabolic abnormalities in YAC128 HD mice (Lopes et al., 2013), respectively. Indeed, intranasal administration IGF-1 in YAC128 HD mice activated Akt and concomitantly increased phosphorylation of mHtt on Ser421 (Lopes et al., 2013),

which was previously described to be decreased in the striatum of YAC128 HD mice (Warby et al., 2005), to reduce mHtt nuclear inclusions (Humbert et al., 2002), and to compensated anterograde and retrograde transport defects in HD cortical neurons (Zala et al., 2008), suggesting that mHtt phosphorylation by Akt is decreased in HD. In fact, in HD patients, a shorter inactive caspase-3 cleaved form of Akt was detected (Humbert et al., 2002; Colin et al., 2005), which can be associated with decreased p-(Ser473)Akt/Akt ratio in lymphoblasts and lymphocytes derived from HD patients (Colin et al., 2005), in ST*Hdh*$^{Q111/Q111}$ cells (Maglione et al., 2010), in HEK293 cells expressing mHtt with 68 CAG repeats (Nagata et al., 2011), and in the striatum of 12 week-old R6/2 HD mice (Ju et al., 2011). Furthermore, Akt and also Erk were previously described to protect against mHtt toxicity (Apostol et al., 2006; Lievens et al., 2008), suggesting that besides Akt, also Erk could be deactivated in HD. Indeed, decreased Erk activity was observed in ST*Hdh*$^{Q111/Q111}$ cells, and in the striatum of pre- and symptomatic R6/2 HD mice, whereas striatal cholinergic interneurons, the most resistant interneuron subtype in HD, showed increased p-Erk (Fusco et al., 2012; Ju et al., 2011; Sarantos et al., 2012), suggesting that Erk activation could be a target for the delay in disease progression.

1.3.3 Targeting energy metabolism

Energy metabolism is deregulated in HD as described earlier in this thesis. Therefore, the use of stabilizers or enhancers of energy metabolism have been tested in HD. Here we will focus on the most studied compounds in HD that revealed an improvement in energy metabolism.

Creatine has been shown to have both metabolic and antioxidant proprieties (Lawler et al., 2002). Althought creatine did not significantly reduce the levels of H_2O_2 or lipid peroxidation, this compound displayed a significant antioxidant scavenging capacity through its ability to remove 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS+) (a peroxidase substrate), $O_2^{\bullet-}$, and ONOO− (Lawler et al., 2002). In early HD patients, creatine treatment decreased 8-OHdG levels, increased serum and brain creatine levels and was shown to be safe and well tolerated (Hersch et al., 2006). In R6/2 and N171-82Q HD mouse models, creatine administration improved motor performance, extended mice survival, increased body and brain weight, decreased brain atrophy, delayed striatal neuron atrophy and the onset of diabetes, reduced the number of mHtt aggregates, and increased striatal ATP and creatine levels (Andreassen et al., 2001;

Dedeoglu et al., 2003; Ferrante at al., 2000; Yang et al., 2009), thus regulating metabolic dysfunction in the context of HD. The Huntington Study Group (HSG) and Massachusetts General Hospital (Steven M. Hersch, MD, PhD) are conducting a Phase 3 randomized, double-blind, placebo-controlled clinical trial in HD (CREST-E; clinicaltrials.gov).

CoQ_{10} is an antioxidant and component and enhancer of respiratory chain function that is under phase 3 clinical trial for HD (2CARE; clinicaltrials.gov), and was reported to be well tolerated in HD patients (Huntington Study Group Pre2CARE Investigators, 2010). Brain levels of CoQ_{10} were significantly lower in R6/2 mice, and the treatment with CoQ_{10} elevated the levels of CoQ_{10} and ATP in the brain, and CoQ_{10} levels in plasma while reducing 8-OHdG concentrations, significantly extended survival, improved motor performance and grip strength, reduced weight loss, brain atrophy, and mHtt inclusions in R6/2 HD mice (Smith et al., 2006). Furthermore, although creatine or CoQ_{10} treatment alone produced significant protection, combined treatment of CoQ_{10} and creatine showed an addictive effect by improving motor performance and extending survival in the R6/2 HD mice (Yang et al., 2009). Moreover, in rats treated with 3-NP, creatine or CoQ_{10}, or their combined supplementation decreased striatal volume damage, increased GSH, but not GSSG levels, and decreased MDA levels, whereas only creatine or CoQ_{10} combination decreased 8-OHdG levels in the striatum of 3-NP treated rats (Yang et al., 2009), suggesting that the combination treatment is more effective in reducing striatal damage induced by 3-NP. Therefore, targeting energy metabolism with creatine or CoQ_{10} or their combination might be effective in HD. In fact, both compounds are in clinical trials for HD.

1.3.4 Targeting transglutaminase

Transglutaminases are a family of ubiquitous thiol and calcium-dependent enzymes that catalyze post-translational modifications and cross-linking (transamidation) acyl transfer reaction between Q residues and the epsilon-amino group of lysine (K) residues between or within proteins, leading to the formation of epsilon-(gamma-glutamyl)-lysine isopeptide bound (Folk, 1983; Greenberg et al., 1991). They also catalyze a reaction between gamma-carboxamide group of a polypeptide-bound Q and a polyamine to form an (gamma-glutamyl)-polyamine isopeptide bound (polyamination) and the deamination of the gamma-carboxamide group of a Q residue within proteins (Folk, 1983; Greenberg et al., 1991). There are nine members of transglutaminase family,

which includes tissue transglutaminase or transglutaminase 2 (TG2) described to be highly active and expressed in brain, particularly in neurons (Appelt et al., 1996; Bailey and Johnson, 2004; Johnson et al., 1997; Lesort et al., 1999). TG2 has multiple enzymatic activities. Besides being a transamidating enzyme (Greenberg et al., 1991), TG2 can also be a signal transducing GTP-binding protein (Nakaoka et al., 1994), display protein disulfide isomerase activity (Hasegawa et al., 2003) and protein kinase activity (Mishra and Murphy, 2006). TG2 has been implicated in several cellular processes, including apoptosis, cell proliferation and differentiation, cell-cell and cell-extracellular matrix interactions, axonal growth and regeneration, signal transduction, transcription and regulation of mitochondrial function (Fesus and Piacentini 2002; Park et al., 2010). Moreover, TG2 has been implicated in several disorders, including neurodegenerative diseases (Grosso and Mouradian, 2012).

In HD, increased epsilon-(gamma-glutamyl)-lysine cross-links and TG2 levels and activity were verified in the brain of HD patients (Dedeoglu et al., 2002; Jeitner et al., 2001; Karpuj et al., 1999; Lesort e t al., 1999), and in the R6/2 (Dedeoglu et al., 2002), R6/1 (Mastroberardino et al., 2002), and YAC128 (Van Raamsdonk et al., 2005) HD mouse models. Moreover, polyQ repeat domains and mHtt were described to be substrates for TG2 (Kahlem et al., 1996; 1998; Cooper et al., 1997a,b; Zainelli et al., 2003), which induced cross-links in mHtt leading to the formation of insoluble aggregates (Zainelli et al., 2003; 2004), whereas genetic deletion of TG2 improved motor performances and survival in R6/2 and R6/1 HD mouse models (Bailey and Johnson, 2005; Mastroberardino et al., 2002), increased mitochondrial function in ST*Hdh*$^{Q111/Q111}$ cells, and protected against NMDA-induced toxicity in medium-sized spiny neurons from YAC128 HD mice (McConoughey et al., 2010). These data clearly indicates that TG2 is involved in mHtt-induced cytotoxicity and that inhibition of TG2 activity might be significant to delay HD progression.

One of the most used protective compounds in HD is cystamine. Cystamine is a linear aliphatic diamine composed of a disulfide bridge, generated from the oxidation of two cysteamine ($HS\text{-}CH_2\text{-}CH_2\text{-}NH_2$) residues. Besides being metabolized to cysteine, hypotaurine and taurine, oral or systemic cystamine administration resulted in significant increased neuronal cysteamine levels, where it prevents the formation of TG2-catalyzed cross-linking of substrate proteins by acting as a competitive amine inhibitor (Bousquet et al., 2010; Gibrat and Cicchetti, 2011; Pinto et al., 2009). Indeed, cystamine administration decreased transglutaminase activity in both R6/2 and YAC128 HD mouse models

(Dedeoglu et al., 2002; Karpuj et al., 2002; Van Raamsdonk et al., 2005), improved motor performance, body and brain weight, and survival (Dedeoglu et al., 2002; Karpuj et al., 2002), and decreased brain atrophy and the number of protein aggregates in striatal and cortical sections (Dedeoglu et al., 2002; Van Raamsdonk et al., 2005). However, some results suggest that TG2 might not be the primary target of cystamine. Cystamine-induced neuroprotection in R6/2 HD mice was maintained in TG2(-/-) R6/2 HD mice, reinforcing the hypothesis that cystamine-induced neuroprotection in HD was not related with TG2 inhibition (Bailey and Johnson, 2006). In fact, cystamine appears to have anti-apoptotic and antioxidant characteristics. Cystamine was described to inhibit caspase-3 activity *in vitro* in a dose-dependent and TG2-independent manner. Cystamine also protected against proteasome inhibitor MG132 and the oxidative stress inducer H_2O_2-increased caspase-3 activation and increased total glutathione levels in human neuroblastoma SH-SY5Y cells (Lesort et al., 2003). Furthermore, cystamine increased in a dose-dependent manner the levels of GSH and L-cysteine, but not GSSG, and decreased the number of aggregates in cell line models expressing mHtt (Fox et al., 2004), which can be associated with its ability to induce Nrf2 transcription and concomintantly protected against against 3-NP-induced lesions (Calkins et al., 2010). The Raptor Pharmaceutical Corp. is currently collaborating with the Centre Hospitalier Universitaire (CHU) d'Angers in France in a clinical trial for the study of cysteamine bitartrate formulation (RP103) in HD named "CYST-HD Multicentre study of treatment of Huntington’s Disease with cysteamine, a randomized, controlled, double-blind multicenter phase II-III trial vs placebo" (http://www.raptorpharma.com).

1.4 Hypothesis and specific aims of the present work

Considering the evidence of oxidative damage in HD and the controversial studies conducted so far, this thesis aimed to clarify the redox imbalance pathways that occur following expression of full-length mHtt and detail the changes in the antioxidant status, namely the glutathione redox system. Furthermore we aimed to evaluate the possible protective role of cystamine and creatine as well as insulin and IGF-1 pathways against oxidative stress in HD. For this purpose, we used striatal cells derived from HD knock-in mice expressing full-length mHtt with 111 Q (ST*Hdh*$^{Q111/Q111}$; mutant cells) *versus* wild-type striatal cells (ST*Hdh*$^{Q7/Q7}$). Importantly, HD striatal cells may represent initial stages of this neurodegenerative disease due to the absence of cell death or visible aggregates of mHtt (Trettel et al., 2000).

The specific aims of the present work were:

1. To thoroughly analyse the regulation of the glutathione redox system;

2. To explore the antioxidant response induced by exposure to H_2O_2, an oxidative stress inducer, or STS, an apoptotic inducer, and further evaluate the protective effect of two compounds that are currently used in HD clinical trials, namely cystamine and creatine;

3. To evaluate the role of insulin and IGF-1 signaling cascades on neuroprotection and oxidative stress in HD knock-in striatal cells, namely through the regulation of Nrf2 transcription factor.

We hypothesised that glutathione redox cycle and its synthesis were disrupted and that decreased antioxidant response caused by stress inducers could be prevented by cystamine and creatine in HD striatal cells; moreover, restoration of insulin and IGF-1 signaling pathways was hypothesized to rescue striatal cells from oxidative stress and cell death caused by full-length mHtt.

The present thesis reveals important mechanisms related with the production of reactive oxygen species and the impact on relevant cellular antioxidants (e.g. glutathione-dependent pathways) in HD striatal cells and determines the role of cystamine and creatine, as well as protective intracellular signaling pathways activated by insulin and IGF-1 that might help to ameliorate oxidative stress and prevent the aggravation of HD.

Chapter 2 – Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM) culture medium, Fetal Bovine Serum (FBS), penicillin/streptomycin and geneticin were purchased by GIBCO (Paisley, UK). 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid (MK-571) sodium salt, 5-sulfosalicylic acid dihydrate, ATP, Alamar Blue (resazurin sodium salt) dye, anti-β-actin, anti-α-tubulin, apocynin, calcein-AM, creatine monohydrate, cystamine, DL-dithiothreitol (DTT), γ-Glu-Cys, gamma-glutamylcysteine (γ-GC), glucose-6-phosphate, GSHee, glycylglycine, GRed, GSH, GSSG, GST assay kit, H_2O_2, L-glutamic acid, L-glutamine, lucigenin, maleimide, NADH, NAD, NADPH, NADP, NDA (2,3-naphthalenedicarboxaldehyde), NEM (N-ethylmaleimide), OPA (*ortho*-phthaldialdehyde), PMSF (phenylmethanesulfonyl fluoride), protease inhibitors (chymostatin, pepstatin, A, leupeptin and antipain), pyruvate, staurosporine from Streptomyces, and *tert*-butyl hydroperoxide (*t*-BHP) were from Sigma Chemical Co. (St Louis, MO, USA). γ-glutamyl-7-amino-4-methyl-coumarin (γ-glutamyl-AMC) was purchased from Bachem (Bubendorf, Switzerland). The fluorescence probes 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), Amplex® Red Xanthine/Xanthine Oxidase Assay Kit, dihydroethidium (DHE), Hoechst 33342, MitoSOX™ Red, propidium iodide, and trizol reagent were obtained from Molecular Probes/Invitrogen (Eugene, OR, USA). N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) (Caspase-3 Substrate VII, Fluorogenic) and tetramethylrhodamine methyl ester ($TMRM^+$) were obtained from Calbiochem (USA). Anti-Akt was from BD Biosciences (USA). LY294002 and antibodies against Erk1/2 (p44/42 MAPK) and P(Thr202/Tyr204)Erk1/2, were from Cell Signaling Technology (Beverly, MA, USA). Anti-Ac(K68)SOD2, anti-GCLc, anti-GRed, anti-Nrf2, anti-P(Thr308)Akt, anti-P(Ser40)Nrf2, Anti-SOD1, anti-SOD2, and anti-TBP were from Abcam (Cambridge, UK). Secondary antibodies conjugated to alkaline phosphatase (anti-mouse and anti-rabbit) were purchased from Amersham Biosciences (Buckinghamshire, UK). Enhanced ChemiFluorescence reagent (ECF), anti-rabbit IgG (from goat), anti-mouse IgG+IgM (from goat) were from GE Healthcare (Little Chalfort, UK). First Strand cDNA Synthesis Kit (AMV) was obtained from Roche (Mannheim, Germany). GoTaq Flexi DNA polymerase and dNTP Mix were from Promega (Madison, WI, USA).

2.2 Cell culture

Striatal cells derived from knock-in mice expressing normal Htt (ST*Hdh*$^{Q7/Q7}$ or wild-type cells; clone 2aA5) or homozygous to mHtt with 111 Q (ST*Hdh*$^{Q111/Q111}$ or mutant cells; clone 109-1A) were kindly donated by Dr. Marcy E. MacDonald (Department of Neurology, Massachusetts General Hospital, Boston, USA). The cells were maintained in an incubation chamber at 33ºC with 5% CO_2, in DMEM culture medium supplemented with 10% FBS, 2 mM glutamine, 1% penicillin/streptomycin and 400 µg/mL geneticin (G-418), as described previously (Trettel et al., 2000). Striatal cells were plated on poly-L-lysine coated glass coverslips, multiwell chambers or flasks 48 h before the experiments in order to allow the desired confluence. In some experiments, cells were incubated with cystamine (0.1 and 0.25 mM), creatine (0.1 and 1 mM), GSHee (0.05, 0.25 or 1 mM) or 50 µM MK-571, for 24 h. In other experiments, cells were pre-incubated for 30 min with 25 µM LY294002 before incubation with insulin or IGF-1 (0.1-10 nM) for 24h. Also, 15 h before some experiments, cells were incubated with STS (0.1-50 nM), a classic inducer of apoptosis, or H_2O_2 (1-100 µM) during 15 h.

2.3 Cell extractions

2.3.1 Total extracts and subcellular fractionation

Nuclear-enriched fractions were obtained from cells that have been washed twice in ice-cold phosphate buffered saline (PBS) and scrapped in lysis buffer 1 (in mM: 10 HEPES, 10 NaCl, 3 $MgCl_2$, 1 EGTA, 0.1% Triton X-100, pH 7.5) supplemented with 50 mM NaF, 1.5 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF and 1 µg/mL protease inhibitor cocktail (chymostatin, pepstatin A, leupeptin and antipain). The cellular homogenate was incubated at 4ºC (on ice) for 40 min and then centrifuged at 4,700 rpm, for 12 min (4ºC). The pellet was resuspended in buffer 2 (in mM: 25 HEPES, 300 NaCl, 5 $MgCl_2$, 1 EGTA, 20% glycerol, pH 7.4) supplemented with 50 mM NaF, 1.5 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF and 1 µg/mL protease inhibitor cocktail, incubated on ice for 60 min, and further centrifuged at 10,600 rpm for 20 min (4°C). The resulting pellet (nuclear-enriched fraction) was resuspended in buffer 2. The samples were then frozen and thaw three times in liquid nitrogen, sonicated for 30 sec and then the samples were stored at −80 °C. To obtain the mitochondrial-enriched fractions, striatal cells were washed with PBS and lysed at 4ºC using a sucrose buffer (pH 7.5) composed by (in mM): 250 sucrose, 20

HEPES, 100 KCl, 1.5 $MgCl_2$, 1 EDTA and 1 EGTA, 1 DTT, 0.1 PMSF, and 1 μg/mL protease inhibitor cocktail, homogenized by using a potter (120 strokes). Then, extracts were centrifuged at 500 g in order to remove nuclear debris and the resulting supernatant was centrifuged at 12,000 g for 20 min in order to obtain an enriched mitochondrial fraction, which was further resuspended in sucrose buffer supplemented with 0.05% Triton X-100.

2.3.2 Total RNA extraction, cDNA conversion and reverse-transcriptase PCR

RNA from different samples was obtained with Trizol Reagent according to the manufacturer's protocol. Briefly, striatal cells were lysed with Trizol and were homogeneized with a pipette. The RNA was precipitated with isopropyl alcohol and the final pellet was resuspended with water DEPC (diethylpyrocarbonate) 0.01% (v/v). Before performing reverse-transcriptase polymerase chain reaction (RT-PCR), the quality of RNA was verified through an electrophoresis in 1% agarose gel to check the ribosomal RNA subunits 18S and 28S. Then, the samples were incubated at 65ºC for 15 min, to avoid extensive secondary structure that may interfere with the annealing step and then were chilled on ice for 5 min. The concentration of RNA was measured and 1 μg was used to transcribe RNA into cDNA with First Strand cDNA Synthesis Kit (AMV). The reaction was as follow: 10 min at +25°C and 60 min at +42°C, for primer annealing and cDNA synthesis, respectively, 5 min at +99°C, for the denaturation of reverse transcriptase and then the samples were cooled to +4°C for 5 min.

2.4 Cell viability and death methods

2.4.1 Alamar Blue assay

The reducing capacity of the cells was evaluated by the Alamar Blue assay. This method is based on the detection of the metabolic activity of the cells, largely depending on the activity of dehydrogenases that produce reduced forms of coenzymes (e.g. NADH). The active compound of Alamar Blue (resazurin) is a nontoxic cell permeable compound that is reduced inside the cells to the pink fluorescent resorufin, whenever they are metabolically active (O'Brien et al., 2000) (Figure 2.1). After medium removal, cells

were incubated with 200 µL Alamar Blue (0.1 mg/ml) for 2 h at 33 ºC, and the absorbance was measured at 570 and 600 nm in a Spectramax Gemini EM spectrophotometer (Molecular Devices, Union City, CA). Cellular function based on the reducing capacity was calculated as a percentage of wild-type cells or control.

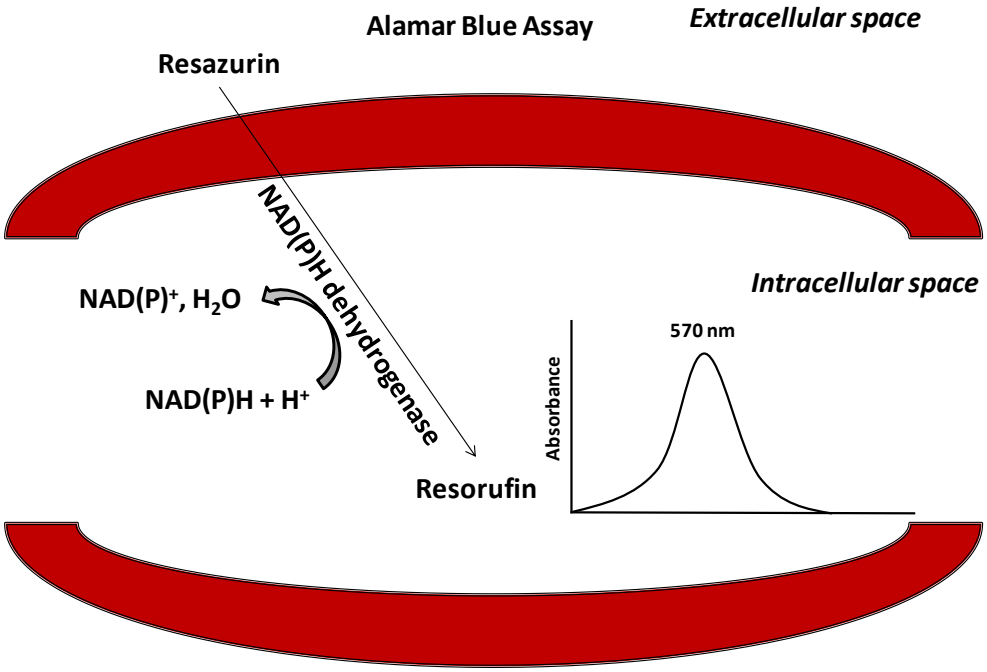


Figure 2.1 – Alamar Blue Assay. Resazurin is a cell permeable compound that has a maximum absorption at ~600 nm and when inside the cells is reduced by NAD(P)H dehydrogenases to the pink fluorescent resorufin that has a maximum absorption at ~570 nm and fluorescence excitation/emission of ~570/585 nm.

2.4.2 LDH release assay

After exposure to H_2O_2 or STS during 15h, striatal cells were used to determine cell viability by monitoring the release of LDH to the extracellular medium, which evaluates plasma membrane integrity, indicator of necrosis cell death. Briefly, the extracellular medium was collected and cells were washed twice with PBS and lysed in 10 mM HEPES (pH 7.4) plus 0.02% Triton X-100 at 4ºC and then collected and submitted to 3 cycles of freezing. Then, all the samples (extra and intracellular fractions) were centrifugated at 14,000 rpm (Eppendorf Centrifuge 5417R), for 10 min at 4ºC. The pellet was discarded and LDH was determined spectrophotometrically, by following the rate of conversion of reduced nicotinamide adenine dinucleotide (NADH) to oxidized NAD^+ at 340 nm (Bergmeyer and Bernt, 1974) (Figure 2.2). LDH released into the

extracellular medium was expressed as a percentage of the total LDH activity in the cells [% of LDH released = extracellular LDH / (extracellular LDH + intracellular LDH)] and then expressed as percentage of wild-type cells or control.

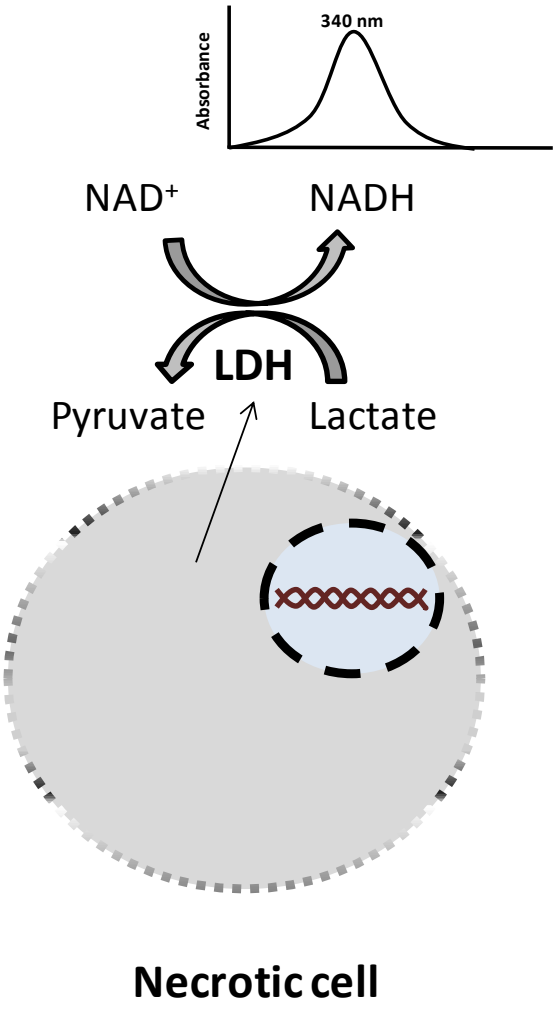


Figure 2.2 – LDH release assay. Under conditions of disrupted cell membrane (necrosis), lactate dehydrogenase (LDH) is released to the extracellular space. If substrates are given, it catalyzes the interconversion of lactate and NAD+ to pyruvate and NADH. NADH in turn absorbs light at 340 nm.

2.4.3 Caspase-3 activity assay

Caspase-3-like activity was determined using a fluorimetric substrate Ac-DEVD-AFC. The DEVD sequence is cleaved by caspase-3 releasing the fluorogenic AFC which is quantified by fluorimetry (Figure 2.3). Striatal cells were washed twice with PBS and then lysed at 4ºC with a lysis buffer composed by (in mM): 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, 2 DTT, 0.1 PMSF, and 1:1000 protease inhibitors and 0.04% Triton X-

100 (pH 7.5). After scraping, a reaction buffer composed by (final concentrations in the well) 25 mM HEPES, 10% sucrose, 0.1% CHAPS, 2 mM DTT and 15 µM Ac-DEVD-AFC (pH 7.5), was added to the cell samples. The fluorescence was taken during 1 h at 33ºC with 400 nm excitation and 505 nm emission. After the readings, cell samples were used to determine protein content by Bio-Rad protein assay. The values were obtained as RFU (Relative Fluorescence Units) per minute per mg protein for each condition and then expressed as percentage of wild-type or control cells.

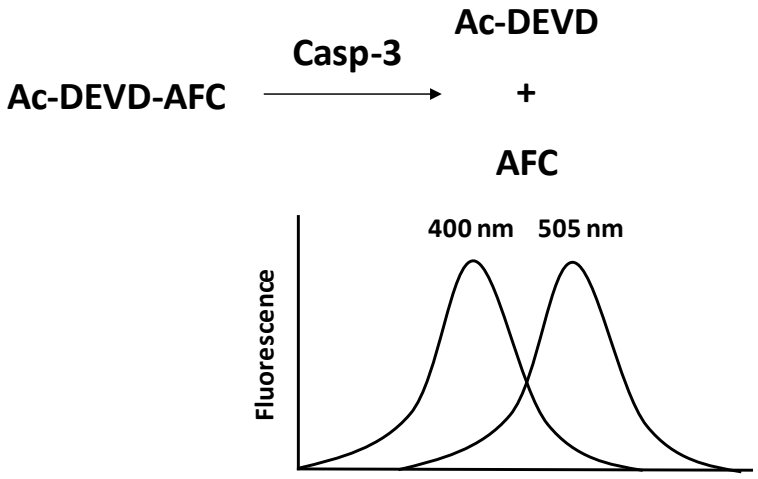


Figure 2.3 – Caspase-3 activity assay. The amino acid DEVD sequence of the caspase-3 (Casp-3) fluorimetric substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) is cleaved by caspase-3 releasing the AFC which has a fluorescence excitation/emission maxima of ~400/505 nm.

2.4.4 Fluorescence microscopy

Cells were washed with microscopy buffer (120 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM HEPES, 5 mM $NaHCO_3$, 1.2 mM Na_2SO_4 and 15 mM glucose) supplemented with 1.2 mM $MgCl_2$ and 1.3 mM $CaCl_2$, pH 7.4. Cells were further incubated with Hoechst 33342 (2 µg/mL, a blue permeable fluorescent dye, which binds to DNA, to determine fragmented/condensed nucleus) and propidium iodide (4 µg/mL, a non-membrane permeable red fluorescent dye that binds to DNA, for necrotic cell evaluation) (Figure 2.4), during 8 and 3 min, respectively, in the dark. Live cells were analyzed by fluorescence microscopy. Images from five different fields in each coverslip were acquired in a fluorescence microscope Axioskop 2 Plus (Zeiss, Jena, Germany) and cells were counted using ImageJ Launcher (version 1.44p, NIH). Fragmented/condensed

nucleus labeled with Hoechst 33342 (apoptotic-like cells) and cell nucleus labeled with propidium iodide (necrotic cells) were determined as a percentage of total cells (stained with Hoechst).

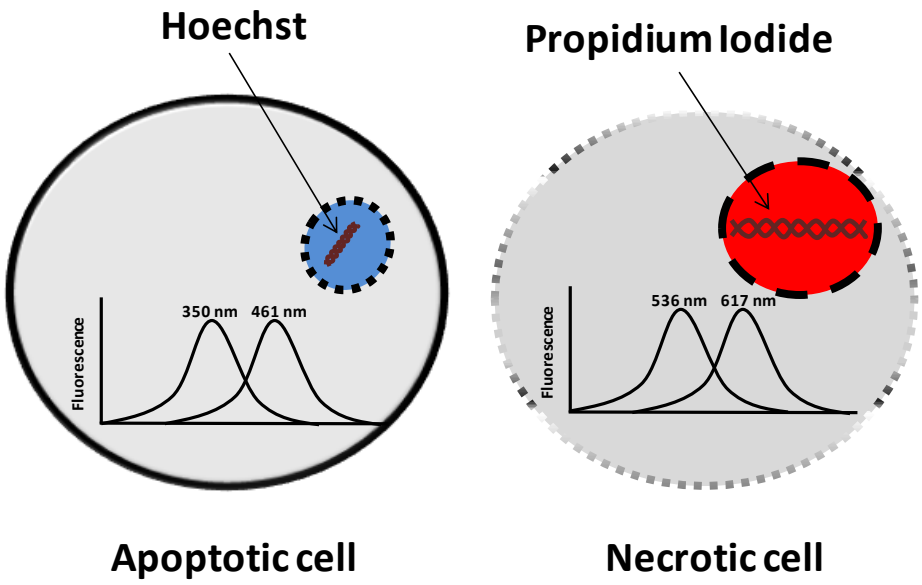


Figure 2.4 – Cell death analysis by fluorescence microscopy. Striatal cells were incubated with Hoechst 33342, a blue permeable fluorescent dye, which binds to DNA, to determine fragmented/condensed nucleus, and has a fluorescence excitation/emission maxima of ~350/461 nm, and propidium iodide, a non-membrane permeable red fluorescent dye that binds to DNA, for necrotic cell evaluation, and has a fluorescence excitation/emission maxima of ~536/617 nm.

2.5 Analysis of mitochondrial membrane potential

The ΔΨm was determined using the cationic fluorescent probe $TMRM^+$, widely used as a fluorescent probe for measuring in situ ΔΨm (Nicholls et al., 2012) (Figure 2.5). The experiments were performed accordingly to (Ferreira et al., 2010), with minor modifications. Briefly, cells were cultured in a 96-well plate during 48 h, at 33ºC. Then, striatal cells were washed twice in PBS and 300 nM $TMRM^+$ (diluted in Krebs medium) was incubated during 1 h, at 33ºC. After incubation, the basal fluorescence was taken during 5 min using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA) (540 nm excitation and 590 emission). FCCP (2.5 µM) and oligomycin (2 µg/ml), which produced maximal mitochondrial depolarization, were added to cells and the fluorescence was taken during another 5 min. Results were expressed as the difference between the increase in $TMRM^+$ fluorescence upon addition of FCCP plus oligomycin and basal fluorescence values, and converted to percentage of wild-type (control) cells.

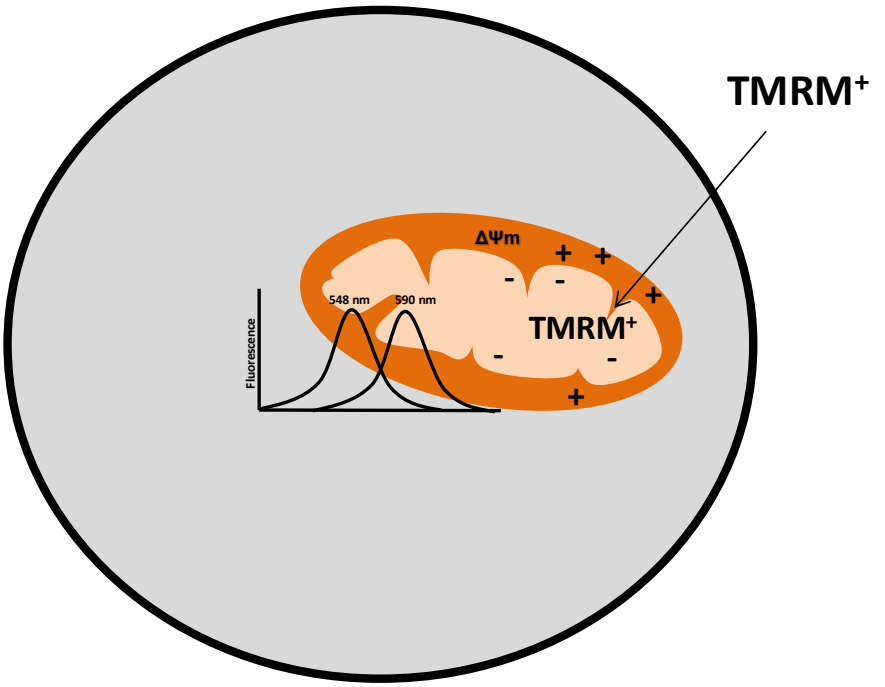


Figure 2.5 – M itochondrial membrane potential assay. The cell-permeable cationic fluorescent probe tetramethylrhodamine methyl ester (TMRM+) is rapidly sequestered by active mitochondria where it accumulates in the highly negatively charged matrix and has a fluorescence excitation/emission maxima of ~548/590 nm. Thus, altered fluorescence signal of TMRM+ can be directly co-related to changes of mitochondrial membrane portential (ΔΨm).

2.6 Intracellular ROS levels and production measurements

2.6.1 Total intracellular and mitochondrial ROS levels measurements

After brief washing with PBS, striatal cells were incubated for 30 min with 20 µM H_2DCFDA, a stable non-fluorescent cell permeable compound, at 33ºC, in modified Krebs medium (in mM: 135 NaCl, 5 KCl, 0.4 KH_2PO_4, 1.8 $CaCl_2$, 1 $MgSO_4$, 20 HEPES and 5.5 glucose) at pH 7.4. H_2DCFDA is incorporated by the cells and hydrolysed by esterases to form H_2DCF, which is then converted to dichlorofluorescein (DCF) by intracellular ROS (LeBel et al., 1992) (Figure 2.6). Intracellular ROS levels were measured by following DCF fluorescence (488 nm excitation, 530 nm emission) at 33ºC, continuously, for 1 h, using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). In order to correct the DCF fluorescence values for variations in total intracellular protein content in each well was quantified by the BioRad protein assay (Bradford method). The values were obtained as RFU per minute per mg protein for each condition and then expressed as percentage of wild-type cells. $O_2^{\bullet-}$ formation was

determined by using the fluorescence probe dihydroehidium (DHE) (Molecular Probes, Invitrogen). DHE is permeable to cell membrane and in cytoplasm it is converted to ethidium (E^+) by $O_2^{\bullet-}$. Then, E^+ binds to DNA and emits fluorescence (Bindokas et al., 1996) (Figure 2.7). Briefly, 5 µM DHE dissolved in Krebs medium was incubated in cells during 1 h at 33ºC. Fluorescence measurements were taken during 1 h (518 nm excitation; 605 nm emission). Mitochondrial $O_2^{\bullet-}$ levels were measured by using the fluorescent probe MitoSOX^{TM} Red (Figure 2.7). Briefly, striatal cells were washed in PBS, and then incubated with 2.5 µM MitoSOX^{TM} Red in Krebs medium during 10 min, at 33ºC. The fluorescence was taken during 1 h and 30 min, at 33ºC, with 510 nm excitation and 580 nm emission. In order to correct the DCF, DHE and MitoSOX^{TM} Red fluorescence values for variations in total protein content in the wells, cell protein in each well was quantified by the BioRad protein assay. The values were obtained as RFU per minute per mg protein for each condition and then expressed as a percentage of wild-type cells or control.

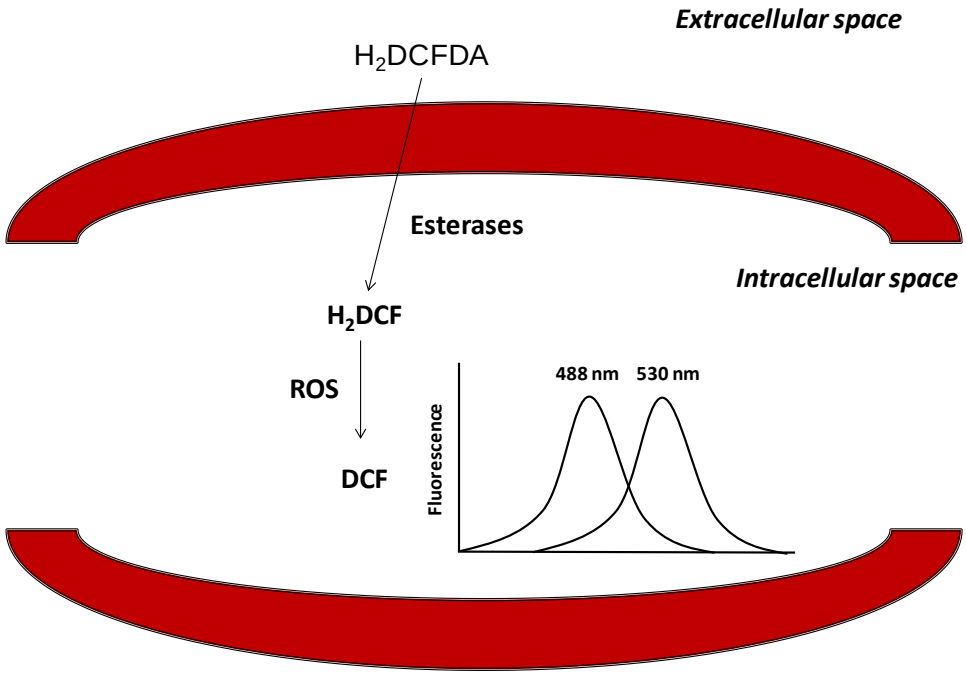


Figure 2.6 – Determination of ROS levels by H_2DCFDA. 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), a stable non-fluorescent cell permeable compound, is hydrolysed by intracellular esterases to form H_2DCF, which is then converted by intracellular ROS to dichlorofluorescein (DCF) and has fluorescence excitation/emission maxima of ~488/530 nm. Thus, increased fluorescence signal of DCF has been directly co-related to intracellular ROS levels. However, it was recently described that H_2DCFDA is very inespecific probe for ROS detection. It can be oxidized by several oxidants, including ONOO−, •OH or hypochlorous acid, and redox-active metals such as iron (Kalyanaraman et al., 2012).

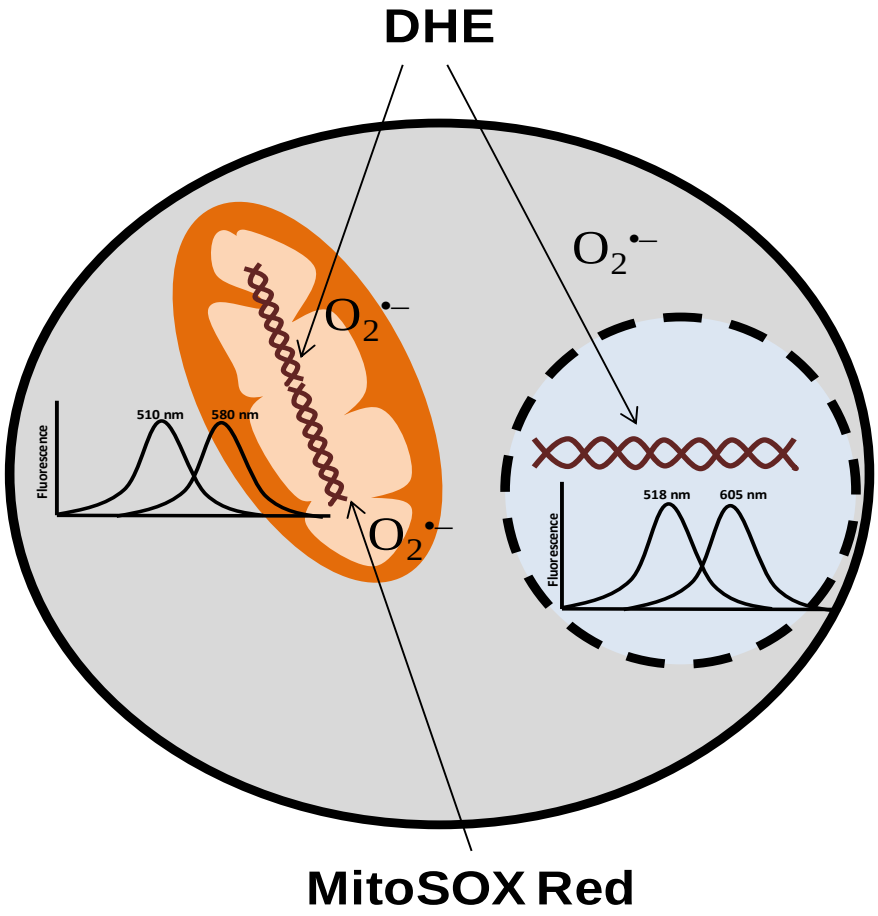


Figure 2.7 – Determination of mitochondrial ROS levels by DHE and MitoSOX^{TM} Red. Dihydroethidium (DHE) is permeable to cell membrane and in cytoplasm it is converted to ethidium (E^+) by $O_2^{\bullet-}$. Then, E^+ binds to DNA and emits fluorescence (excitation/emission: 518/605 nm). The cell-permable MitoSOX^{TM} Red reagent is a hydroethidium (HE) derivate with a triphenylphosphonium (TPP) moiety for mitochondrial targeting. It is rapidly oxidized by $O_2^{\bullet-}$ and binds to mitochondrial DNA where it has a fluorescence excitation/emission maxima of ~510/580 nm. Thus, increased fluorescence signal of DHE and MitoSOX^{TM} Red can be directly co-related to increased $O_2^{\bullet-}$ levels. However MitoSOX^{TM} Red may also non-specifically oxidize (Kalyanaraman et al., 2012).

2.6.2 Cytosolic and mitochondrial ROS production measurements

2.6.2.1 NOX activity assay

NOX activity was determined using the chemiluminescent probe lucigenin, as described previously (Li et al., 1998) with some modifications (Figure 2.8). Briefly, striatal cells were washed twice with PBS and lysed with 25 mM Tris-HCl (pH 7.4). Then, 30 µg protein were added to a 96-well plate containing 100 µM NADPH and 5 µM lucigenin in a final volume of 200 µL (adjusted with 25 mM Tris-HCl). Luminescence

readings were performed during 15 min and results were calculated as relative luminescence units (RLU) per min per mg protein and expressed as percentage of wild-type cells or control. Apocynin, an inhibitor of NOX was used as a control of the experiment.

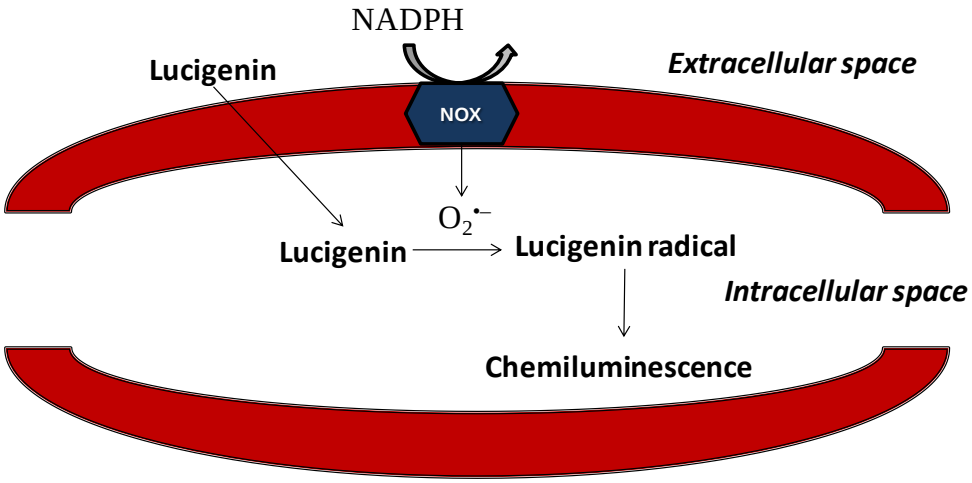


Figure 2.8 – NOX activity assay. The chemiluminescent probe lucigenin is oxidized by $O_2^{\bullet-}$ that can be produced by NADPH oxidase (NOX) after NAD(P)H supply. The final product lucigenin radical can emit light. Thus, increased luminescence signal of lucigenin can be directly co-related to increased NOX activity.

2.6.2.2 **XO activity and hypoxanthine levels**

XO activity and hypoxanthine levels were determined as described in the manual provided by the Amplex® Red Xanthine/Xanthine Oxidase Assay Kit (Figure 2.9). The results were expressed as percentage of wild-type cells or control.

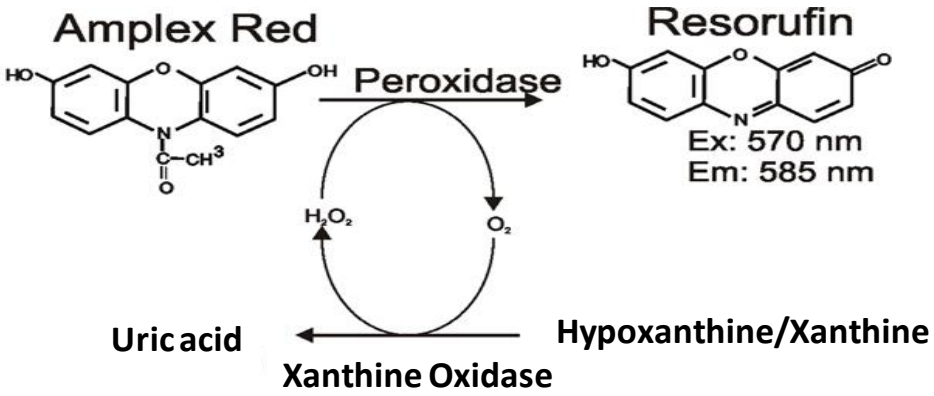


Figure 2.9 – XO activity assay. The Amplex® Red Xanthine/Xanthine Oxidase Assay is based on the conversion by xanthine oxidase (XO) of purine bases, hypoxanthine or xanthine, to uric acid and superoxide ($O_2^{\bullet-}$) which is spontaneously degraded to hydrogen peroxide (H_2O_2), which in

the presence of horseradish peroxidase (HRP) reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin; this has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively.

2.6.2.3 **Mitochondrial complexes I and III ROS production**

For the mitochondrial complexes I and III ROS formation, 5 µM DHE dissolved in Krebs medium was incubated in cells during 1 h at 33ºC. Fluorescence measurements were taken during 1 h (518 nm excitation; 605 nm emission) in the presence of 10 µM rotenone, a mitochondrial complex I inhibitor, or antimycin A, a mitochondrial complex III inhibitor.

2.7 Cellular antioxidant systems measurements

2.7.1 Measurement of antioxidant enzymes activities

2.7.1.1 **Measurement of SOD activity**

Determination of SOD activity was performed accordingly to the SOD Assay Kit (Sigma) (Figure 2.10). In order to measure SOD2 activity, 2 mM potassium cyanide (KCN) (which inhibits SOD1) was added.

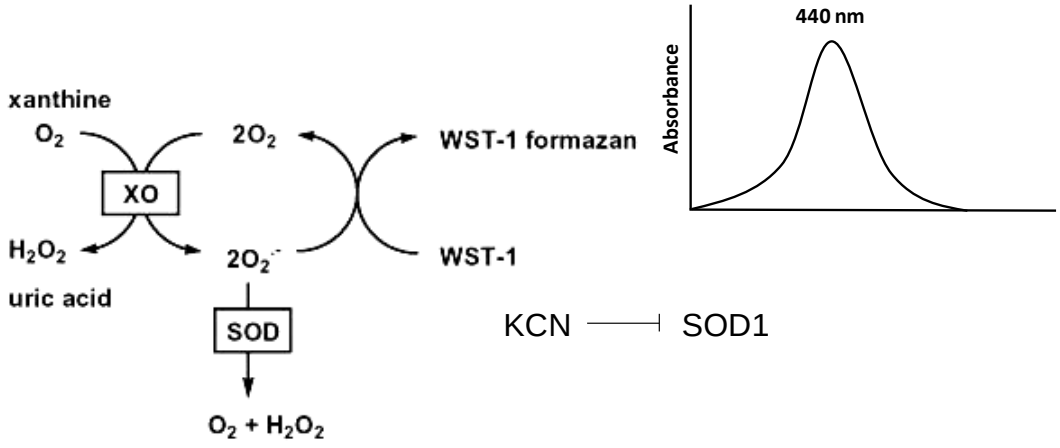


Figure 2.10 – SOD activity assay. The superoxide dismutase (SOD) Assay Kit is based on the conversion by superoxide ($O_2^{\cdot-}$) of WST-1 (2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt) into a water-soluble formazan dye (WST-1 formazan) which has an absorption maxima of ~440 nm. The rate of the WST-1 conversion to WST-1 formazan is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, Thus, increased SOD activity will present decreased WST-1 formazan formation. Specific

SOD2 activity can be measured in the presence of potassium cyanide (KCN) , since it inhibits SOD1 activity.

2.7.1.2 **Measurement of GRed and GPx activities**

The cells were washed twice in PBS and lysed in 25 mM Tris-HCl (pH 7.4) and then centrifuged at 20,800×*g* for 10 min, at 4ºC (Eppendorf Centrifuge 5417R). The supernatant was used for protein quantification using the BioRad protein assay (Bradford method) and for measuring GRed and GPx activities, spectrophotometrically, at 340 nm, through the analysis of NADPH oxidation, as described previously (Paglia and Valentine, 1967) with some modifications (Figure 2.11). Briefly, the activity of GPx in samples was measured upon a 5 min incubation, in the dark, with phosphate buffer containing 0.25 M KH_2PO_4, 0.25 M K_2HPO_4 and 0.5 mM EDTA, pH 7.0, 10 mM GSH and GRed (1 unit). The reaction occurred after the addition of 2.5 mM NADPH and 12 mM *t*-BHP. For the activity of GRed, each sample was incubated with a phosphate buffer containing 0.2 M K_2HPO_4 and 2 mM EDTA, pH 7.0, plus 2 mM NADPH. The measurements were initiated with the addition of 20 mM GSSG. GRed and GPx activities were determined using a Microplate Spectrophotometer SpectraMax Plus^{384} (Molecular Devices, USA). Results were calculated milliunits (mU) per mg protein and expressed as percentage of wild-type cells or control.

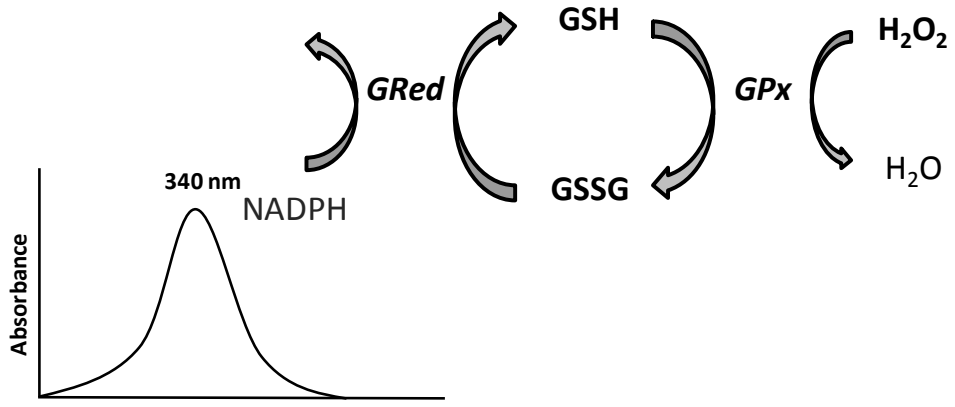


Figure 2.11 – Determination of GPx and GRed activities. Glutathione peroxidase (GPx) activities measured in the presence of glutathione reductase (GRed), NADPH, the reduced form of glutathione (GSH), and *tert*-butyl hydroperoxide (*t*-BHP). GRed activity is measured in the presence of NADPH and the oxidized form of glutathione (GSSG). The conversion of NADPH (which absorbs at 340 nm) into $NADP^+$ is linearly related to GPx and GRed activities.

2.7.1.3 **Measurement of G6PD and 6PGD activities**

G6PD activity was determined according to Choo and collaborators (2005), with some minor modifications (Figure 2.12). Briefly, the cells were washed twice in PBS and lysed in 25 mM Tris-HCl (pH 7.4) and then centrifuged at 20,800×*g* for 10 min, at 4ºC (Eppendorf Centrifuge 5417R). The supernatant was used for protein quantification using the BioRad protein assay (Bradford method) and cell samples (30 µg) were mixed with a reaction buffer containing (in mM): 0.38 NADP, 6.3 $MgCl_2$, 3.3 G6P, and 5 maleimide (an inhibitor of 6-phosphogluconate dehydrogenase (6PGD) activity) in 50 mM Tris-HCl (pH 7.5). For 6PGD activity determination, experiments were performed in the absence of maleimide, corresponding, therefore, to the total NADPH production (G6PD + 6PGD) by the pentose phosphate pathway; thus 6PGD activity = total NADPH production – G6PD activity. The NADPH production was continuously monitored at 340 nm using a Microplate Spectrophotometer SpectraMax Plus^{384} (Molecular Devices, USA) at 37ºC and the results were calculated in milliunits (mU) per mg protein and expressed as percentage of wild-type cells.

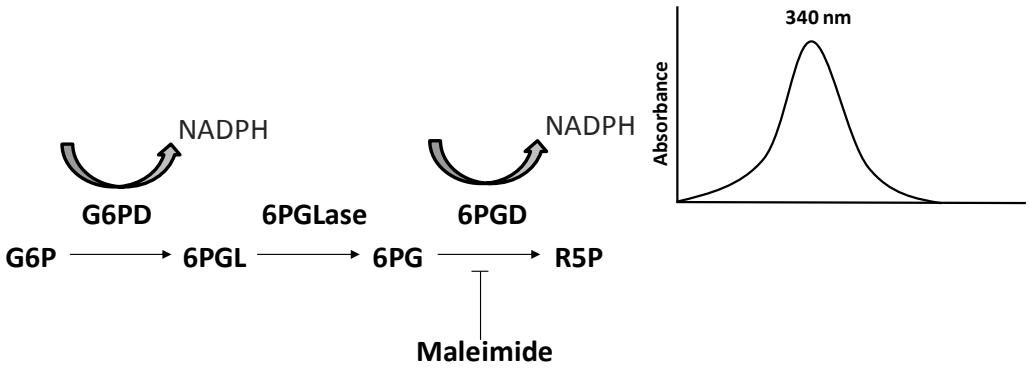


Figure 2.12 – Determination of G6PD and 6PGD activities. Since both glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) activities can produce NADPH, maleimide, which inhibits 6PGD activity, is used to measure G6PD activity. Besides maleimide, G6PD is measured after addition of glucose-6-phosphate (G6P), and $NADP^+$, both substrates of G6PD, resulting in NADPH and 6-phospho-D-glucono-1,5-lactone (6PGL) formation; the latter is a substrate for 6-phosphogluconolactonase (6PGLase), producing 6-phosphogluconate (6PG), which is then converted to ribulose-5-phosphate (R5P) by 6PGD. The rate of NADPH formation is followed at 340 nm. 6PGD activity is measured without maleimide supply.

2.7.1.4 **Determination of GCL and GS activities**

GCL and glutathione synthetase (GS) activities were measured according to White and collaborators (2003), with some minor modifications (Zhu et al., 2006) (Figure 2.13). Cells were washed twice with PBS and lysed with 25 mM Tris-HCl (pH 7.4) plus 0.1 mM EDTA. Cellular extracts were centrifuged at 20,800×*g* for 10 min at 4ºC, and protein content analysed by the BioRad protein assay (Bradford method). For GCL activity, 50 µL of sample was added to 50 µL of GCL reaction buffer containing (in mM): 100 Tris, 10 ATP, 20 L-glutamic acid, 2 EDTA, 20 sodium borate, 2 serine, and 40 $MgCl_2$, and incubated at 37ºC during 5 min. The GCL reaction was initiated by adding 50 µL of 2 mM L-cysteine. For GS activity, L-glutamic acid and L-cysteine were replaced with 30 mM glycine and 3 mM γ-GC, respectively. After 20 min incubation, the GCL and GS reactions were stopped by adding 50 µL of 200 mM 5-sulfosalicylic acid. Samples were vortexed, incubated on ice for 20 min, and centrifuged at 660×*g* at 4°C, for 5 min. Then, 20 µL of supernatant were transferred into a 96-well plate and 180 µL of NDA derivatization solution (50 mM Tris, pH 10, 0.5 M NaOH and 10 mM NDA in dimethyl sulfoxide (DMSO), v/v/v 1.4/0.2/0.2, respectively) was then added to each well to form NDA-γ-GC or NDA-GSH fluorescent complexes. The fluorescence was detected at 472 nm (excitation) and 528 nm (emission), using a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). The results were calculated as RFU per mg protein and expressed as percentage of wild-type cells.

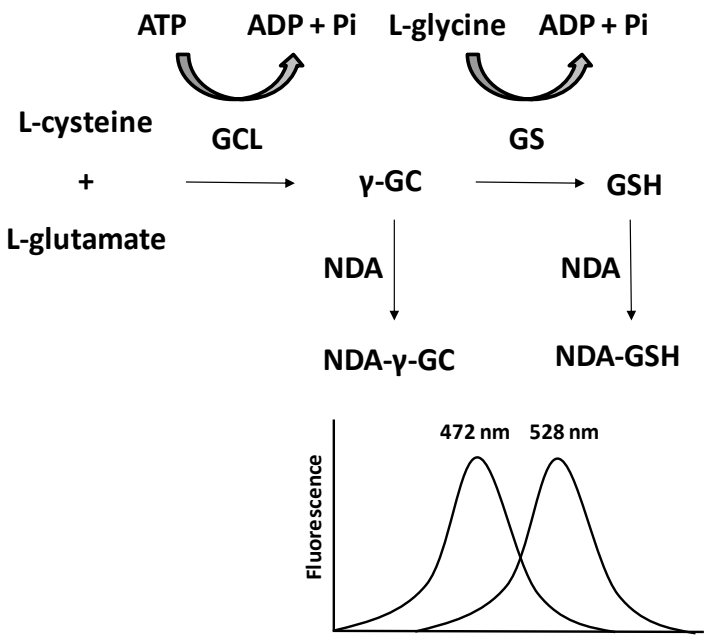


Figure 2.13 – Determination of GCL and GS activities. Glutamate-cysteine ligase (GCL) activity is measured after addition of the substrates ATP, L-cysteine and L-glutamate producing γ-glutamylcysteine (γ-GC), which forms fluorescent adducts with 2,3-naphthalenedicarboxaldehyde (NDA) (excitation/emission: 472/528 nm). Glutathione synthetase (GS) activity is measured after addition of the substrates γ-GC and L-glycine, producing the reduced form of glutathione (GSH) that also forms fluorescent adducts with NDA (excitation/emission: 472/528 nm).

2.7.1.5 **Measurement of GST activity**

Determination of GST activity was performed accordingly to the manual provided by the GST Assay kit (Sigma). Briefly, after a washing step in PBS cell samples were mixed in a 96-well plate with a substrate solution containing 1-chloro-2,4-dinitrobenzene (CDNB) and GSH and the absorbance was followed at 340 nm during 6 min (Figure 2.14). The results were calculated in µmol per min per mg protein and expressed as percentage of wild-type cells.

2.7.1.6 **Measurement of γ-GT activity**

The cells were lysed at 4ºC in 100 mM Tris-HCl, 1 mM EDTA and 0.1% Triton X-100; pH 7.6 and γ-glutamyl transpeptidase (γ-GT) activity was measured accordingly to Zhu and collaborators (2006) with some modifications (Figure 2.14). Briefly, 50 µL of cell samples were added to 50 µL of a reaction mixture containing 100 mM Tris–HCl (pH 7.6), 40 mM glycylglycine, 40 µM γ-glutamyl-7-amino-4-methyl-coumarin (γ-glutamyl-AMC), and 0.1% (v/v) Triton X-100, in a 96-well plate. The fluorescence was

measured with excitation at 370 nm and emission at 440 nm, for 45 min at 33ºC. The values were obtained as RFU per minute per mg protein for each condition and then expressed as percentage of wild-type cells.

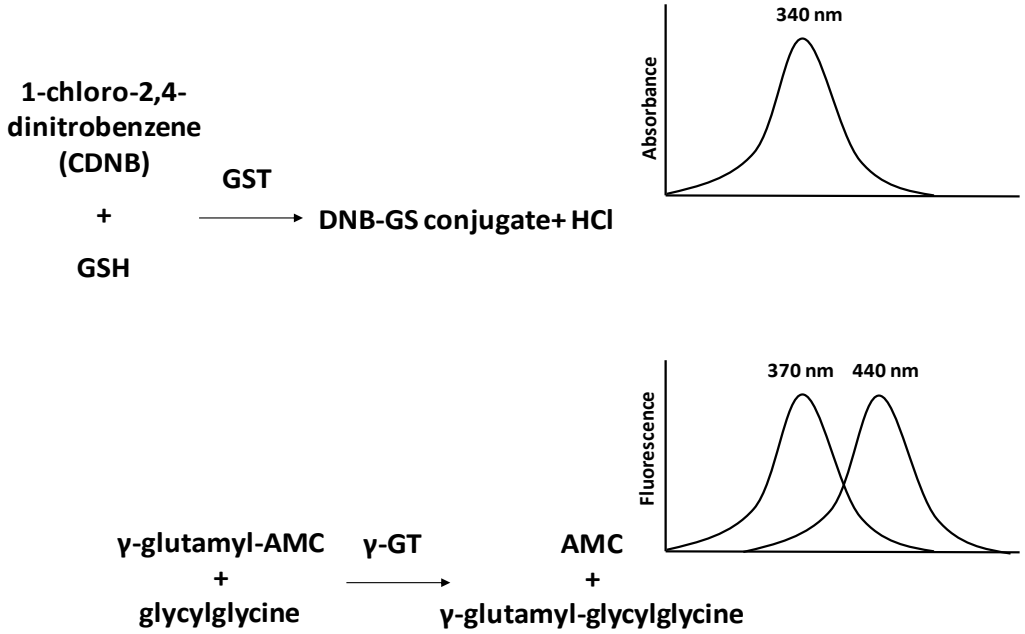


Figure 2.14 – Determination of GST and γ-GT activities. Glutathione S-transferase (GST) activity assay is based on the GST-catalyzed reaction between the reduced form of glutathione (GSH) and the GST substrate, CDNB (1-chloro-2,4-dinitrobenzene), producing DNB-GS, which absorbs at 340 nm. γ-Glutamyl transpeptidase (γ-GT) activity is measured in the presence of glycylglycine and gamma-glutamyl-7-amino-4-methyl-coumarin (γ-glutamyl-AMC), producing γ-glutamyl-glycylglycine and AMC that has a fluorescence excitation/emission maxima of ~370/440 nm.

2.7.1.7 **Analysis of Mrp1 activity based on calcein fluorescence**

Mrp1 activity was determined based on calcein fluorescence, since Mrp1 is involved in the transport of calcein to the extracellular space (Dogan et al., 2004) (Figure 2.15). Striatal cells were cultured during 24 h and then briefly washed in PBS. After the washing step, the cells were incubated with 5 µM calcein-AM in modified Krebs medium during 1 h, at 33ºC. Calcein-AM diffuses into cells where it is cleaved by intracellular esterases, resulting in fluorescent calcein. After incubation, striatal cells were washed twice in PBS and intracellular calcein fluorescence was analysed using excitation at 494 nm and emission at 517 nm. The results were calculated as RFU per mg protein and expressed as a percentage of wild-type cells.

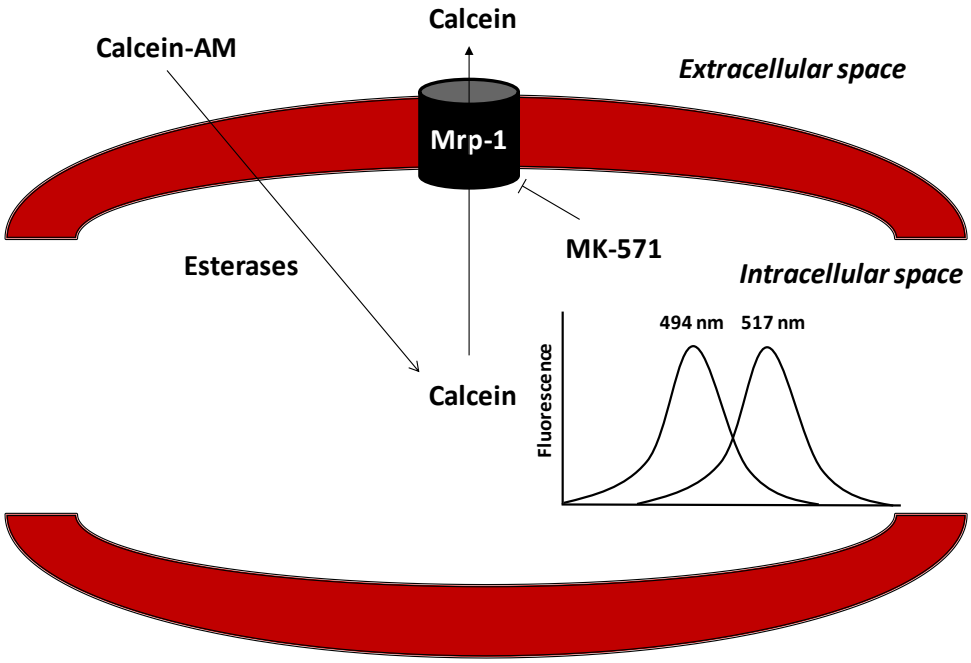


Figure 2.15 – Mrp1 activity assay. Multidrug resistance-associated protein 1 (Mrp1) activity is determined based on calcein fluorescence, since Mrp1 is involved in the transport of calcein to the extracellular space. Calcein-AM diffuses into cells where it is cleaved by intracellular esterases, resulting in fluorescent calcein (excitation/emission: 494/517 nm). MK-571 is an inhibitor of Mrp1 activity and is used as a positive control.

2.7.1.8 **Analysis of Nrf2/ARE activity based on luciferase gene reporter assay**

Nrf2 transcription activity was measured based on its capability of binding ARE region, promoting luciferase gene transcription. Striatal cells plated on 12-well plates (90,000 cells/well) were transfected with the reporter construct [pGL4.37[luc2P/ARE/Hygro] (Promega) using Fugene 6 (Promega) by adding 1.1 µg plasmid. After 24 h, cells were exposed to insulin (0.1 nM) for another 24 h. 3 days after transfection, cells were washed with PBS and incubated at -80ºC for 2 h in Lysis Buffer (1.15 M Tris, 1 mM EDTA, 8 mM $MgCl_2$, 15% glycerol, 1 mM DTT, 1% Triton X-100, pH 7.4). Cells were then scraped and samples were centrifuged (5 min, 4ºC at 14,000 rpm). 50 µl of the supernatant were transferred to a white opaque 96-wells plate. The luciferase activity was measured in a Microplate Luminometer Reader LMax 384 (Molecular Devices, USA) using two different buffers: Reading Buffer (1.15 M Tris, 1 mM EDTA, 8 mM $MgCl_2$, 15% glycerol, 1 mM DTT, 2 mM ATP, pH 7.4) and Luciferase Buffer (167 µM; prepared in water with pH 8). In order to correct the

luciferase activity, the protein content in each well was measured by the Bio-Rad protein assay (Bradford method). The percentage of luciferase activity was then normalized to controls (wild-type cells transfected without any treatment).

2.7.2 Measurement of antioxidant systems levels

2.7.2.1 **Measurement of glutathione and protein levels**

2.7.2.1.1 Measurement of glutathione levels by fluorimetry

The levels of GSH and GSSG were measured as previously described (Hissin and Hilf, 1976) (Figure 2.16). Briefly, striatal cells were cultured during 48h at 33ºC. After incubation, the cells were washed twice in PBS and lysed in 15 mM Tris, pH 7.4, with 0.2% Triton X-100. GSH levels were measured in samples after the addition of OPA (1 mg OPA/ml methanol) and 100 mM NaH_2PO_4. After 15 min incubation, the fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The experimental procedure for GSSG was similar, although the samples were mixed during 30 min with NEM (5 mg NEM/ml methanol) which forms adducts with GSH. Then, the mixture was incubated for 15 min in 100 mM NaOH plus OPA (1 mg OPA/ml methanol), and the fluorescence was measured with excitation at 350 nm and emission at 420 nm in a Microplate Spectrofluorometer Gemini EM (Molecular Devices, USA). The results were calculated as RFU per mg protein and expressed as percentage of wild-type cells, control, or as a percentage of control (untreated) conditions in the case of MK-571 incubation.

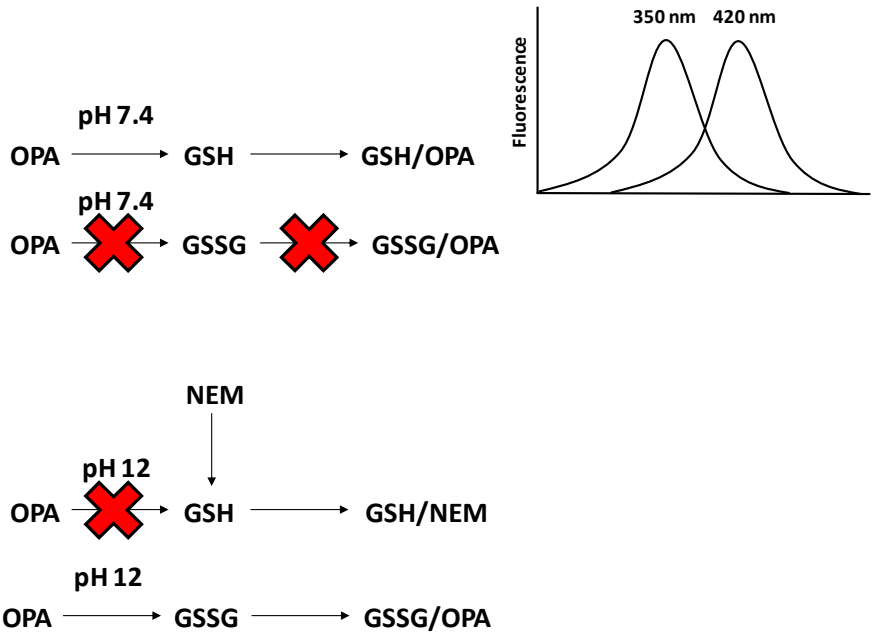


Figure 2.16 – Determination of GSH and GSSG levels by fluorimetry. The levels of the reduced form of glutathione (GSH) are determined based on the capacity of GSH to form fluorescent adducts with *ortho*-phthaldialdehyde (OPA) (excitation/emission: ~350/420 nm) at neutral pH. The levels of the oxidized form of glutathione (GSSG) are based on the capacity of GSSG to form fluorescent adducts with OPA at pH 12. N-ethylmaleimide (NEM), which forms non-fluorescent adducts with GSH, is added to the samples to prevent GSH-OPA fluorescent adducts formation.

2.7.2.1.2 Measurement of intra- and extracellular levels of total glutathione by HPLC

Extracellular cultured medium was collected, whereas the cells were washed with PBS and further lysed in 1 M NaOH for determination of intra- and extracellular levels of total glutathione (GSH+GSSG). Both fractions were centrifuged at 20,800×*g*, at 4ºC for 10 min, to remove cell debris, and the supernatants were used for total glutathione analysis in a Gilson-ASTED HPLC system. Samples were separated on a Hichrom ACE type column (150 × 4.6 mm, 5 µM C18) at a flow rate of 2.5 mL/min for 45 min, using a ternary solvent system consisting of solvent A [37.5 mM sodium phosphate, 50 mM propionic acid, 7% acetonitrile, and 3% DMSO (pH 6.2)], solvent B (40% acetonitrile, 33% methanol, and 7% DMSO), and solvent C [62.5 mM sodium phosphate, 50 mM propionic acid, 7% acetonitrile, and 3% DMSO (pH 5.5)]. Total glutathione was detected as a fluorescent derivative after precolumn derivatization with OPA/2-mercaptoethanol (MCE), using a Gilson fluorescent detector model 121, with excitation at 340 nm and emission at 410 nm. Total glutathione levels were determined by comparison with peak

areas of GSH standards, calculated in nanomoles per milligram of protein and expressed as a percentage of wild-type cells. Protein levels were determined by the BioRad protein assay (Bradford method).

2.7.2.1.3 Western Blotting

Striatal cells were washed twice in PBS and extracted with lysis buffer containing 20 mM Tris-HCl (pH 7), 1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1 mM PMSF, 50 mM NaF, 1.5 mM Na_3VO_4 and 1 μg/mL protease inhibitor cocktail. Total extracts were centrifuged at 14,000 rpm for 10 min in order to discard debris, and protein content determined by the Bio-Rad method. Samples were denaturated in 50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 5% glycerol, 0.01% bromophenol blue and 100 mM DTT at 95ºC, for 5 min. Equivalent amounts of 30-60 μg of protein were separated on 12% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were further blocked with 5% fat-free milk and incubated with anti-GCLc (1:1000), anti-GRed (1:2,000), anti-SOD1 (1:1000), anti-Ac(Lys68)SOD2 (1:1000), anti-SOD2 (1:1000), anti-Akt (1:1000), anti-p-(Thr308)Akt (1:1000), anti-Erk (1:1000), anti-p-(Thr202/Tyr204)Erk (1:1000), anti-Nrf2 (1:1000) and anti-p-(Ser40)Nrf2 (1:1000). Anti-α-tubulin (1:1000) or anti-β-actin (1:5000) were used as loading controls for total cellular fractions, and anti-TBP (TATA binding protein) (1:1000) was used as loading control of nuclear fractions. Immunoreactive bands were visualized by alkaline phosphatase activity after incubation with ECF reagent, by using a BioRad Versa Doc 3000 Imaging System.

2.7.2.2 **Measurement of mRNA levels**

2.7.2.2.1 Quantification of G6PD, GCLc, HO-1 and Mrp1, NQO1 mRNA levels

The concentration of the resulting single-stranded cDNA was determined, and 10 ng was used to each further reaction. The cDNA of each sample was amplified using sequence-specific primers to to G6PD, forward (5'-3') ATG GCA GAG CAG GTG GCC; reverse (5'-3') GCA CTG TTG GTG GAA GAT G; GCLc: forward (5'-3') ATG GGG CTG CTG TCC CAA G; reverse (5'-3') GTA TGA GAG GAT CAC CCT AG; HO-1: forward (5'-3') GCT CAC GGT CTC CAG TCG CC; reverse (5'-3') CAC TGC

CAC TGT TGC CAA C; Mrp1, forward (5'-3') CTG CAC AAC CTG CGC TTC; reverse (5'-3') GGT GCC AGA GGC CAG AC; NQO1: forward (5'-3') AGG CTC AGC TCT TAC TAG C; reverse (5'-3') ATT CAT TTT GTT GTT ATG GCA G. The normalization of the amplified product was performed in relation to the product of actin obtained in the same samples using as primers (5'-3') GGA GAC GGG GTC ACC CAC AC and (5'-3') AGC CTC AGG GCA TCG GAA CC, forward and reverse respectively. All reactions were performed using GoTaq Flexi DNA Polymerase (5x kit) and 10 mM dNTP Mix. The amplification reaction mixture (50 μl) contained 10 μl of the cDNA template, 1.5 mM of $MgCl_2$, 0.2 μM of each primer, 0.05 U Taq Pol. The thermal cycling conditions included 3 min at 94ºC, proceeding with 40 cycles of 94ºC for 30 s, 57ºC for 30 s and 72ºC for 45 s, followed by 72ºC for 10 min. The size of the PCR products was visualized in a 1.7% agarose gel and analyzed afterwards using a BioRad Gel Doc 3000 Imaging System. G6PD, GCLc, HO-1, Mrp1, or NQO1 mRNA levels were expressed in relation to actin.

2.8 Statistical analysis

Statistical significance was determined by one-way or two-way ANOVA followed by the Bonferroni post-hoc test for multiple groups or by the Student's *t*-test for comparison between two Gaussian populations, as described in figure legends. Data were expressed as the mean ± S.E.M. of the number of experiments indicated in figure legends. Significance was accepted at $p<0.05$.

Chapter 3 – Results

3.1 Glutathione redox cycle dysregulation in Huntington's disease knock-in striatal cells[106]

3.1.1 Summary

HD is a CAG repeat disorder affecting the *HTT* gene, which encodes for Htt and is characterized by prominent cell death in the striatum. Oxidative stress was previously implicated in HD neurodegeneration, but the role of the major endogenous antioxidant system, the glutathione redox cycle, has been less studied following expression of full-length mHtt. Thus, in this work we analysed the glutathione system in striatal cells derived from HD knock-in mice expressing mHtt *versus* wild-type cells. Mutant cells showed increased intracellular ROS and caspase-3 activity, which were significantly prevented following treatment with GSHee. Interestingly, mutant cells exhibited an increase in intracellular levels of both GSH and GSSG, and enhanced activities of GPx and GRed. Furthermore, GST and γ-glutamyl transpeptidase (γ-GT) activities were also increased in mutant cells. Nevertheless, GCL and GS activities and levels of GCLc were decreased in cells expressing full-length mHtt, highly suggesting decreased *de novo* synthesis of glutathione. Enhanced intracellular total glutathione, despite decreased synthesis, could be explained by decreased extracellular glutathione in mutant cells. This occurred concomitantly with decreased mRNA expression levels and activity of the multidrug resistance-associated protein 1 (Mrp1), a transport protein that mediates cellular export of glutathione disulfide and glutathione conjugates. Additionally, inhibition of Mrp1 enhanced intracellular GSH in wild-type cells only. These data suggest that full-length mHtt affects the export of glutathione by decreasing the expression of Mrp1. Data further suggest that boosting of GSH-related antioxidant defence mechanisms induced by full-length mHtt is insufficient to counterbalance increased ROS formation and emergent apoptotic features in HD striatal cells.

[106] Based on the following publication: Ribeiro, M.; Rosenstock, T. R.; Cunha-Oliveira, T.; Ferreira, I. L.; Oliveira, C. R.; Rego, A. C. Glutathione redox cycle dysregulation in Huntington's disease knock-in striatal cells. Free Radic. Biol. Med. **53**:1857-1867; 2012.

3.1.2 Introduction

Oxidative stress is one of several dysfunctional mechanisms described in HD (Browne et al., 1997; 1999; Chen et al., 2007; Goswami et al., 2006; Stoy et al., 2005), which may result from impaired mitochondrial function, namely due to interaction of the organelle with mHtt (Panov et al., 2002) and/or imbalanced levels of antioxidants. Altered oxidative parameters have been observed in both central and peripheral samples of HD patients and in some animal models of the disease. In HD post-mortem tissues, levels of 8-OHdG, a DNA oxidative damage marker, and MDA, a lipid peroxidation marker, were increased in CN and parietal cortex (Browne et al., 1997; Polidori et al., 1999). In HD patients blood plasma, elevated levels of 8-OHdG were observed (Chen et al., 2007; Hersch et al., 2006). Also, increased levels of 8-OHdG (Chen et al., 2007) and MDA (Stoy et al., 2005) were previously observed, respectively, in leukocytes and serum of HD patients. Concordantly, in R6/2 HD mice (expressing the exon-1 of the human *HTT* gene), 8-OHdG levels were shown to be increased in striatum, urine and plasma (Bogdanov et al., 2001).

The levels of antioxidants also vary depending on the type of tissue and possibly the stage of HD pathology. In striatum and cortex of HD post-mortem samples a proteomic analysis revealed an induction of antioxidant proteins Prdx's 1, 2 and 6 and GPx1 and -6, and an increase in SOD2 and catalase activities, when compared to control individuals (Sorolla et al., 2008). Furthermore, increases in mRNA levels of GPx1, catalase and SOD1 were observed in striatal cells expressing full-length mHtt (Lim et al., 2008); however these studies did not analyze the enzymatic activities of the antioxidant systems. Conversely, in erythrocytes from HD patients, GPx and SOD1 activities were found to be decreased, compared to unaffected subjects (Chen et al., 2007), suggesting an impairment of the antioxidant defenses in HD peripheral samples.

Concordantly with increased levels of antioxidant proteins in HD human brain, a significant increase in the levels of GSH was observed in striatum of 8 to 12 week-old R6/2 HD mice (Tkac et al., 2007). Enhanced GSH levels were also detected in isolated mitochondria from both cortex and striatum of R6/2 HD mice; however, GCL, an enzyme involved in the synthesis of GSH, was reported to be actively decreased in both striatum and cortex of R6/2 HD mice, not explaining the increase in GSH levels (Choo et al., 2005). Moreover, in the same study, G6PD activity, an enzyme that produces NADPH essential for GRed activity, was also reported to be decreased in the striatum of R6/2 HD

mice (Choo et al., 2005). Together, these observations show some discrepancies in the antioxidant redox systems in HD, namely associated with the glutathione redox cycle, evidencing the need to clarify the regulation of antioxidant levels in striatal cells expressing full-length mHtt. Thus, in this study, we detailed the changes in GSH antioxidant system in striatal cells derived from HD knock-in mice. Our results indicate that expression of full-length mHtt is accompanied by heightened of GSH-related antioxidant levels and activity due to reduced functional activity of Mrp1, although they appear to be insufficient, as HD cells exhibit increased ROS generation and cell death features.

3.1.3 Results

3.1.3.1 ROS production and caspase-3 activation in HD striatal mutant cells – influence of GSHee

In order to investigate ROS production in HD striatal cells derived from knock-in mice, we used the fluorescent probe H_2DCFDA which has been reported to measure ROS, including H_2O_2, ONOO− and HOCl in viable cells (LeBel et al., 1992; Whiteman et al., 2005). A significant increase in ROS formation was observed in mutant cells when compared to wild-type cells (Figure 3.1.1A). Treatment with GSHee, a cell permeable ester of GSH (0.05, 0.25 and 1 mM) significantly decreased ROS levels in mutant cells to similar values of wild-type (control) cells (Figure 3.1.1A). Moreover, an increase in caspase-3 activity was observed in mutant cells (Figure 3.1.1B), which might be related to the increase in ROS formation, as described previously by Gil and Rego, (2008). GSHee treatment also decreased caspase-3 activity in mutant cells (Figure 3.1.1B), which was significant for 0.25 mM GSHee. These results suggested that intracellular levels of GSH might be insufficient to deal with ROS produced in cells expressing mHtt. Thus, in the following experiments we determined the changes in components of the glutathione redox cycle in HD striatal cells.

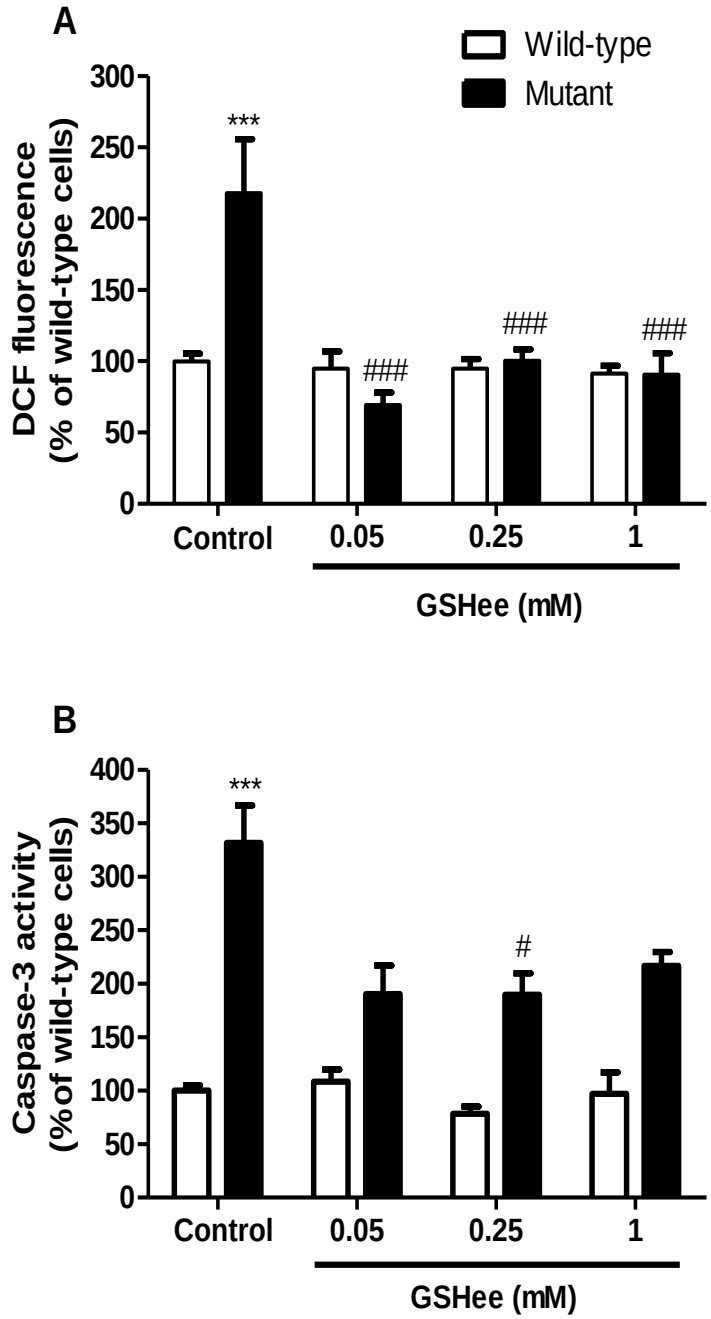
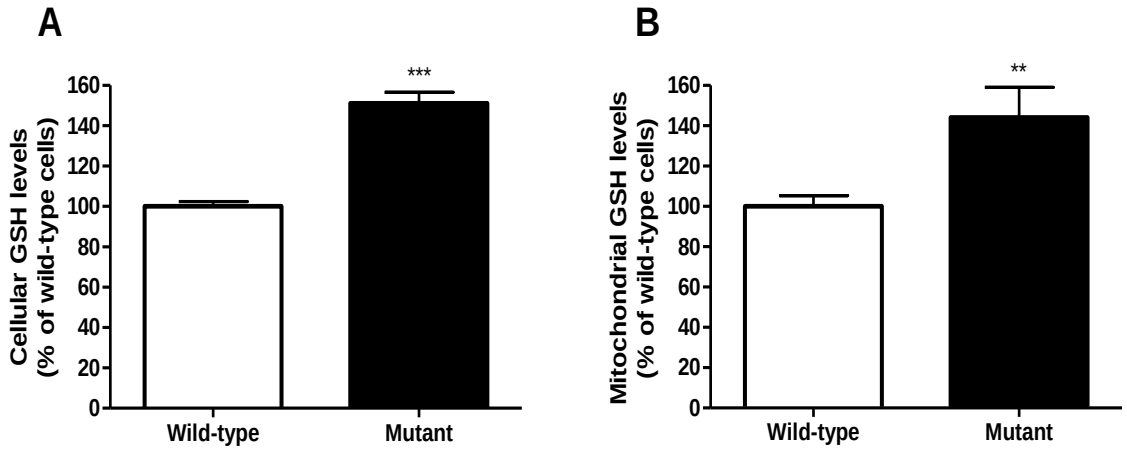


Figure 3.1.1 – Effect of GSH ethyl ester on ROS generation and caspase-3 activity in HD knock-in striatal cells. ROS formation and caspase-3 activity were measured in wild-type and mutant cells before and after incubation with 0.05, 0.25 or 1 mM GSH ethyl ester (GSHee) for 24 h. (A) DCF fluorescence shows that mutant cells produce significant more peroxides than wild-type cells (696.6 ± 54.9 RFU/min/mg protein); GSHee supplementation significantly reduced ROS formation in mutant cells, when compared to non-treated conditions. (B) Caspase-3 activity is increased in mutant cells and 0.25 mM GSHee protected against caspase-3 activation (caspase-3 activity in untreated wild-type cells: 404.1 ± 19.2 RFU/min/mg protein). Data are presented as mean ± S.E.M. of 3 to 5 independent experiments performed in duplicates, triplicates or 6 replicates. Statistical analysis was performed by two-way ANOVA followed by Bonferroni post-

test for analysis of multiple groups: ***P<0.0001 when compared to wild-type cells; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.0001 when compared to non-treated conditions.

3.1.3.2 **Glutathione redox cycle is affected upon expression of mutant huntingtin**

GSH has been shown to be essential to detoxify cells against H_2O_2. In order to investigate the levels of the glutathione antioxidant defense systems present in striatal mutant and wild-type cells, we measured the intracellular levels of GSH and GSSG. Surprisingly, a significant increase in the levels of GSH (Figure 3.1.2A,B) and GSSG (Figure 3.1.2C,D) were found in total (cellular) and mitochondrial fractions of mutant cells, compared to wild-type cells. These data suggested an alteration of the glutathione redox cycle enzymes in HD knock-in striatal cells. Thus, we further determined GPx and GRed activities. GPx converts GSH and H_2O_2 into GSSG and H_2O, respectively. GRed uses GSSG and NADPH to produce GSH and $NADP^+$. Concomitantly with the alterations in GSH and GSSG, we observed a significant increase in total and mitochondrial GPx activity in mutant cells, when compared to wild-type cells (Figure 3.1.2E and F). Moreover, we observed that total and mitochondrial GRed activities were increased in mutant cells, as compared to wild-type cells (Figure 3.1.2G and H).



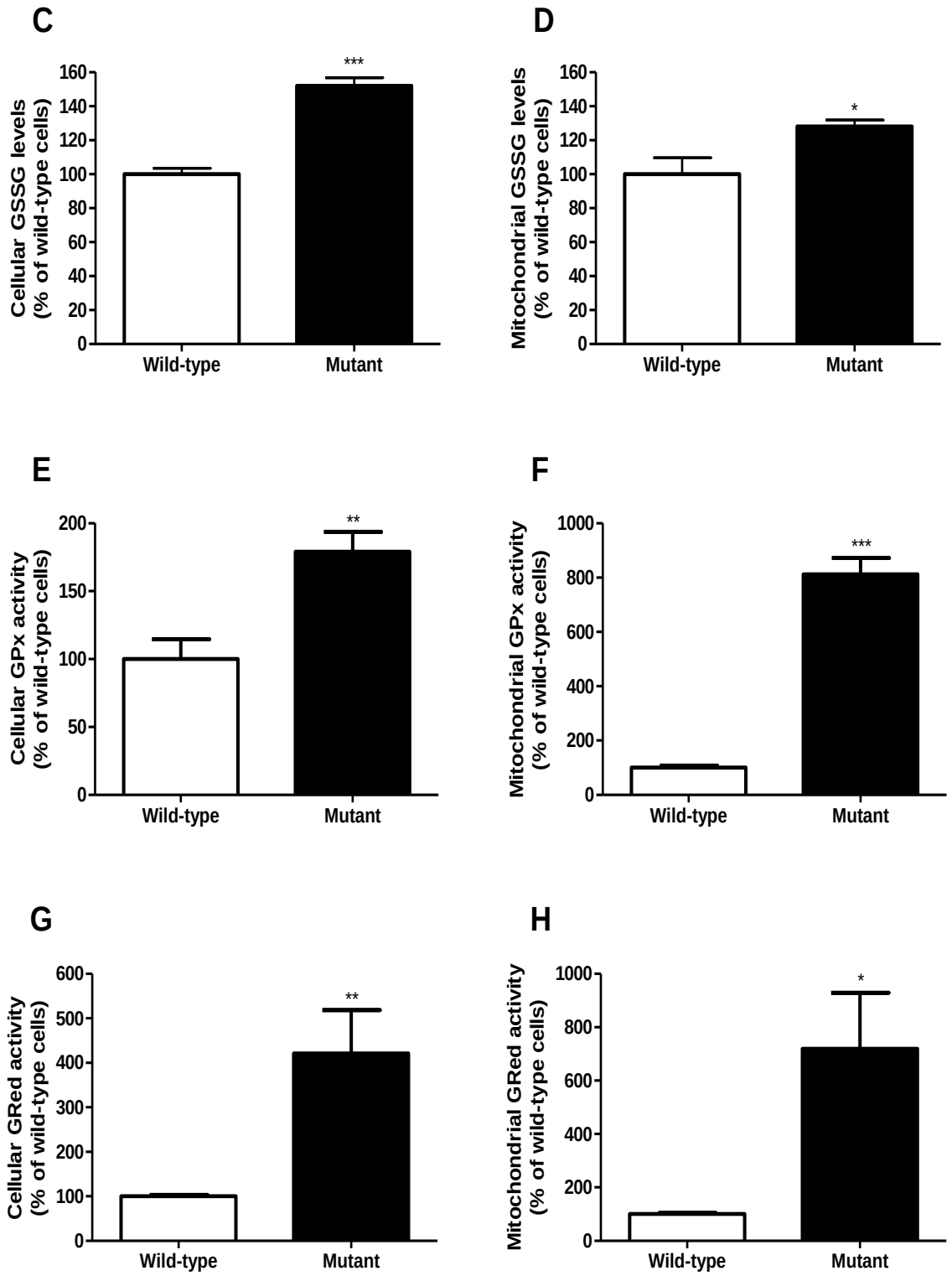
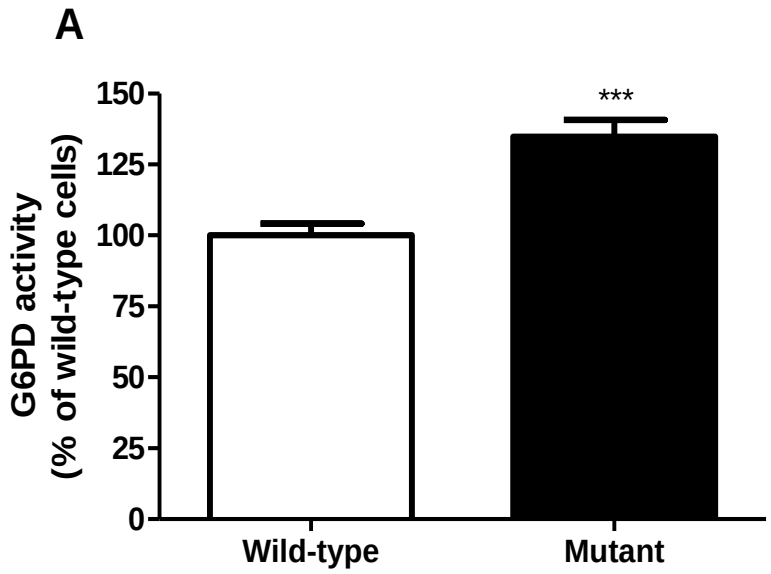


Figure 3.1.2 – Glutathione redox cycle upon expression of full-length mutant huntingtin. Striatal cells were cultured during 48 h and then used to determine reduced (GSH) and oxidized (GSSG) glutathione levels, and the activities of glutathione peroxidase (GPx) and glutathione reductase (GRed). Levels of total (A,C) and mitochondrial (B,D) GSH (A,B) and GSSG (C,D) were significantly increased in mutant cells, when compared to wild-type cells (cellular GSH: 464600 ± 12140 RFU/mg protein; mitochondrial GSH: 53586 ± 3693 RFU/mg protein; cellular GSSG:

63805 ± 2350 RFU/mg protein; mitochondrial GSSG: 8965 ± 590 RFU/mg protein). GPx (E,F) and GRed (G,H) activities were also examined in total (E,G) and mitochondrial (F,H) fractions. Data show significantly increased GPx and GRed activities in mutant cells, when compared to wild-type cells (in wild-type cells: cellular GPx activity: 110.2 ± 17.1 mU/mg protein; mitochondrial GPx activity: 61.2 ± 18.3 mU/mg protein; cellular GRed activity: 36.1 ± 3.2 mU/mg protein; mitochondrial GRed activity: 21.4 ± 2.7 mU/mg protein). Results are expressed as the mean ± S.E.M. of 3 to 4 independent experiments performed in triplicates. Statistical analysis was performed by Student's *t*-test: *P<0.05; **P<0.01 and ***P<0.0001 when compared to wild-type cells.

3.1.3.3 NADPH and pentose-phosphate NADPH producing enzymes are increased in mutant cells

In order to explain the increase in activity of GRed in mutant cells we further measured the activities of the pentose phosphate pathway enzymes, which are mainly responsible for producing the NADPH necessary for GRed activity, namely G6PD and 6PGD. Our results showed significant increases in G6PD and in 6PGD activities in HD mutant cells, compared to wild-type cells (Figure 3.1.3A and B), corroborating our previous findings. However, no changes in mRNA levels of G6PD were found in striatal cells (Figure 3.1.3C).



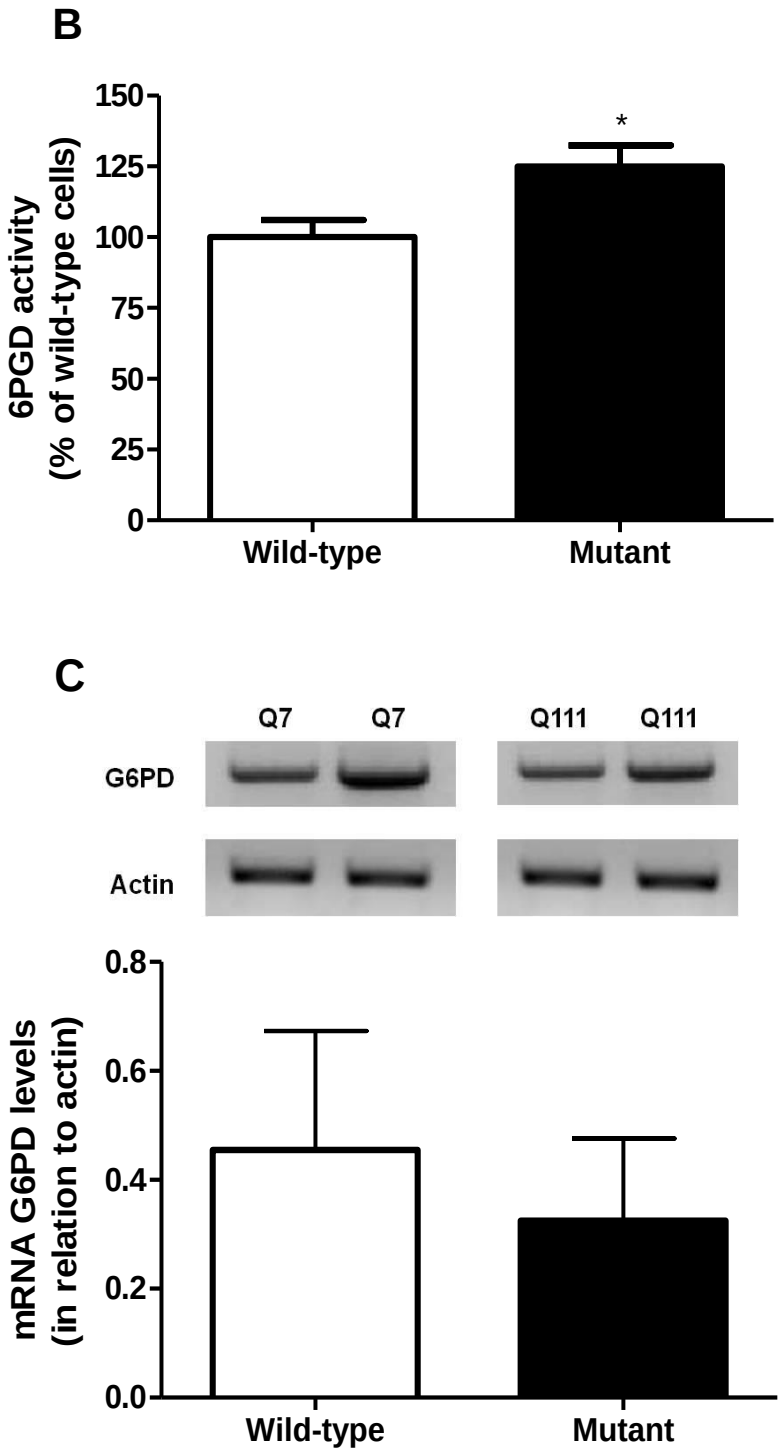
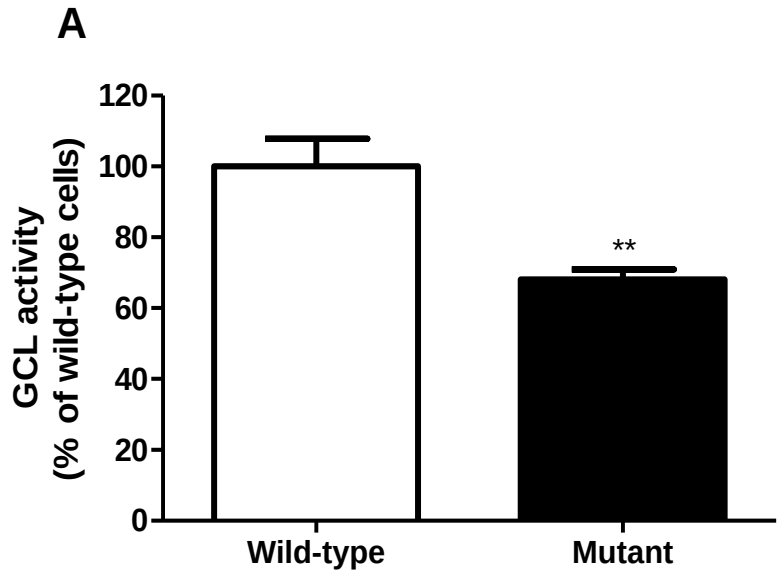


Figure 3.1.3 – NADPH-producing pentose phosphate pathway enzyme activities in striatal mutant cells. HD mutant striatal cells from knock-in mice and wild-type cells were used for analysis of glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) activities and G6PD mRNA levels. G6PD (A) and 6PGD (B) activities were significantly increased in mutant cells, when compared to wild-type cells, whereas G6PD mRNA levels (C) were unchanged in mutant cells (G6PD activity in wild-type cells:89.4 mU ± 11.2 mU/mg protein; 6PGD activity in wild-type cells: 52.3 ± 10.1 mU/mg protein). The results are expressed as the mean ± S.E.M. from 3 to 4 independent experiments performed in triplicates. Statistical

analysis was performed by the Student's *t*-test: *P<0.05 and ***P<0.0001 when compared to wild-type cells.

3.1.3.4 **Decreased GSH producing enzymes and increased activities of gamma-glutamyl transpeptidase and glutathione S-transferase in mutant cells**

Because increased glutathione levels could result from increased GSH synthesis, we also studied GCL and GS activities and analyzed the protein expression levels of GCLc. Unexpectedly, we observed a significant decrease in GCL and GS activities in mutant cells, compared to wild-type cells (Figure 3.1.4A and B), suggesting decreased GSH synthesis. We also observed a slight, although significant, decrease in the protein levels of GCLc in mutant cells when compared to wild-type cells (Figure 3.1.4C), contributing to the observed decrease in GCL activity (Figure 3.1.4A). Since these results did not explain the higher GSH levels observed in Figure 3.1.2A and B, we measured the activities of γ-GT, an enzyme involved in GSH degradation, and GST, which also participates in glutathione metabolism. Our results demonstrate an increase in the activity of γ-GT in mutant cells when compared to wild-type cells (Figure 3.1.5A). Moreover, GST activity was also increased in total and mitochondrial (although not significant) fractions obtained from mutant cells, compared to wild-type cells (Figure 3.1.5B and C), thus not explaining the intracellular accumulation of GSH.



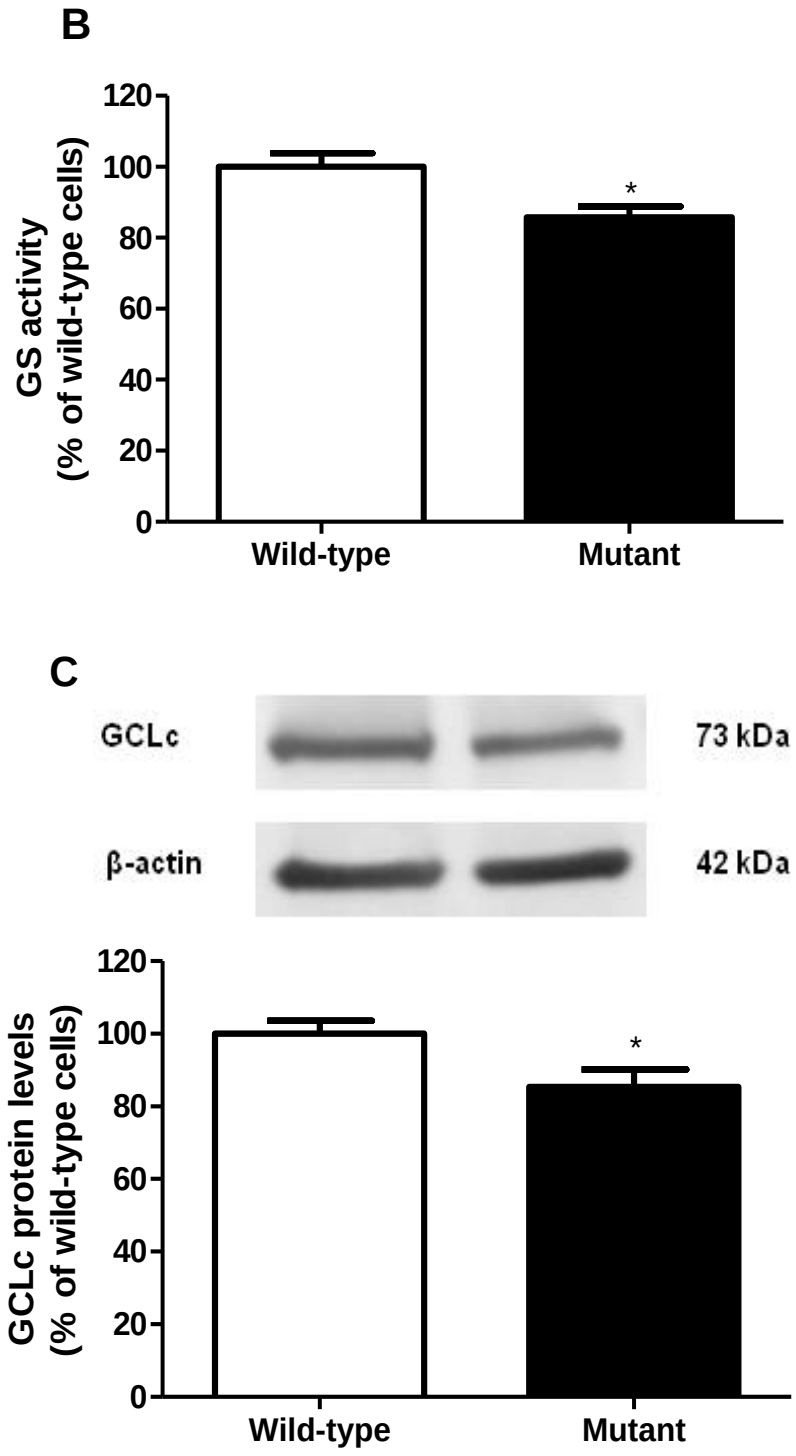
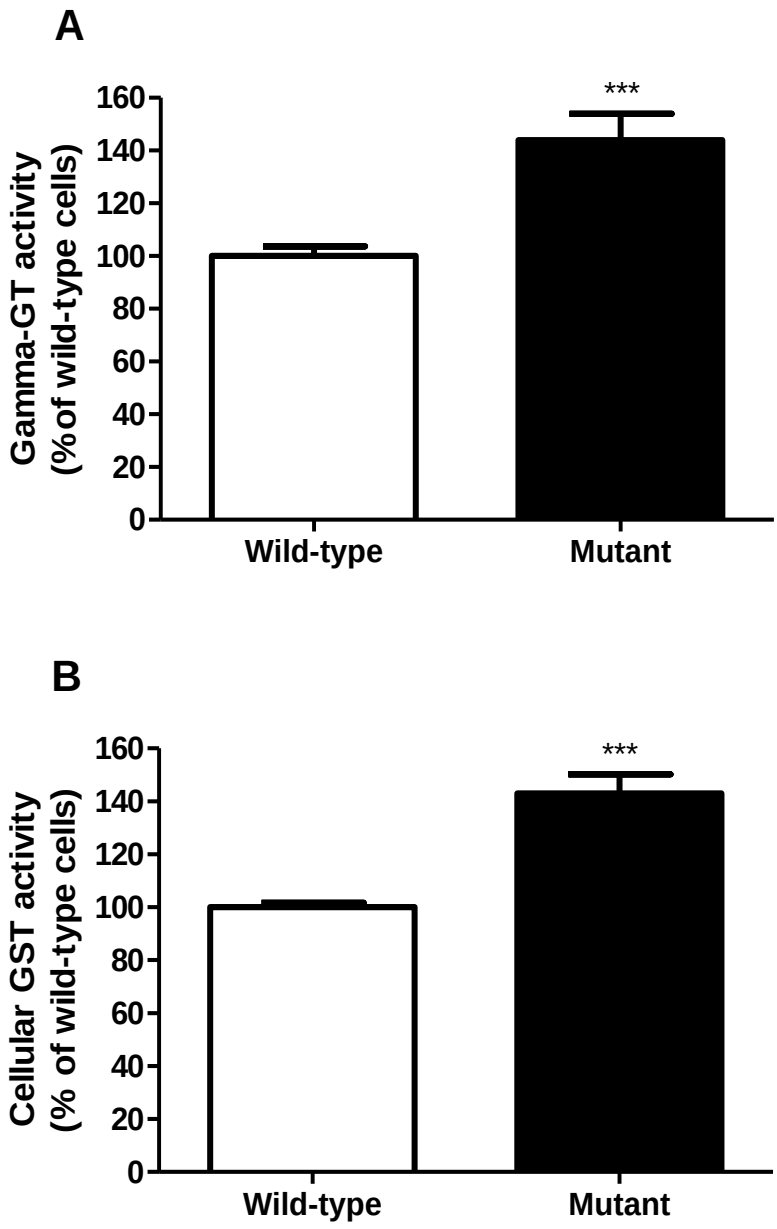


Figure 3.1.4 – Activity and protein expression levels of enzymes involved in GSH synthesis in mutant cells. GSH synthesis was determined through the analysis of (**A**) glutamate-cysteine ligase (GCL) and (**B**) glutathione synthetase (GS) activities, and (**C**) catalytic subunit of GCL (GCLc) protein levels, which were decreased in mutant *versus* wild-type cells (GCL activity: 3981 ± 138.4 RFU/mg protein; GS activity: 5764 ± 223.7 RFU/mg protein). The results are expressed as the mean ± S.E.M. from 3 to 6 independent experiments performed in duplicates. Statistical analysis was performed by Student's *t*-test: *P<0.05 or **P<0.01 when compared to wild-type cells.


A
Gamma-GT activity
(%of wild-type cells)
160
140
120
100
80
60
40
20
0
***
Wild-type
Mutant
B
Cellular GST activity
(% of wild-type cells)
160
140
120
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Wild-type
Mutant

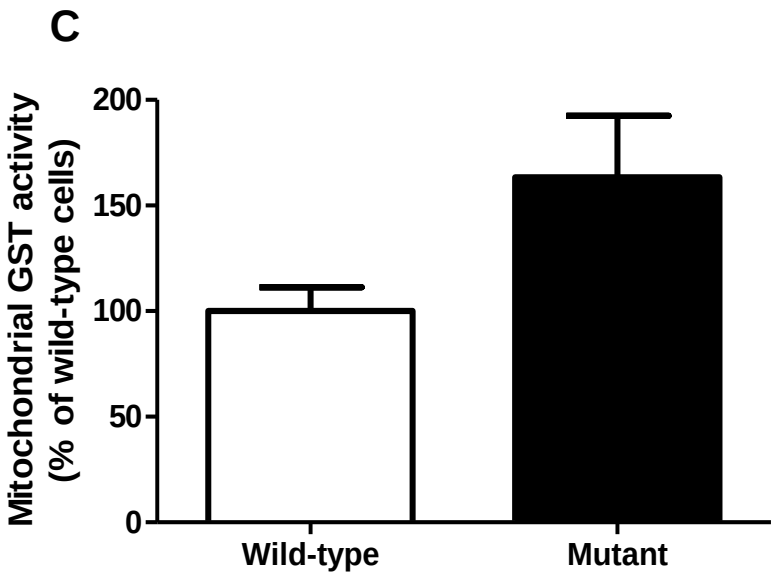


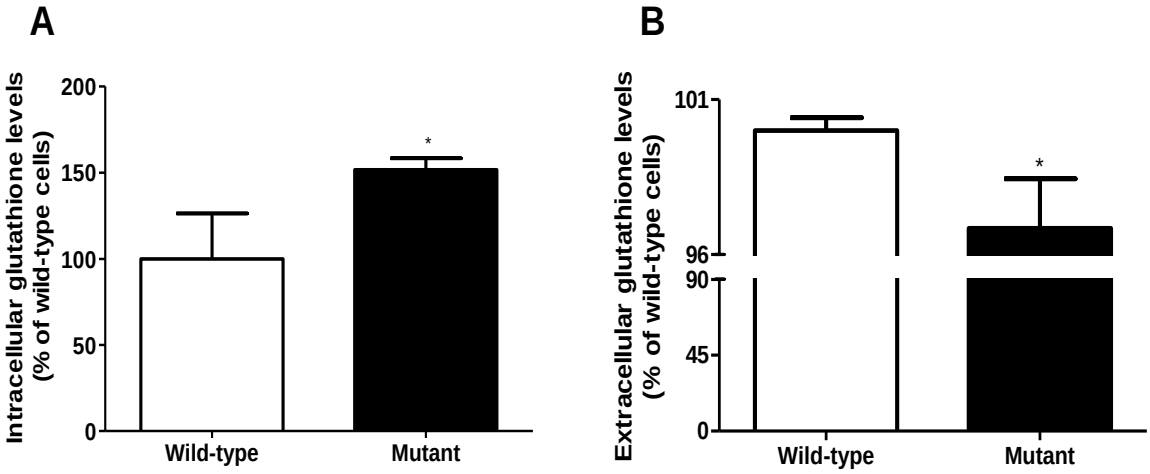
Figure 3.1.5 – Activities of gamma-glutamyl transpeptidase and glutathione S-transferase in HD knock-in striatal cells. Gamma-glutamyl transpeptidase (γ-GT) (A) and glutathione-S-transferase (GST) (B,C) were measured in total lysates (A,B) and mitochondrial fractions (C) obtained from HD mutant and wild-type striatal cells. γ-GT and GST activities were increased in mutant cells when compared to wild-type cells, although statistically significant results compared to wild-type cells were observed in total fractions only (γ-GT activity in wild-type cells: 372 ± 29.5 RFU/min/mg protein; cellular and mitochondrial GST activity in wild-type cells: 78.2 ± 5.6 µmol/min/mg protein and 42.4 ± 2.3 µmol/min/mg protein, respectively). The results are expressed as the mean ± S.E.M. from 3 independent experiments performed in duplicates. Statistical analysis was performed by Student's *t*-test: ***P<0.0001 when compared to wild-type cells.

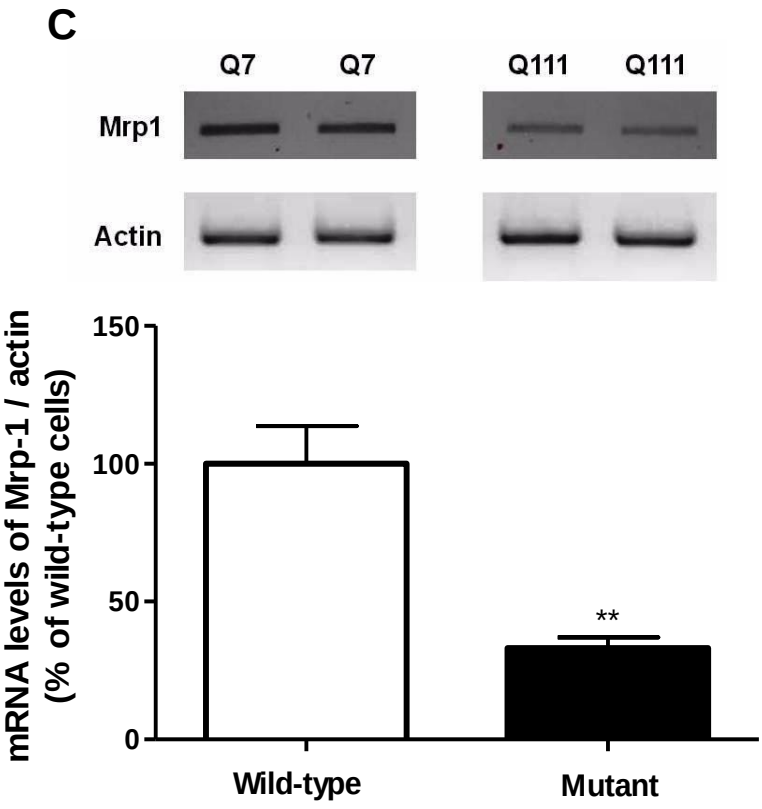
3.1.3.5 **Decreased extracellular glutathione levels and Mrp1 mRNA levels upon expression of mutant huntingtin**

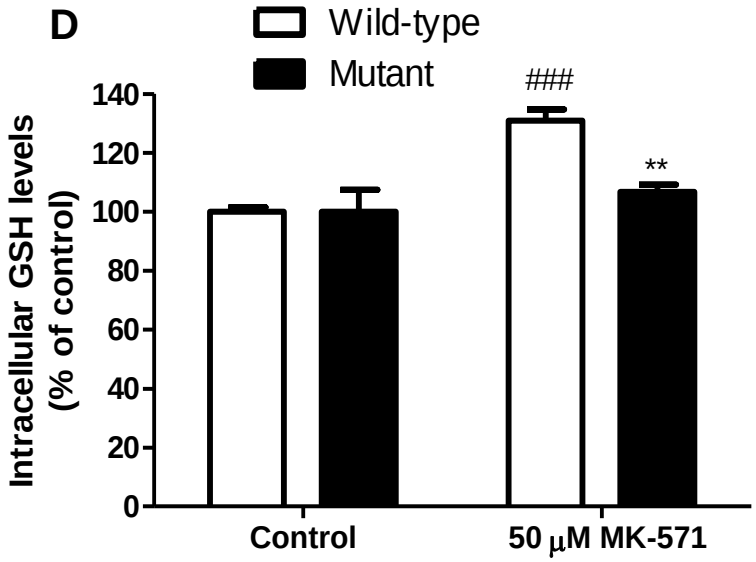
In order to explain the higher levels of GSH in mutant cells, we also evaluated the levels of glutathione by HPLC, which gives information about total glutathione (GSH plus GSSG) levels. Concordantly with data shown in Figure 3.1.2, we also observed a significant increase in the intracellular total glutathione levels in mutant cells (Figure 3.6A). Because the activities of GSH metabolizing and converting enzymes, γ-GT and GST, were increased (Figure 3.1.5), the rise in GSH in mutant cells was still unexplained. Thus, we analyzed whether enhanced intracellular GSH pool resulted from cellular retention by evaluating the extracellular levels of total glutathione in the culture medium of striatal cells maintained for 72 h in culture. Data in Figure 3.1.6B show a slight, but significant, decrease in the accumulation of extracellular total glutathione (about 8%

decrement of GSH plus GSSG levels present in the culture medium, containing FBS) in the mutant striatal cells, suggesting a deficit in its release.

In order to explain the decrease in glutathione release we determined the mRNA expression of Mrp1, a transport protein that mediates cellular export of glutathione and glutathione conjugates (e.g. Müller et al., 1994). Importantly, Mrp1 expression was largely and significantly decreased in mutant cells (Figure 3.1.6C), suggesting that expression of full-lenght mHtt induces alterations in the glutathione dynamics due to altered expression of Mrp1. This result was supported by a significant increase in intracellular GSH levels in wild-type cells after Mrp1 inhibition with MK-571 (at 50 µM, for 24 h), whereas no changes were detected in mutant cells (Figure 3.1.6D). Additionally, we determined the activity of Mrp1 based on the fluorescence of calcein. Mrp1 is involved in the cellular export of calcein and therefore an increase in calcein fluorescence may correlate to decreased Mrp1 activity (Dogan et al., 2004). In accordance with decreased expression of the protein, we determined a significant increase in intracellular calcein fluorescence in mutant cells, when compared to wild-type cells, indicating a decrease in Mrp1 activity (Figure 3.1.6E).




C
Q7
Q7
Q111
Q111
Mrp1
Actin
mRNA levels of Mrp-1 / actin
(% of wild-type cells)
150
100
50
0
**
Wild-type
Mutant


D
Wild-type
Mutant
Intracellular GSH levels
(% of control)
140
120
100
80
60
40
20
0
###
**
Control
50 μM MK-571

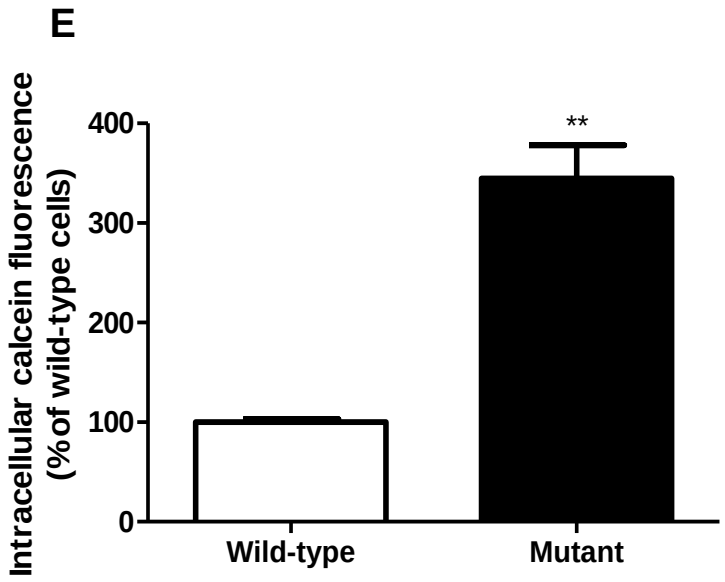


Figure 3.1.6 – Intracellular and extracellular glutathione and Mrp1 mRNA levels upon expression of mHtt. Striatal cells were incubated and the culture medium was collected after 72 h in culture for analysis of extracellular glutathione. Cells were lysed and extra- and intracellular total glutathione (GSH+GSSG) levels were determined by HPLC. (A) Intracellular glutathione levels were increased, whereas (B) extracellular glutathione levels were significantly decreased in mutant cells (intracellular glutathione levels in wild-type cells: 24.82 ± 4.854 nmol/mg protein; extracellular glutathione levels in wild-type cells: 26.06 ± 4.955 nmol/mg protein). In (C) mRNA levels of Mrp1 were also significantly reduced in mutant cells, when compared to wild-type cells. (D) Intracellular GSH levels (in wild-type cells: 482354 ± 9859 RFU/mg protein) measured before and after incubation with 50 μM MK-571; exposure to MK-571 increased GSH levels in wild-type cells only. (E) Mrp1 activity based on cellular export of fluorescent calcein (Mrp1 activity: 76790 ± 2548 RFU/mg protein in wild-type cells). Results are expressed as the mean ± S.E.M. from 3 to 4 independent experiments performed in duplicates. Statistical analysis was performed by Student's *t*-test: *P<0.05 or **P<0.01 when compared to wild-type cells. For analysis of multiple groups (D) statistical analysis was performed by two-way ANOVA followed by Bonferroni post-test: ***P*<0.01 when compared to wild-type cells; $^{###}$P<0.0001 when compared to non-treated conditions.

3.1.4 Discussion

In this work we found evidences for oxidative stress in cells expressing full-lenght mHtt. Increased ROS occurs concomitantly with enhanced intracellular glutathione levels and activity of the glutathione redox cycle, despite the downward effects of mHtt on the activity and expression of enzymes involved in GSH synthesis. Importantly, decreased expression of Mrp1 favours the intracellular accumulation of glutathione.

Previous studies reported significantly higher ROS formation in the striatum of R6/1 HD mice expressing the exon-1 of mHtt compared to control mice (Perez-Severiano

et al., 2004). In HD knock-in striatal cells, mitochondrial dysfunction (Milakovic and Johnson, 2005; Oliveira et al., 2006; Quintanillaet al., 2008) may related to increased formation of $O_2^{\bullet -}$ and other cellular ROS, as shown in this study. Moreover, in Wistar rats subjected to the mitochondrial complex II inhibitor 3-NP, known to mimic some characteristics of HD, higher peroxide formation was detected when compared to untreated rats (Tunez et al., 2006). Although HD knock-in striatal cells evidence increased ROS generation, they do not exhibit massive cell death and large or visible mHtt aggregates (Trettel et al., 2000), thus mimicking initial HD cytopathological features. Nevertheless, these cells exhibit features of apoptosis, as demonstrated by increased caspase-3 activation (Lim et al., 2008; Rosenstock et al., 2011).

We further show that striatal cells expressing full-lenght mHtt present higher levels of GSH compared to wild-type cells, although this is not sufficient for preventing the rise in ROS. Indeed, treatment with GSHee was effective in preventing ROS generation, revealing that mutant cells are under a reversible state of oxidation. Also, caspase-3 activation was ameliorated by treatment of mutant striatal cells with GSHee, further suggesting that increased intracellular accumulation of GSH is insufficient to rescue HD cells from features of apoptotic cell death. Previously, treatment of HD striatal cells with GSHee significantly prevented 3-NP-mediated decrease in $\Delta\Psi m$ (Mao et al., 2006).

Although HD knock-in striatal cells, expressing full-lenght mHtt, showed increased levels of glutathione, a decrease in GSH levels was previously described in the cortex of HD patients (Beal et al., 1992). In addition, decreased GSH levels and higher lipid peroxidation were observed in the plasma of HD symptomatic and asymptomatic individuals, compared to age and sex-matched controls, suggesting that oxidative stress may occur before the onset of HD symptoms (Klepac et al., 2007). Conversely, the levels of mitochondrial GSH were increased in the striatum and cortex of R6/2 HD mice compared to wild-type animals, suggesting a compensatory mechanism to counteract the increase in mitochondrial ROS generation (Choo et al., 2005), which may also help to explain our data. R6/2 mice are one of the most studied models of HD. However, R6/2 mice present a rapid progression of the disease due to only express the exon-1 of mHtt and present some phenotypes not typically associated with HD (Gil and Rego, 2008). Therefore, studies with models that express full-length mHtt are important to understand the mechanisms associated with the occurrence of oxidative stress in the disease.

Despite increased intracellular glutathione, here we demonstrate, for the first time, a decrease in GCL and GS activities in striatal cell lines expressing full-lenght mHtt, largely suggesting a decrease in GSH synthesis. Additionally, GCLc protein (Figure 3.1.4C) and mRNA (author's unpublished data) expression levels are significantly decreased in mutant cells. In agreement, a decrease in GCL activity was previously shown in the cortex and striatum of R6/2 HD, compared to wild-type mice (Choo et al., 2005). In the present work we show that this defect is also observed following expression of full-lenght mHtt.

Along with increased cellular GSH in HD striatal cells, we observed an increase in GSSG levels. In contrast, a significant decrease in GSSG (by about 50%) was detected in the CN of HD patients (Sian et al., 1994). We also observed an increase in GPx activity. In accordance with these data, GPx1 transcription levels were shown to be increased in three clones of striatal cells expressing mHtt, compared to wild-type cells (Lim et al., 2008). Nevertheless, decreased GPx activity was found in erythrocytes of HD patients compared to control individuals (Chen et al., 2007), whereas in striatal tissue of R6/1 HD mice GPx activity was not different (Perez-Severiano et al., 2004), and GPx transcription was unaltered in R6/2 HD mice (Fox et al., 2004), compared with wild-type mice. Concordantly with our study, an increase in GRed activity was found in erythrocytes from HD patients, compared to control individuals (Zanella et al., 1980) and increased GRed transcription was found in the basal ganglia of R6/2 HD mice (Fox et al., 2004), suggesting a repository mechanism of GSH levels. We also observed increased activity of NADPH producing-enzymes of the pentose-phosphate pathway (G6PD and 6PGD), which is relevant since NADPH is an important cofactor of GRed. Changes in G6PD activity in HD models are controversial. G6PD activity was found to be increased in erythrocytes from HD patients (Zanella et al., 1980), but decreased in the striatum from R6/2 mice (Choo et al., 2005). Moreover, hexokinase, a prime enzyme in glycolysis and in the pentose-phosphate pathway upstream G6PD, was found to be actively increased in the brain of 3-NP-treated and in the HD transgenic N171-82Q mouse models (Olah et al., 2008).

Despite decreased GSH synthesis in HD striatal cells, we show that glutathione was accumulated intracellularly; this was not explained by alterations in GST or γ-GT (both involved in glutathione metabolism), since their activities were increased in mutant cells. Conversely, increased intracellular glutathione could be due to decreased release of

the endogenous antioxidant to the extracellular milieu by Mrp1, which mediates the export of GSH and GSH conjugates in an ATP-dependent manner (Müller et al., 1994; Rappa et al., 1997), thus also requiring a functional metabolic status of the cells. Mrp1 is a 190 kDa protein involved in cellular xenobiotics metabolite detoxification, namely carcinogens, pesticides, herbicides or metalloids. Mrp1 is highly overexpressed in many drug-resistant tumour cell lines and expressed at moderate levels in most normal tissues. In the brain it is part of the drug permeability barrier between blood and the cerebrospinal fluid (Cole and Deeley, 2006). No data describing the Mrp1 involvement in HD has been reported until now. Here, we show that mRNA levels of Mrp1 are decreased in mutant HD striatal cells, which may be related to the negative effects of mHtt on transcription regulation (Cha, 2000). Indeed, mHtt interacts with and sequesters CBP, a co-activator of transcription that forms a complex with CREB to initiate gene transcription (Mayr and Montminy, 2001), within protein aggregates (Steffan et al., 2000) leading to decreased transcription of PGC-1α, a transcriptional regulator of mitochondrial biogenesis and antioxidant enzymes (Cui et al., 2006). Therefore, mHtt may be interacting and/or altering transcription and thus modifying normal Mrp1 expression.

In astrocytes, Mrp1 was previously reported to mediate 60% of the GSH export and to be exclusively responsible for GSSG export (Minich et al., 2006). Moreover, due to its role in the transport of glutathione S-conjugates and GSSG into the extracellular space, proteins of the Mrp family may play an important function in defense mechanisms against oxidative stress (Keppler, 1999). Concordantly with the decreased expression of Mrp1, we observed that Mrp1 activity is affected in mutant cells. Furthermore, we observed a significant increase in intracellular GSH levels in wild-type cells, but not in mutant striatal cells after inhibition of Mrp1 with MK-571, largely evidencing the dysfunction in this transport protein in HD cells. No changes of intracellular GSH levels were observed in mutant cells, reinforcing the hypothesis of decreased Mrp1 function upon expression of full-lenght mHtt. In this perspective, fragments of mHtt may be involved in altering the activity of Mrp1, more studies are required to test this possibility.

3.2 Oxidizing effects of exogenous stressors in Huntington's disease knock-in striatal cells – protective effect of cystamine and creatine[107]

3.2.1 Summary

HD is a polyQ-expansion disease associated to degeneration of striatal and cortical neurons. Previously, we showed that oxidative stress occurs in HD knock-in striatal cells, but little is known regarding cell antioxidant response against exogenous stimuli. Therefore, in the present study we analysed cellular antioxidant profile following H_2O_2 and STS exposure, and tested the protective effect of cystamine and creatine in striatal cells expressing mHtt with 111 Q (ST*Hdh*$^{Q111/Q111}$; mutant cells) *versus* wild-type cells (ST*Hdh*$^{Q7/Q7}$). Mutant cells displayed increased mitochondrial ROS and decreased NOX and XO activities, reflecting lower $O_2^{\bullet-}$ cytosolic generation, along with increased SODs and components of glutathione redox cycle. Exposure to H_2O_2 and STS enhanced ROS in mutant cells and largely increased XO activity; STS further boosted the generation of mitochondrial ROS and caspase-3 activity. Both stimuli slightly increased SOD1 activity, without affecting SOD2 activity, and decreased GRed with a consequent rise in GSSG in mutant cells, whereas H_2O_2 only increased GPx activity. Additionally, creatine and cystamine increased mutant cells viability and prevented ROS formation in HD cells subjected to H_2O_2 and STS. These results indicate that elevation of the antioxidant systems accompanies mitochondrial-driven ROS generation in mutant striatal cells and that exposure to noxious stimuli induces a higher susceptibility to oxidative stress by increasing XO activity and lowering the antioxidant response. Furthermore, creatine and cystamine are efficient in preventing H_2O_2- and STS-evoked ROS formation in HD striatal cells.

[107] Based on the following publication: Ribeiro, M.; Silva, A. C.; Rodrigues, J.; Naia, L.; Rego, A. C. Oxidizing effects of exogenous stressors in Huntington's disease knock-in striatal cells--protective effect of cystamine and creatine. Toxicol Sci **136**:487-499; 2013.

3.2.2 Introduction

The selective death of striatal and cortical neurons is not completely understood. Several cytotoxic mechanisms have been described in HD, including oxidative stress (Browne et al., 1997, 1999; Chen et al., 2007; Goswami et al., 2006; Stoy et al., 2005). Oxidation markers, such as 8-OHdG and MDA were increased in leukocytes and plasma of HD patients, respectively (Chen et al., 2007). Impairment in antioxidant defenses may also underlie enhanced oxidative stress in HD. In erythrocytes from HD patients, GPx and SOD1 activities were decreased (Chen et al., 2007). Conversely, increased levels of Prdx's 1, 2 and 6 and GPx 1 and 6, and increased activities of SOD2 and catalase were observed in HD *post-mortem* striatal and cortical brain samples (Sorolla et al., 2008). Increased levels of GSH were also detected in the striatum of 8 week-old (early symptomatic) R6/2 HD mice (Tkac et al., 2007) and in ST*Hdh*^{Q111} striatal cells derived from HD knock-in mice (Ribeiro et al., 2012), which may represent early phases of HD due to the absence of visible protein aggregates and overt cell death (Trettel et al., 2000). Therefore, treatment with compounds that can inhibit or prevent the oxidation of proteins, lipids and DNA in HD might be a potential target for alleviating disease progression.

PolyQ repeat domains and mHtt are substrates for TG2 (Kahlem et al., 1996; 1998). Cystamine is a competitive inhibitor of TG2, which may inhibit mHtt cross-links induced by TG2, reducing mHtt aggregates. Cystamine administration in R6/2 HD mice increased motor performance, body and brain weight, and survival (Dedeoglu et al., 2002; Karpuj et al., 2002), and decreased brain atrophy and the number of protein aggregates in striatal and cortical sections (Dedeoglu et al., 2002). Cystamine also increased GSH and decreased the number of aggregates in cell lines expressing mHtt (Fox et al., 2004). Cystamine-induced neuroprotection R6/2 HD mice was maintained in TG2(-/-) R6/2 HD mice, suggesting that TG2 inhibition is not the sole target of cystamine in HD (Bailey and Johnson, 2006). Indeed, cystamine was described to inhibit caspase-3 activity (Lesort et al., 2003). Moreover, cystamine protected against H_2O_2-increased caspase-3 activation and increased total glutathione levels in human neuroblastoma SH-SY5Y cells (Lesort et al., 2003).

Energy metabolism is also impaired in HD. Increased lactate levels were previously described in occipital cortex and in striatum of HD patients (Jenkins et al., 1998). Furthermore, impaired mitochondrial respiration and ATP production were reported in ST*Hdh*^{Q111} HD striatal cells (Milakovic and Johnson, 2005) and ATP

depletion was also demonstrated in brain tissues of R6/2 mice (Mochel et al., 2012). Creatine is a substrate of cytosolic and mitochondrial CK's (Woznicki and Walker 1979), which catalyze the reversible phosphorylation of creatine, using ATP as a phosphate donor. Creatine can thereby modulate ATP metabolism by increasing the phosphocreatine content (Woznicki and Walker 1979). Apart from counterbalancing energy metabolism, creatine has been shown to have direct antioxidant proprieties (Lawler et al., 2002). In early HD patients, creatine decreased 8-OHdG levels, increased serum and brain creatine levels and was shown to be safe and well tolerated (Hersch et al., 2006). Creatine is presently under phase III clinical trial for HD (clinicaltrials.gov).

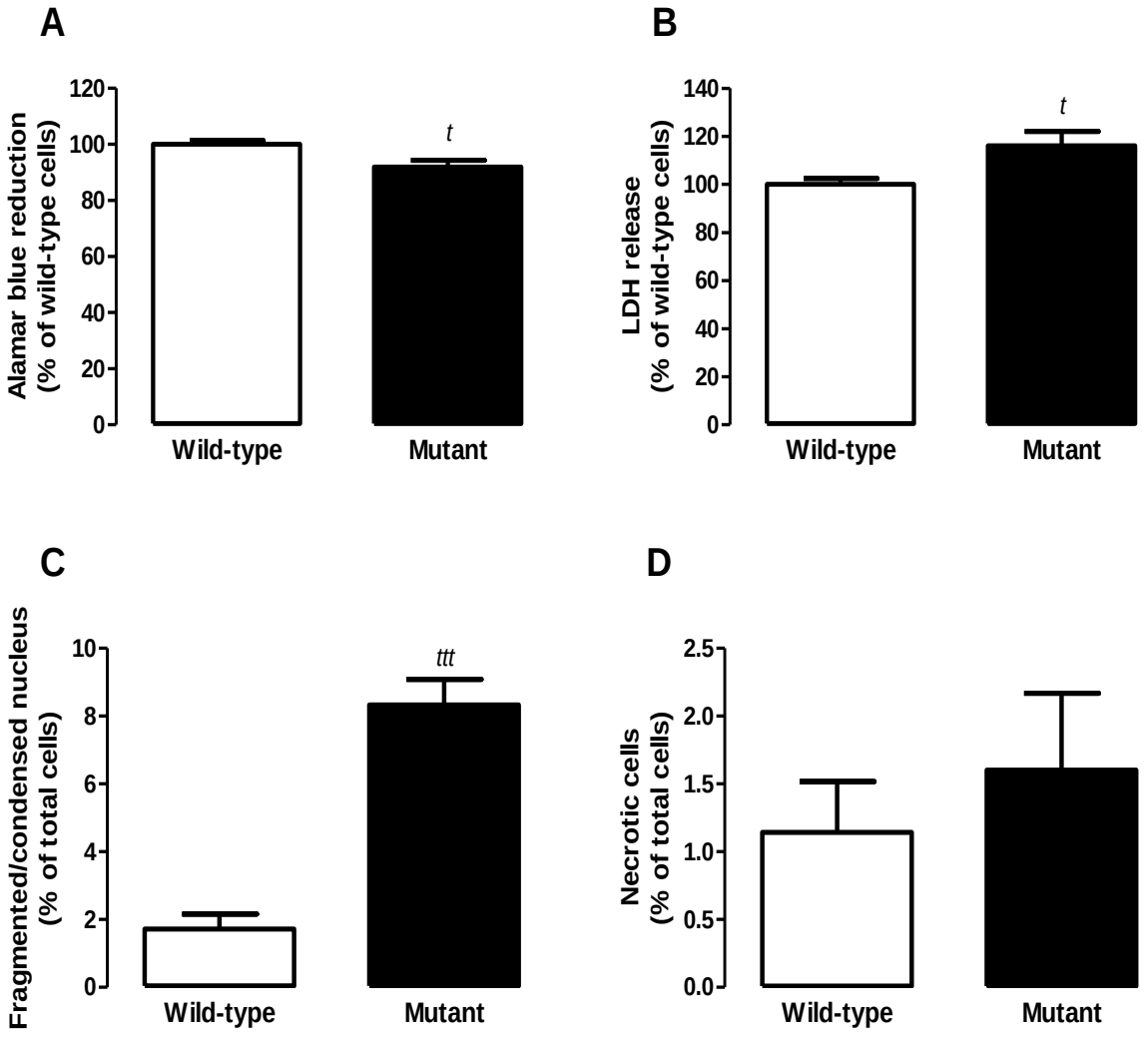
Although some proteins involved in antioxidant defense system have been described to be dysregulated in HD models, namely in striatal cells derived from knock-in mice (e.g. Ribeiro et al., 2012), the efficacy of cell response against exogenous toxic injuries in cells expressing full-length mHtt is not well known. Therefore, in this study we used HD knock-in-derived striatal cell lines, which express endogenous and comparable levels of both wild-type and mHtt, and represent early phases of HD (Trettel et al., 2000), to test the cellular response against exogenous stressors, H_2O_2 and STS, and to evaluate the potential antioxidant effect of cystamine and creatine. We demonstrate that cells expressing mHtt are more susceptible to increased ROS formation after exposure to exogenous stressors by increasing XO activity and lowering the antioxidant response, namely SOD2 and GRed, the latter promoting the levels of GSSG, and that both creatine and cystamine are able to decrease ROS levels, protecting HD striatal cells.

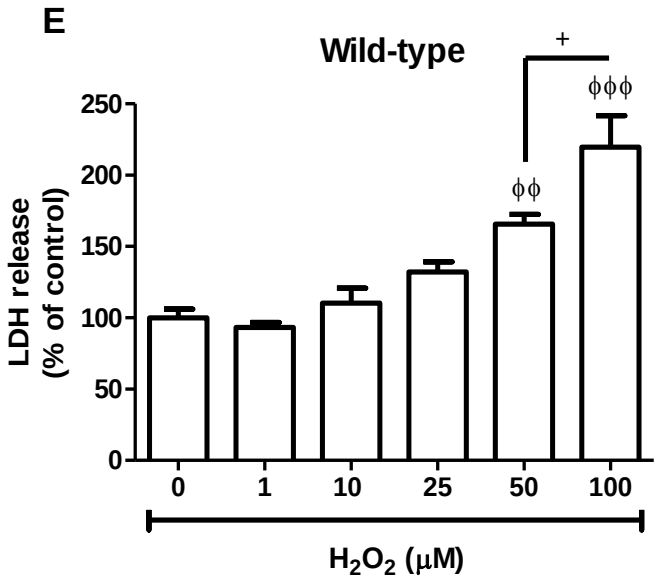
3.2.3 Results

3.2.3.1 Hydrogen peroxide and staurosporine decrease cell viability and increase ROS formation in mutant cells

Striatal cells viability was measured by Alamar Blue (Figure 3.2.1A) and by LDH release (Figure 3.2.1B). A slight decrease in viability was observed by both methods in mutant cells when compared to wild-type cells (Figure 3.2.1A,B). Cell death was analysed by Hoechst 33342 (Figure 3.2.1C) and propidium iodide (Figure 3.2.1C) staining to determine fragmented/condensed nucleus and necrotic cell death, respectively. Mutant cells exhibited significant apoptotic cell death compared to wild-type cells (Figure

3.2.1C), however no significant changes in necrotic cell death were observed between both striatal cell lines (Figure 3.2.1D). In order to analyze the influence of exogenous stressors on striatal cell viability, we incubated striatal cells with different concentrations of H_2O_2 or STS during 15 h followed by LDH release measurement, and compared with non-treated cells (control) (Figure 3.2.1E-H). H_2O_2 induced cell death at concentrations of 50 µM and 100 µM in both striatal cells (Figure 3.2.1E,F). STS induced cell death in wild-type cells only at concentrations of 25 and 50 nM (Figure 3.2.1G,H). For each stress inducer, we chose a concentration that did not cause necrotic cell death, namely 25 µM H_2O_2 and 10 nM STS.




E
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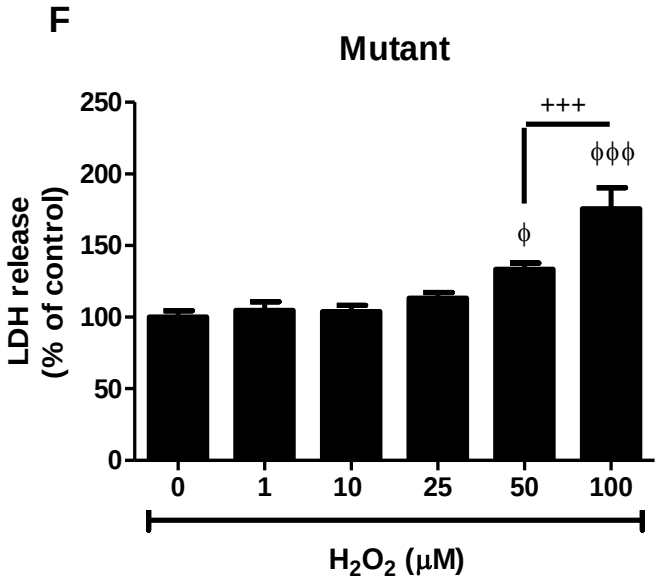
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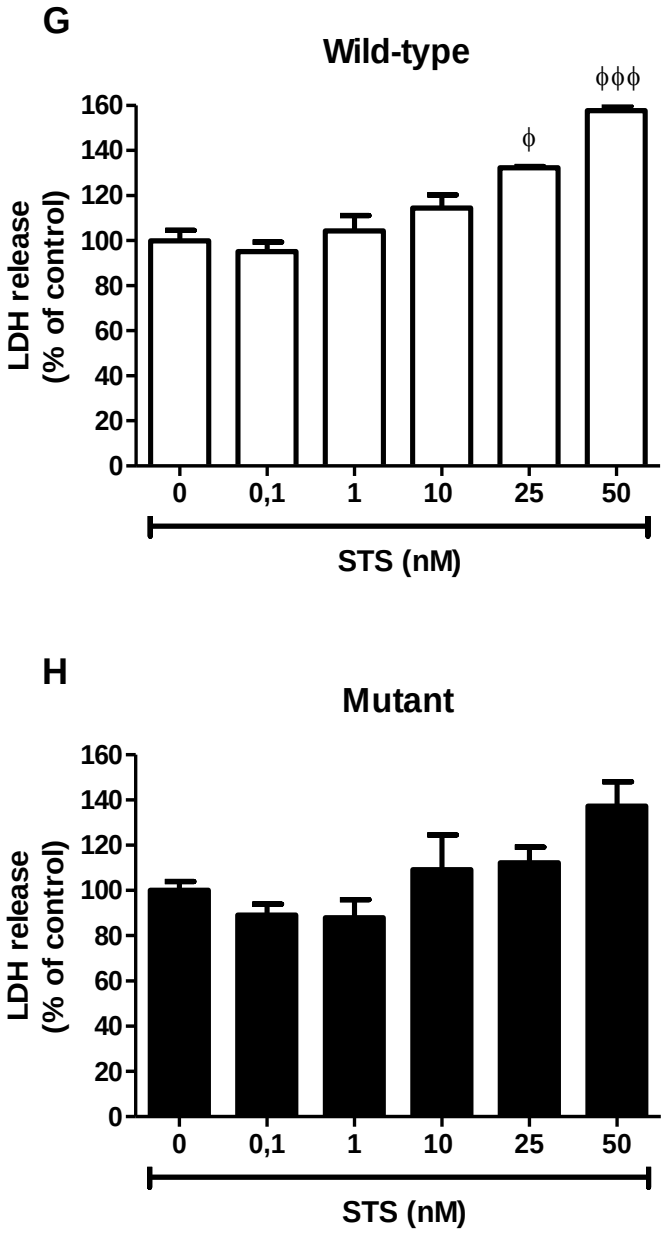
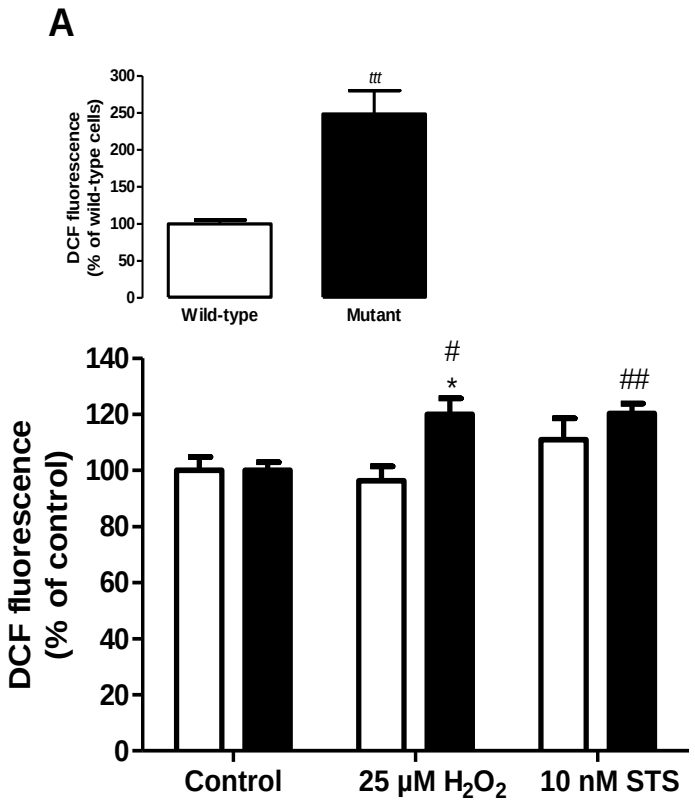


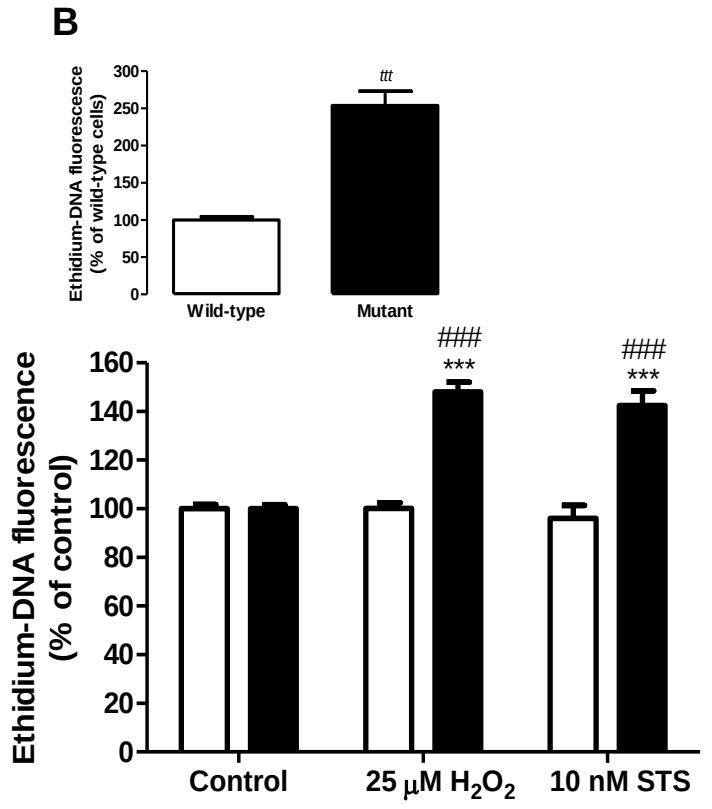
Figure 3.2.1 – mHtt-induced decreased cellular viability - influence of H_2O_2 and STS. Cell viability was assessed by Alamar Blue reduction (**A**) and LDH release (**B,E-H**). Alamar blue reduction was decreased in mutant compared to wild-type cells (**A**) which indicates loss of metabolic function induced by mHtt. LDH release, which indicates loss of cell membrane integrity or necrosis, was increased in mutant (% of total LDH released= 29.28 ± 0.95) *versus* wild-type cells (% of total LDH released 25.21 ± 1.14) (**B**). Cell death was determined by Hoechst 33342 (**C**) and propidium iodide (**D**) staining. Hoechst 33342 staining was increased in mutant compared to wild-type cells (**C**) which indicates increased apoptosis induced mHtt expression. Propidium iodide staining was unchanged between between both striatal cells (**D**) which indicates that necrotic cell death is unchanged in mutant cells. A cellular dose-response effect for H_2O_2 (1-100 µM) (**E,F**) and STS (0.1-50 nM) (**G,H**) was performed during 15h and analysed by LDH release. Wild-type (**E**) and mutant (**F**) cells presented loss of cell viability in the presence of 50 and 100 µM H_2O_2. 25 and 50 nM STS-induced loss of viability in wild-type (**G**) but not in mutant (**H**) cells. Data are the mean ± S.E.M. of 3 independent experiments performed

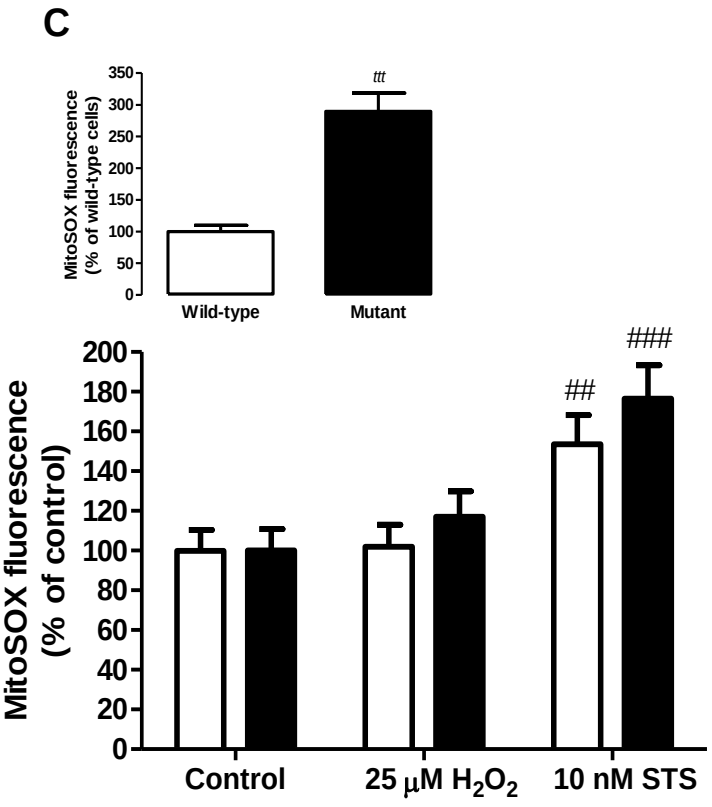
in duplicates. Statistical analysis: $^{t}P<0.05$ and $^{ttt}P<0.0001$ by Student's *t*-test when comparing mutant *versus* wild-type cells; $^{\phi}P<0.05$, $^{\phi\phi}P<0.01$ and $^{\phi\phi\phi}P<0.0001$ by one-way ANOVA when comparing treated *versus* non-treated (control) cells; $^{+}P<0.05$ and $^{+++}P<0.0001$ by one-way ANOVA when comparing 50 *versus* 100 µM H_2O_2.

We measured intracellular ROS by using the fluorescent probe H_2DCFDA, which can be oxidized by ROS, including ONOO−, $^{\bullet}$OH or HOCl, among other oxidants, and redox-active metals such as iron (e.g. Kalyanaraman et al., 2012). Under basal conditions, overall ROS formation was significantly higher in mutant cells when compared to wild-type cells (Figure 3.2.2A-insert). After exposure to H_2O_2 or STS, we observed a potentiation effect on ROS formation in mutant cells (Figure 3.2.2A). We also measured the generation of $O_2^{\bullet-}$ using the fluorescent probe DHE (Figure 3.2.2B). The red fluorescence formed from the two-electron oxidation product, E^+, is usually used to measure intracellular $O_2^{\bullet-}$ formation. However, DHE is oxidized by $O_2^{\bullet-}$ to form 2-hydroxyethidium (2-OH-E^+) and also with ONOO− and $^{\bullet}$OH to form E+ and dimers, which difficult a specific analysis (e.g. Kalyanaraman et al., 2012). A significant increase in $O_2^{\bullet-}$ levels were observed in mutant cells, when compared to wild-type cells (Figure 3.2.2B-insert). Incubation with H_2O_2 or STS provoked a similar but more pronounced increase in $O_2^{\bullet-}$ formation in mutant cells (Figure 3.2.2B). We evaluated if the $O_2^{\bullet-}$ production was mitochondrial-dependent using the selective mitochondrial $O_2^{\bullet-}$ fluorescent probe MitoSOX Red, a triphenylphosphonium (TPP) cation conjugated with hydroethidine, which appears to readily react with $O_2^{\bullet-}$ (Robinson et al, 2008), although it may also non-specifically oxidize (Kalyanaraman et al., 2012). We observed a 2-fold rise in mitochondrial $O_2^{\bullet-}$ in mutant cells, when compared to wild-type cells (Figure 3.2.2C-insert), revealing a high susceptibility of mutant cells to produce mitochondrial ROS. When treated with H_2O_2 or STS, mitochondrial $O_2^{\bullet-}$ levels increased in both striatal cells following STS exposure only. Caspase-3 activation was previously observed in HD striatal cells (Lim et al., 2008; Ribeiro et al., 2012; Rosenstock et al., 2011) and can be activated via the intrinsic mitochondrial apoptotic pathway, which was described to be activated in HD patients and in R6/2 HD mice (Kiechle et al., 2002). Therefore, we tested if mutant striatal cells were more vulnerable to apoptosis after incubation with the stress inducers H_2O_2 and STS (Figure 3.2.2D). We observed a significant increase in caspase-3-like activity in mutant cells under basal conditions, when compared to wild-type cells (Figure 3.2.2D-insert). A slight increase in caspase-3-like activity was observed after H_2O_2 (25 µM) treatment in mutant cells when compared with untreated/control cells

(*P*<0.01 by Student's *t*-test). On the other hand, STS induced a significant rise in caspase-3-like activity in both striatal cells, particularly in mutant cells when compared to wild-type cells (Figure 3.2.2D), coincident with a higher susceptibility of HD striatal cells to apoptotic cell death (e.g. Rosenstock et al., 2011). These data suggest that the major source of ROS induced by mHtt might be driven by mitochondria; indeed, STS seems to stimulate both mitochondrial and cytosolic ROS generation, whereas H_2O_2 appears to stimulate cytosolic ROS formation in HD striatal cells.




B
Ethidium-DNA fluorescesce (% of wild-type cells)
300
250
200
150
100
50
0
ttt
Wild-type
Mutant
Ethidium-DNA fluorescence (% of control)
160
140
120
100
80
60
40
20
0
###
***
###
***
Control
25 μM H2O2
10 nM STS


C
MitoSOX fluorescence (% of wild-type cells)
350
300
250
200
150
100
50
0
ttt
Wild-type
Mutant
MitoSOX fluorescence (% of control)
200
180
160
140
120
100
80
60
40
20
0
##
###
Control
25 μM H2O2
10 nM STS

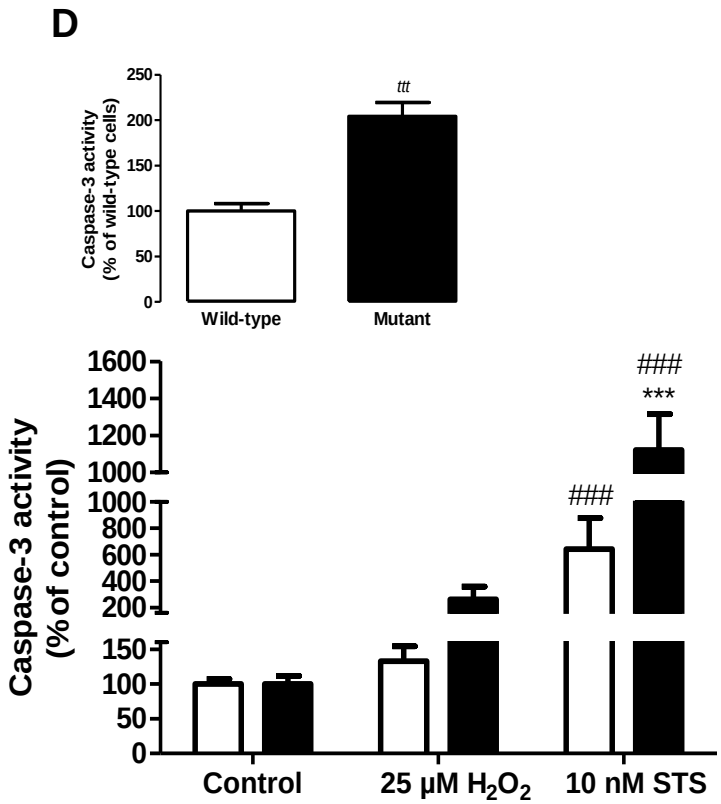


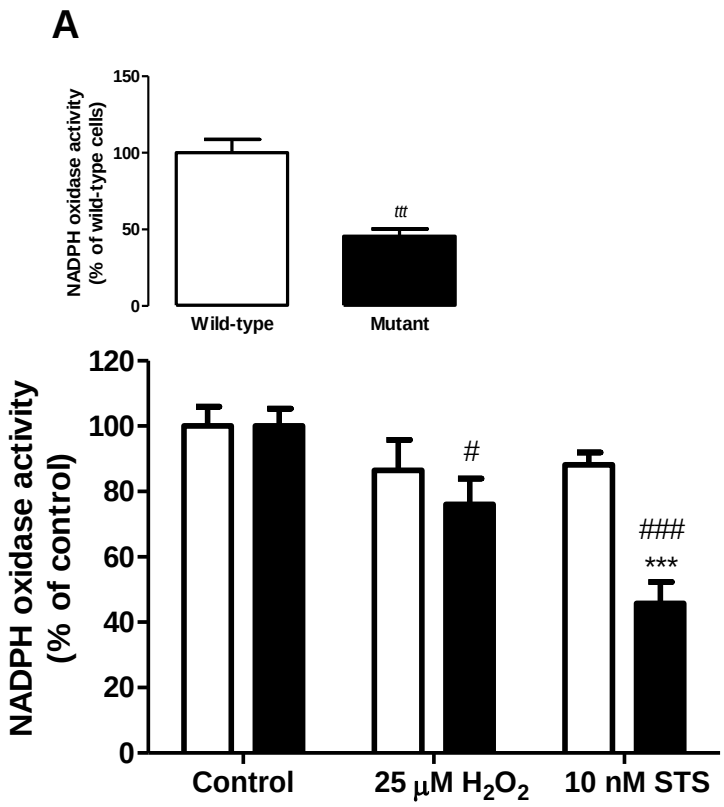
Figure 3.2.2 – Increased ROS formation and caspase-3 activity in striatal cells expressing mHtt – effects of H_2O_2 and STS. ROS formation is increased in HD knock-in striatal cells compared to wild-type cells under basal conditions (insert graphs show basal conditions only) or after exposure to H_2O_2 or STS during 15h, as measured by (A) DCF fluorescence using H_2DCFDA and (B) Ethidium (E^+)-DNA fluorescence using DHE, showing significant $O_2^{\bullet-}$ generation. (C) MitoSOX^{TM} Red fluorescence, which evaluates the generation of mitochondrial $O_2^{\bullet-}$, increases under basal conditions in mutant cells and after exposure to 10 nM STS in both striatal cells. (D) Caspase-3-like activity is increased in mutant cells compared to wild-type cells and 10 nM STS induced caspase-3 activation in both striatal cells with a greater increase in mutant cells compared to wild-type cells. Data are the mean ± S.E.M. of 3-10 independent experiments. Statistical analysis: $^{ttt}P<0.0001$ by Student's *t*-test when comparing mutant *versus* wild-type cells; $^{*}P<0.05$ and $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.0001$ by two-way ANOVA when comparing treated *versus* non-treated (control) cells.

3.2.3.2 **Hydrogen peroxide and staurosporine modify NADPH oxidase and xanthine oxidase activities in mutant cells**

To verify if the higher ROS formation in mutant cells was dependent on cytosolic oxidase activities, we measured NOX and XO activities, two major sources of $O_2^{\bullet-}$ in the cytosol. A significant decrease in NOX and XO activities was observed in untreated mutant cells, when compared to wild-type cells (Figure 3.2.3A and B-inserts).

Considering the decrease in XO activity in mutant cells, we also measured the levels of hypoxanthine, a XO substrate. However, no significant changes in hypoxanthine levels were observed (Figure 3.2.3C-insert), suggesting that altered enzyme activity may not be caused by insufficient substrate levels. These results suggested lower cytosolic ROS formation in HD mutant cells, as a result of decreased NOX and XO activities, emphasizing the contribution of mitochondria under control conditions.

However, after exposure to H_2O_2 or STS, NOX activity decreased (Figure 3.2.3A), while XO activity largely increased (Figure 3.2.3B) in mutant cells, suggesting that XO contributes to ROS formation induced by the stress inducers. Interestingly, hypoxanthine levels increased significantly in wild-type cells after H_2O_2 and STS stimulation, but decreased following STS exposure in mutant cells, when compared to untreated conditions (Figure 3.2.3C), implicating that the loss of substrate accompanies enhanced XO activity.



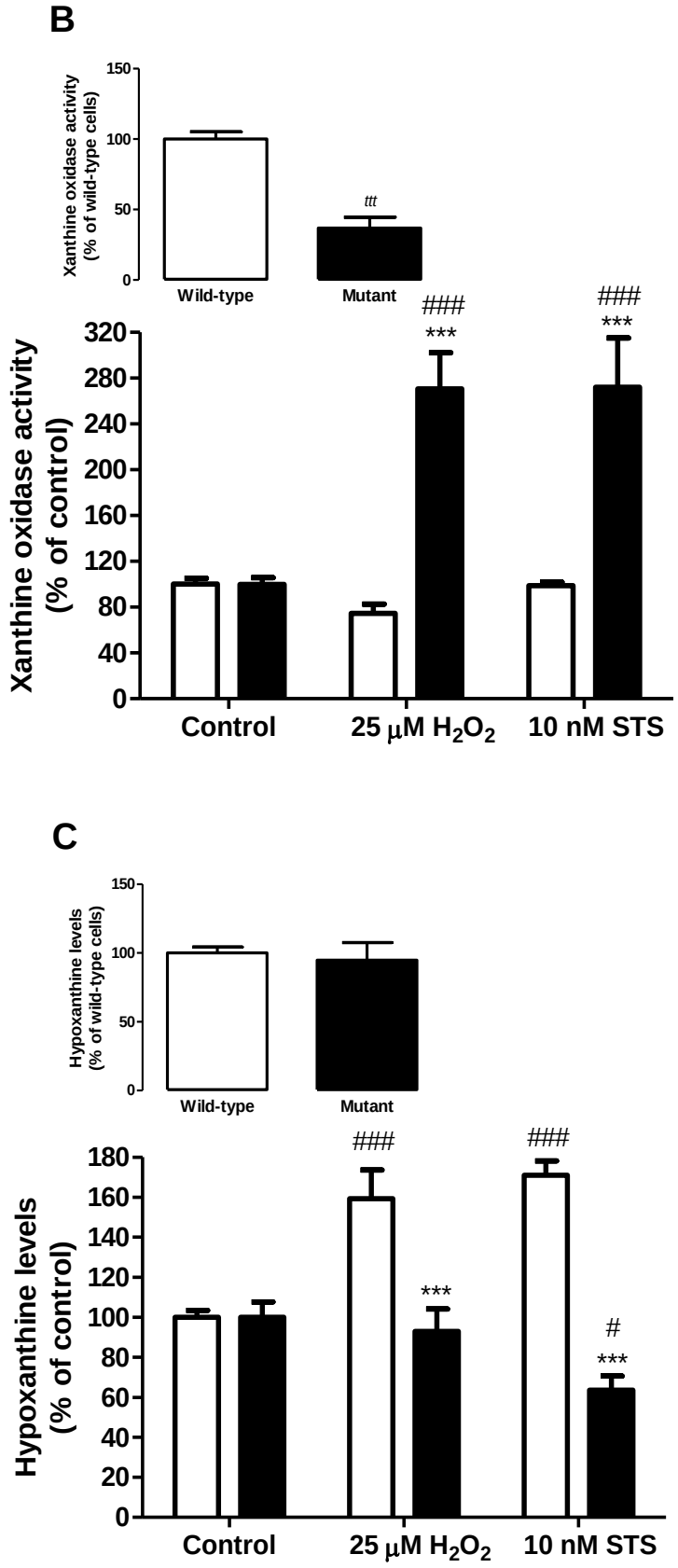
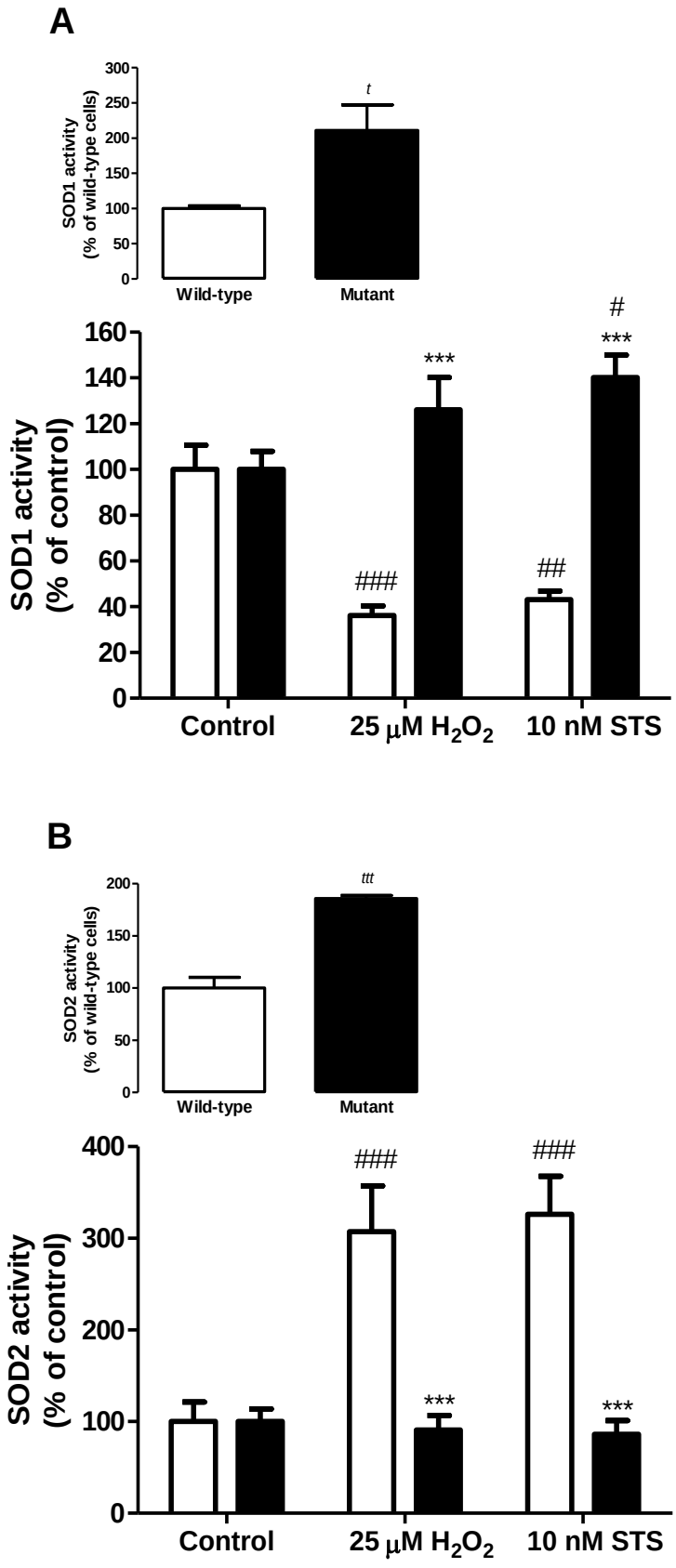


Figure 3.2.3 – Cytosolic oxidase activities in HD mutant striatal cells under basal conditions and after exposure to H_2O_2 and STS. NADPH oxidase (NOX) (A) and xanthine oxidase (XO) (B)

activities were measured in wild-type and mutant striatal cells, under basal conditions (basal conditions shown in insert graphs) and after exposure to H_2O_2 or STS during 15h, to evaluate their contribution in generating cytosolic $O_2^{\bullet-}$. NOX and XO activities were significantly decreased in mutant when compared to wild-type cells under basal conditions. After exposure to 25 µM H_2O_2 or 10 nM STS NOX activity decreased, whereas XO activity increased in mutant cells. (C) Hypoxanthine levels are unaltered in mutant cells under basal conditions. However hypoxanthine levels increased in wild-type cells after incubation with 25 µM H_2O_2 or 10 nM STS (15h), and decreased in mutant cells after 10 nM STS exposure only. Results are the mean ± S.E.M. of 4 independent experiments. Statistical: $^{ttt}P<0.0001$ by Student's *t*-test when comparing mutant *versus* wild-type cells; $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$ and $^{\#\#\#}P<0.0001$ by two-way ANOVA when comparing treated *versus* non-treated cells.

3.2.3.3 **Differential response of antioxidant systems between mutant and wild-type cells against exogenous stress stimuli**

In order to determine if increased ROS levels, and in particular $O_2^{\bullet-}$, induced by H_2O_2 or STS was dependent on antioxidant modifications in mutant cells, we determined SOD1, SOD2 and total intracellular SOD activities. A significant increase in SOD1, SOD2 and total intracellular SOD activities was observed in untreated mutant cells, compared to wild-type cells (Figure 3.2.4A ,B and C-inserts), suggesting a compensatory mechanism against higher levels of $O_2^{\bullet-}$. Incubation with H_2O_2 or STS induced a large decrease in SOD1 activity (Figure 3.2.4A) and an increase in SOD2 (Figure 3.2.4B) and total intracellular SOD (Figure 3.2.4C) activities in wild-type cells. Conversely, STS exposure only moderately enhanced SOD1 activity (Figure 3.2.4A), without significantly affecting SOD2 or total SOD activities (Figure 3.2.4B) in mutant cells. These results indicate that HD mutant cells are less able to stimulate SOD activity following stress stimuli.


A
SOD1 activity
(% of wild-type cells)
300
250
200
150
100
50
0
t
Wild-type
Mutant
SOD1 activity
(% of control)
160
140
120
100
80
60
40
20
0
###
***
##
#
***
Control
25 μM H2O2
10 nM STS
B
SOD2 activity
(% of wild-type cells)
200
150
100
50
0
ttt
Wild-type
Mutant
SOD2 activity
(% of control)
400
300
200
100
0
###
***
###
***
Control
25 μM H2O2
10 nM STS

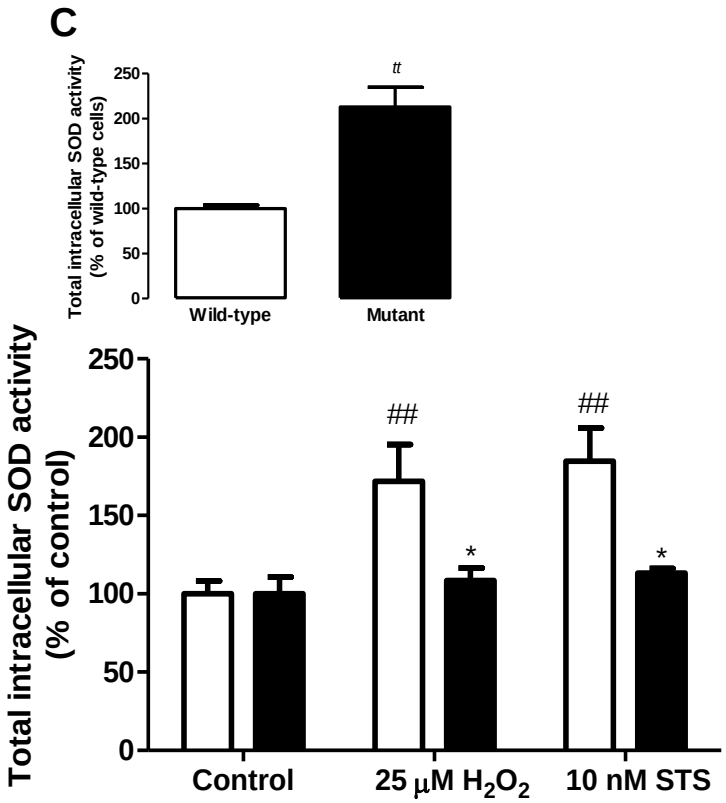
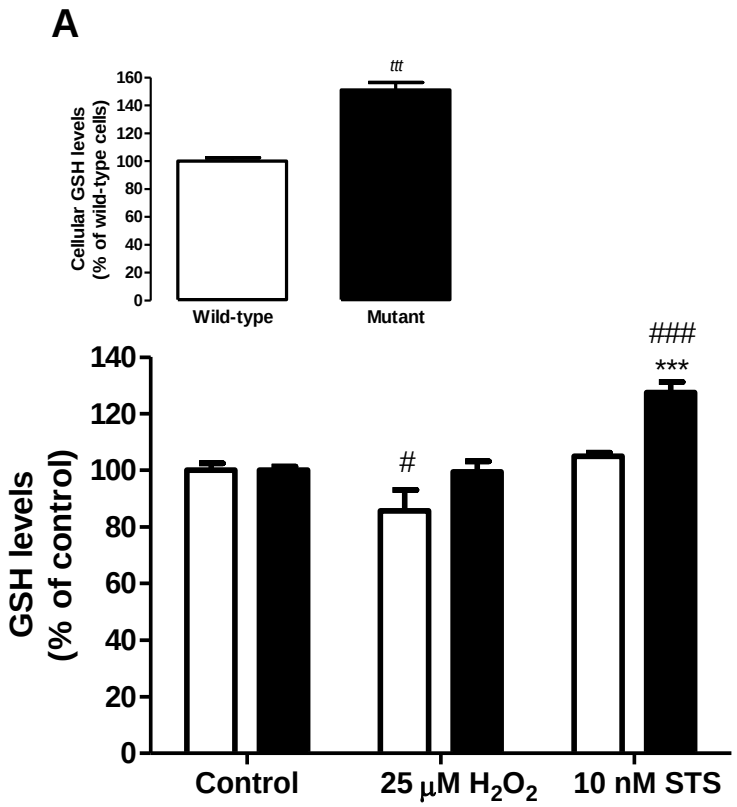
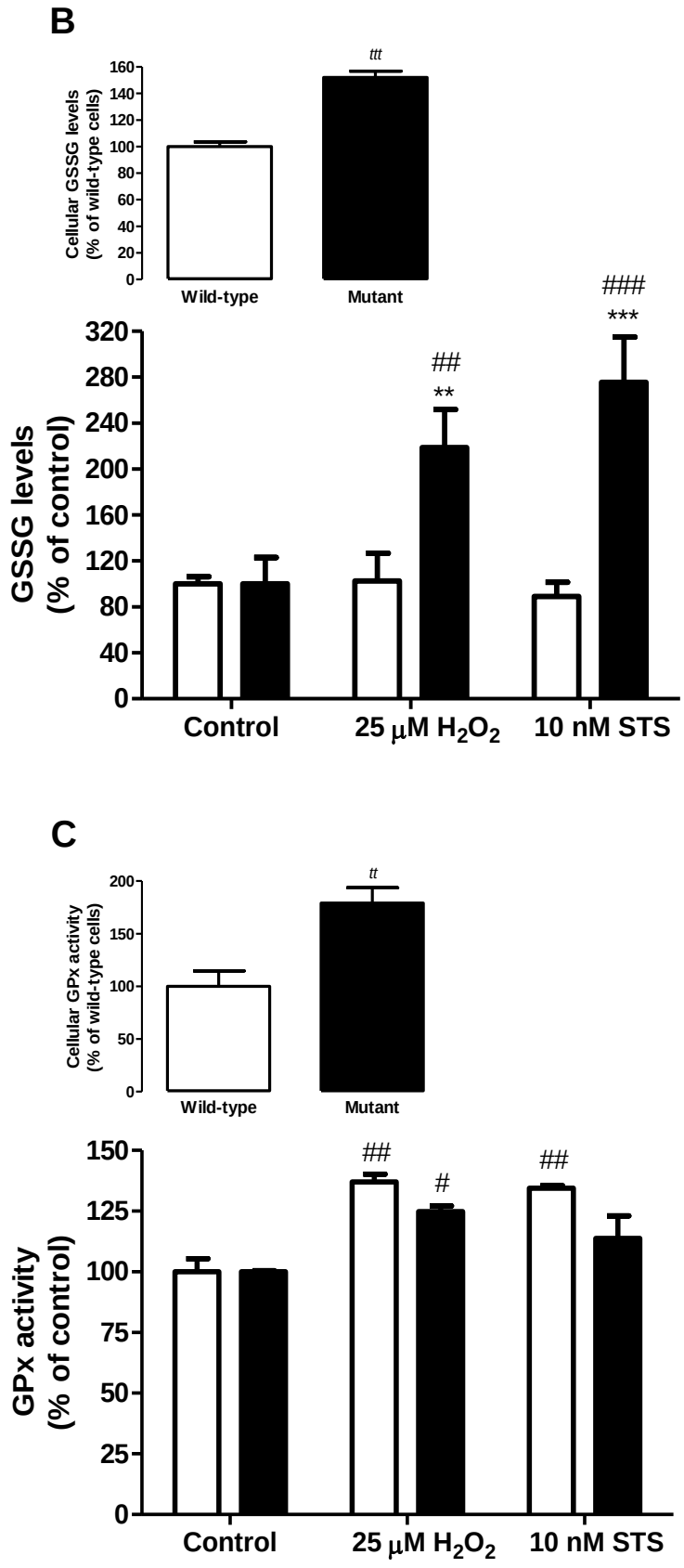


Figure 3.2.4 – Altered SOD 1 and 2 activities in basal mutant cells and after exposure to H_2O_2 and STS. Under basal conditions (shown in insert graphs), SOD1, SOD2 and total intracellular SOD activities increased significantly in HD knock-in striatal cells (A,B,C). After exposure to 25 μM H_2O_2 or 10 nM STS during 15h, (A) SOD1 activity decreased and (B) SOD2 and total intracellular SOD (C) increased in wild-type cells. In mutant cells, only STS increased SOD1 activity (A) with no effects in SOD2 (B) and total intracellular SOD (C) activities. Data are the mean ± S.E.M. of 4 independent experiments. Statistical analysis: $^{t}P<0.05$; $^{tt}P<0.01$ and $^{ttt}P<0.0001$ by Student's *t*-test when comparing mutant *versus* wild-type cells; $^{*}P<0.05$ and $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.0001$ by two-way ANOVA when comparing treated *versus* non-treated cells.

We have previously shown that glutathione redox cycle is dysregulated in HD striatal cells (Ribeiro et al., 2012). Therefore, to verify the capacity of response against exogenous stress in mutant cells, we next analysed the levels of GSH and GSSG and the activities of GPx and GRed after exposure to H_2O_2 or STS. Under basal conditions, GSH (Figure 3.2.5A-insert) and GSSG (Figure 3.2.5B-insert) levels and GPx (Figure 3.2.5C-insert) and GRed (Figure 3.2.5D-insert) activities were increased in mutant cells when compared to wild-type cells, as shown by us (Ribeiro et al., 2012). After exposure to STS, mutant cells displayed an increase in GSH levels with no changes observed in wild-type cells (Figure 3.2.5A). Exposure to H_2O_2 slightly affected wild-type cells only, with a decrease in GSH levels (Figure 3.2.5A). GSH oxidation augmented significantly in the

presence of H_2O_2 and STS in mutant cells (Figure 3.2.5B), confirming the higher susceptibility of mutant cells to undergo oxidative stress following exposure to a noxious stimulus. In the order to evaluate the changes in GSH oxidation after H_2O_2 and STS stimuli in mutant cells, we also measured the activities of GPx and GRed. We observed an increase in GPx activity in wild-type cells after STS or H_2O_2 treatment and only with H_2O_2 in mutant cells (Figure 3.2.5C). GRed activity greatly decreased in mutant cells after H_2O_2 or STS treatment, and a significant rise was detected in STS-treated wild-type cells (Figure 3.2.5D). These results indicate that the increase in ROS formation evoked by H_2O_2 and STS in mutant cells is linked to a striking increase in GSH oxidation possibly due to the large reduction in GRed activity, suggesting that the glutathione defense system is largely compromised in mutant cells exposed to H_2O_2 or STS.




B
Cellular GSSG levels (% of wild-type cells)
ttt
Wild-type
Mutant
GSSG levels (% of control)
##
**
###
***
Control
25 μM H2O2
10 nM STS
C
Cellular GPx activity (% of wild-type cells)
tt
Wild-type
Mutant
GPx activity (% of control)
##
#
##
Control
25 μM H2O2
10 nM STS

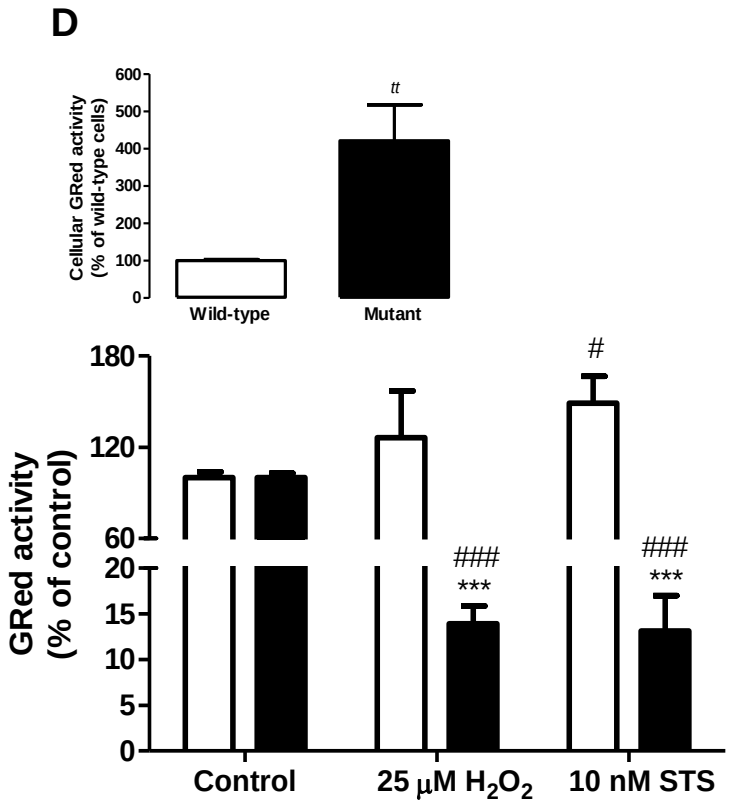


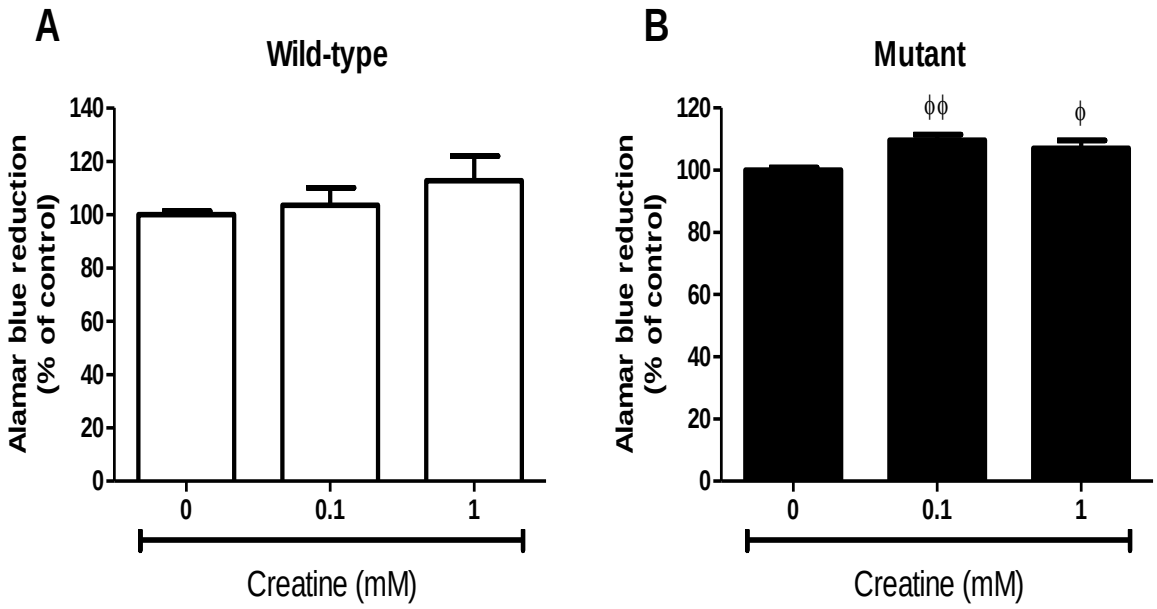
Figure 3.2.5 – Glutathione redox cycle is affected by mutant huntingtin expression and after stress stimuli. Striatal cells were cultured in the absence (basal conditions shown in insert graphs), or in the presence of 25 μM H_2O_2 or 10 nM STS during 15h and then used to determine reduced (GSH) and oxidized (GSSG) glutathione levels, and the activities of glutathione peroxidase (GPx) and glutathione reductase (GRed). (A) GSH levels are increased in mutant cells under basal conditions. STS exposure increased GSH levels in mutant cells. (B) GSSG levels are increased in basal mutant cells. After exposure to H_2O_2 or STS, GSSG levels largely increased in mutant cells. (C) GPx activity is increased in mutant cells under basal conditions. Incubation with H_2O_2 increased GPx activity in both striatal cells, whereas STS exposure only increased GPx activity in wild-type cells. (D) GRed activity is increased in mutant cells under basal conditions. After H_2O_2 and STS exposure GRed activity greatly decreased in mutant cells. Data are the mean ± S.E.M. of 4 independent experiments. Statistical analysis: $^{tt}P<0.01$ and $^{ttt}P<0.0001$ by Student's *t*-test when comparing mutant *versus* wild-type cells; $^{**}P<0.01$ and $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.0001$ by two-way ANOVA when comparing treated *versus* non-treated cells.

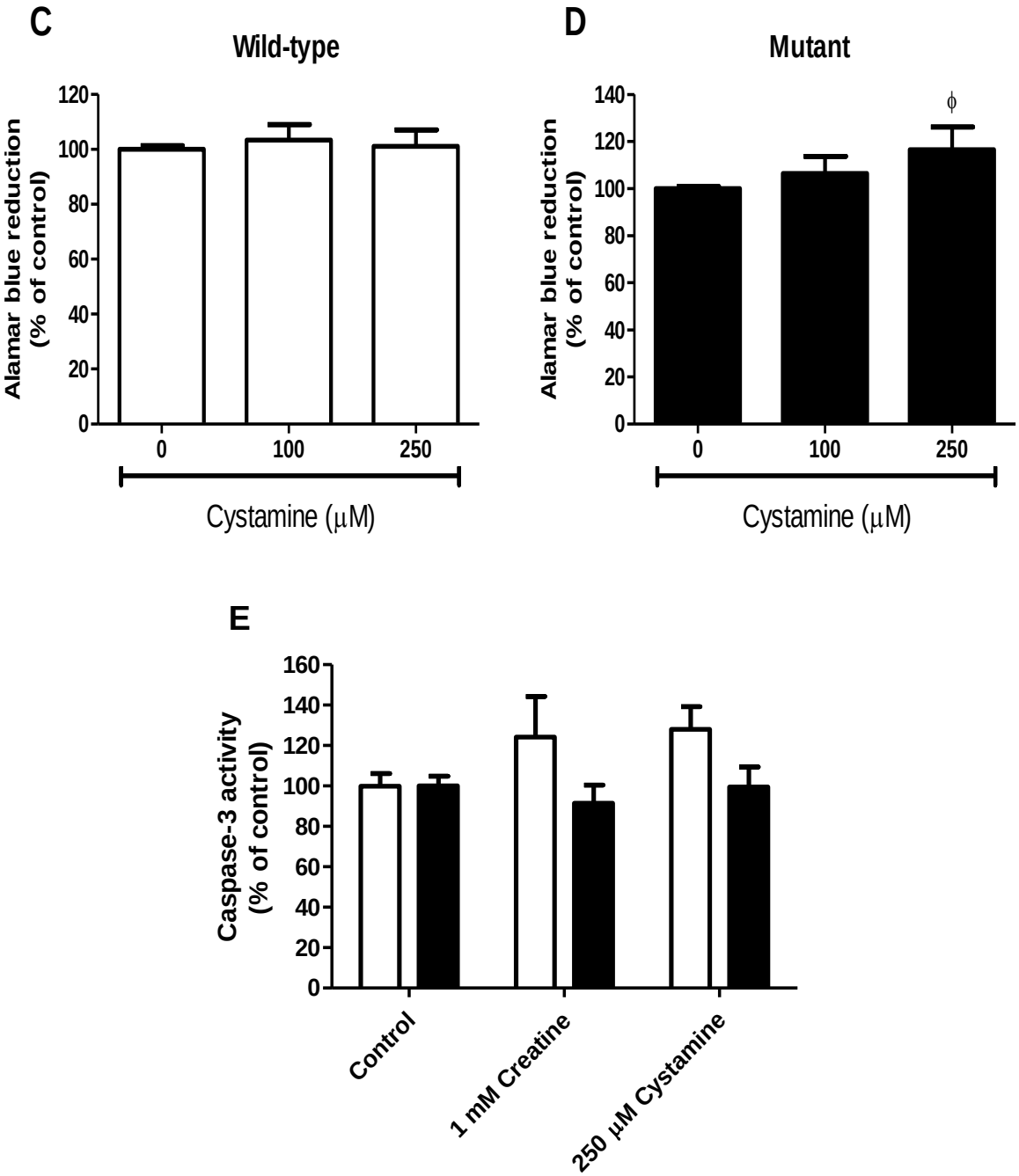
3.2.3.4 **Creatine and cystamine protect against increased ROS levels induced by STS or H_2O_2 in mutant cells**

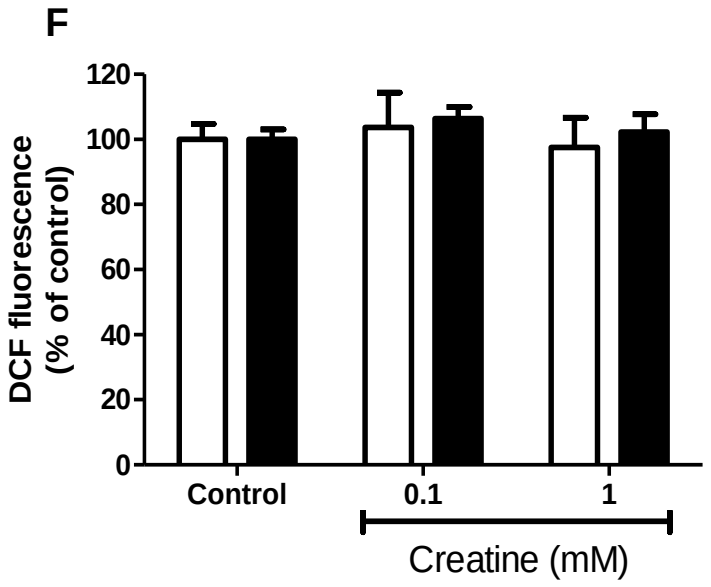
Creatine has been shown to have direct antioxidant proprieties (Lawler et al., 2002) and decreased 8-OHdG levels in serum of HD patients (Hersch et al., 2006), while cystamine increased GSH and L-cysteine, but not GSSG in cells expressing mHtt (Fox et

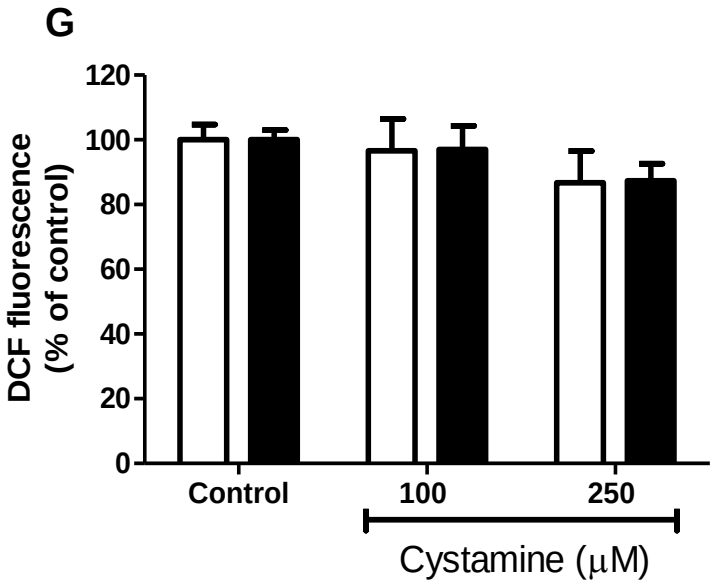
al., 2004). Therefore, we tested whether both compounds affect striatal cells viability and caspase-3 activation and could prevent against ROS formation in HD striatal cells under basal conditions and after exposure to STS or H_2O_2. We observed that creatine (0.1 mM and 1 mM) (Figure 3.2.6A,B) induced a slight and significant increase in mutant cells viability. Also, cystamine (250 µM) induced a slight and significant increase in the viability in mutant cells (Figure 3.2.6C,D). However, no significant changes in caspase-3-like activity were observed after creatine or cystamine treatment in both striatal cell models (Figure 3.2.6E).

In basal conditions neither creatine nor cystamine altered ROS levels in both striatal cell models (Figure 3.2.6F, G). Nevertheless, both 1 mM creatine and 250 µM cystamine significantly decreased ROS levels evoked by H_2O_2 (Figure 3.2.6H) or STS (Figure 3.2.6I) in mutant cells, suggesting a protection of these compounds against oxidative stress in HD striatal cells.




C
Wild-type
Alamar blue reduction (% of control)
120
100
80
60
40
20
0
0
100
250
Cystamine (μM)
D
Mutant
Alamar blue reduction (% of control)
140
120
100
80
60
40
20
0
ϕ
0
100
250
Cystamine (μM)
E
Caspase-3 activity (% of control)
160
140
120
100
80
60
40
20
0
Control
1 mM Creatine
250 μM Cystamine


F
DCF fluorescence (% of control)
120
100
80
60
40
20
0
Control
0.1
1
Creatine (mM)


G
DCF fluorescence (% of control)
120
100
80
60
40
20
0
Control
100
250
Cystamine (μM)

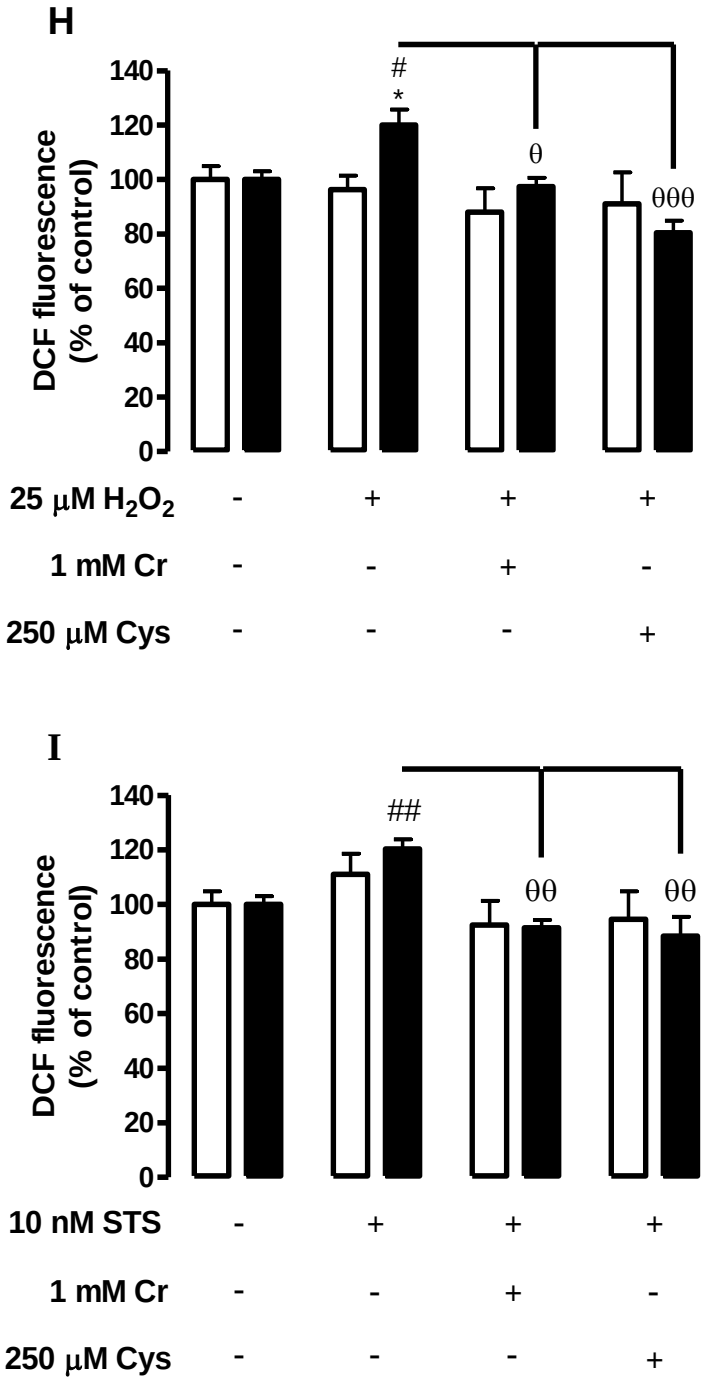


Figure 3.2.6 – Creatine and cystamine increase cell viability and protect from H_2O_2 or STS-induced ROS formation in HD striatal cells. Creatine (0.1 and 1 mM) treatment did not affect wild-type cells viability (A), but increased mutant cells (B) viability, as assessed by alamar blue reduction assay. Cystamine (100 and and 250 μM) treatment did not alter wild-type cells viability (C) but cystamine (250 μM) increased mutant cells viability (D). (E) Caspase-3-like activity was not changed by 1 mM creatine or 250 μM in both striatal cells. Creatine (0.1 and 1 mM) (F) or cystamine (100 and 250 μM) (G) treatment during 24h did not influence ROS formation in both striatal cells under basal conditions. After exposure to 25 μM H_2O_2 (H) or 10 nM STS (I), during 15h, 1 mM creatine and 0.25 mM cystamine decreased ROS formation in mutant cells. Data are the mean ± S.E.M. of 5 independent experiments. Statistical analysis: $^{\phi}P<0.05$, $^{\phi\phi}P<0.01$ and

$^{\phi\phi\phi}P<0.0001$ by one-way ANOVA when comparing treated *versus* non-treated (control) cells; $^{*}P<0.05$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ by two-way ANOVA when comparing treated *versus* non-treated cells; $^{o}P<0.05$ and $^{oo}P<0.01$ by two-way ANOVA when comparing H_2O_2 or STS condition with creatine or cystamine plus H_2O_2 or STS conditions.

3.2.4 Discussion

In this study we analysed the formation of ROS and related defense systems against stress stimuli, H_2O_2 and STS. Striatal cells expressing mHtt presented higher ROS formation, namely $O_2^{\bullet-}$, potentiated by H_2O_2 or STS through XO activity, and decreased antioxidant profile. We also show that exposure to H_2O_2 or STS potentiated mHtt-induced $O_2^{\bullet-}$ formation in mutant cells, but only STS was able to increase mitochondrial-driven $O_2^{\bullet-}$, indicating different cytotoxic mechanisms between H_2O_2 and STS. We previously showed that $O_2^{\bullet-}$ formation was higher in HD cybrid lines following exposure to 3-NP or STS (Ferreira et al., 2010). Higher levels of peroxides were also detected in R6/1 mice striatum (Pérez-Severiano et al., 2004) and in whole brain of rats subjected to 3-NP (Túnez et al., 2006*).*

Heightened NOX activity was described in human HD *post-mortem* cortex and striatum, and in cortical and striatal neurons and synaptosomes from HD(140Q/140Q) mice. Moreover, NOX was involved in striatal damage evoked by QA in adult rats (Maldonado et al., 2010). In contrast, we report that NOX activity is decreased in mutant cells, which is further decreased by H_2O_2 or STS. XO activity, another cytosolic producer of $O_2^{\bullet-}$, was decreased in mutant cells, which could potentially equilibrate mitochondrial $O_2^{\bullet-}$ formation. Concordantly, uric acid levels were reduced in human HD cerebral cortex (Beal et al., 1992). Here we further show that H_2O_2 or STS significantly increased XO activity in mutant cells, contributing for increased ROS formation.

We previously reported that HD striatal cells exhibited features of intrinsic apoptosis (Ribeiro et al., 2012; Rosenstock et al., 2011). Caspase-3 activation can be triggered *via* the intrinsic mitochondrial apoptotic pathway, which was described to be activated in HD patients and in R6/2 HD mice (Kiechle et al., 2002). STS induced pronounced caspase-3 activation, compared with H_2O_2, which may be linked to STS-evoked mitochondrial ROS generation, as cytochrome c release may cause ROS formation (Cai and Jones, 1998). Indeed, both mitochondrial and cytosolic ROS underlie STS-induced retinal cell death involving caspase-3 activation and DNA fragmentation

(Gil et al., 2003). Moreover, we showed that 10 nM STS induced cytochrome c and apoptosis-inducing factor (AIF) release and DNA fragmentation in ST*Hdh*$^{Q111/Q111}$ cells (Rosenstock et al., 2011). STS is a potent inhibitor of protein kinase C (PKC) (Tamaoki et al., 1986) (which is known to phosphorylate and promote the nuclear translocation of Nrf2), abrogating the expression of antioxidant and detoxifying proteins (e.g. Huang et al., 2002). Previous studies showed that treatment of ST*Hdh*$^{Q111/Q111}$ cells with H_2O_2 enhanced the number of apoptotic and necrotic cells, and increased poly(ADP-ribose) polymerase (PARP) cleavage, a hallmark of apoptosis (Ruiz et al., 2012). H_2O_2 also induced caspase-independent apoptosis through AIF release in a PARP1-dependent manner in fibroblast cultures (Yu et al., 2002). Moreover, H_2O_2 was described to enhance mHtt-induced cell death and promote ubiquitin-labeled mHtt aggregates by decreasing proteasome activity (Goswami et al., 2006).

Higher $O_2^{\bullet-}$ formation in HD appears concomitantly with increased antioxidant defenses. Increased total SOD and SOD1 activities were observed in 19 week–old R6/1 mice, although older (35 week-old) mice exhibited decreased SODs activities (Santamaria et al., 2001), suggesting that a failure in antioxidant system occurred with disease progression in HD mice. On the contrary, SOD1 levels remained unchanged in HD patient's brains (Sorolla et al., 2008). Interestingly, overexpression of SOD1 protected against H_2O_2-induced decrease in proteasome activity, and reduced mHtt aggregation and cell death (Goswami et al., 2006). We show increased SOD1/2 and total SOD activities in untreated mutant cells. SOD2 activity and protein levels were increased in both striatum and cortex of HD patients, evidencing an increase in antioxidant defenses as a result of mitochondrial ROS generation (Sorolla et al., 2008), as occurs in untreated mutant striatal cells. SOD2 mRNA was increased in the CN of HD patients, again suggesting a compensatory mechanism against higher ROS (Kim et al., 2010). Moreover, SOD2 mRNA increased in the basal ganglia of R6/2 HD mice (Fox et al., 2004). Wild-type cells exhibited increased total SOD/SOD2 activities in response to stress stimuli, suggesting an adaptive response to stress of mitochondrial antioxidant defenses. In mutant cells subjected to STS, SOD1 activity increased significantly, probably in response to enhanced mitochondrial $O_2^{\bullet-}$ levels, as SOD1 is also located in mitochondria. Nevertheless, SOD2/total SOD activities remained unchanged after H_2O_2 or STS treatment in mutant cells, suggesting a defect in stress response and increased susceptibility of HD striatal cells. Increased ROS formation was reported to stimulate sirtuin (SIRT)3 transcription, causing SOD2 deacetylation and activation (Chen et al.,

2011). However, SIRT3 protein levels were reduced in ST*Hdh*$^{Q111/Q111}$ cells (e.g. Fu et al., 2012), which may result in unchanged SOD2 activity in the presence of exogenous stressors. Interestingly, heterozygous SOD2 knockout (SOD2+/−) mice showed large striatal lesions produced by 3-NP (Andreassen et al., 2001), whereas upregulation of SOD2 was neuroprotective against 3-NP (Madhavan et al., 2008).

Previously we showed increased glutathione levels despite decreased GCLc activity in HD striatal cells. We demonstrated that increased GSH was due to a decrease in the capacity of the Mrp1 to export GSH, along with increased ROS formation (Ribeiro et al., 2012). In agreement, the levels of mitochondrial GSH were increased in the striatum and cortex of R6/2 HD mice (Choo et al., 2005) and total GSH levels increased with age in R6/2 HD mice forebrain (Fox et al., 2004). Increased GSH was also detected in the striatum of 8-week old (early symptomatic) R6/2 HD mice (Tkac et al., 2007). In contrast, a decrease in GSH was observed in HD patient's *post-mortem* brain cortex (Beal et al., 1992). Here we show that GSH levels increased in mutant cells after STS treatment; however, GSSG levels have risen in a great extent after STS and H_2O_2 exposure in mutant cells, indicating a high level of oxidation in HD cells. We previously demonstrated that the activity of enzymes of the glutathione redox cycle, GPx and GRed, was increased in mutant cells (Ribeiro et al., 2012). Concordantly, GPx1 transcription levels were increased in the same HD model (Lim et al., 2008), suggesting mHtt-induced stress response. Moreover, GPx1 and 6 were increased in the striatum of HD patients, and GPx and catalase activities were elevated in HD striatum and cortex (Sorolla et al., 2008). Here we show an increase in GPx activity in wild-type cells subjected to STS or H_2O_2, whereas GPx activity was only increased in mutant cells exposed to H_2O_2, indicating a lower ability of mutant cells to stimulate antioxidant activity when exposed to STS. In agreement with our data, an increase in GRed activity was found in HD patient's erythrocytes (Zanella et al., 1980); also, increased GRed transcription was found in the basal ganglia of R6/2 HD mice (Fox et al., 2004), suggesting an induction at antioxidant defenses by mHtt. However, diminished GRed activity was observed after exposure to stress stimuli in mutant cells. Concordantly, QA administration in rats caused a loss of GRed activity (Cruz-Aguado et al., 2000).

We also show that cystamine increased mutant cells viability and protected against H_2O_2 and STS-induced ROS formation in mutant cells. Cystamine may act as an antioxidant by increasing GSH levels. Indeed, cystamine increased the GSH and L-cysteine, but not GSSG, in cells expressing mHtt (Fox et al., 2004). Furthermore,

cystamine protected against 3-NP lesions *via* induction of Nrf2 (Calkins et al., 2010); importantly, impaired Nrf2 signaling was recently described in ST*Hdh*$^{Q111/Q111}$ cells (Jin et al, 2013). By inhibiting TG2, cystamine protected from oxidative stress-induced cell death in cortical neurons (Basso et al., 2012). Additionally, cystamine may inhibit caspase-3 activity (Lesort et al., 2003) in a TG2-independent manner. Cystamine protected against H_2O_2-increased caspase-3 activation and increased glutathione levels in human neuroblastoma SH-SY5Y cells (Lesort et al., 2003). Nevertheless, we show that cystamine was not able to decrease caspase-3 activity in both striatal cells. A randomized, controlled double-blind multicenter phase II/III clinical trial of cysteamine (FDA-approved reduced form of cystamine) for the potential treatment of HD is currently being conducted using BDNF as a biomarker (www.raptorpharma.com).

Creatine also increased mutant cells viability and protected against H_2O_2 and STS-induced ROS levels in mutant cells. Creatine can have direct antioxidant proprieties by decreasing 8-OHdG levels in serum of HD patients (Hersch et al., 2006), increasing GSH and decreasing MDA levels in rats treated with 3-NP (Yang et al., 2009). Moreover, creatine increased brain phosphocreatine, and protected against 3-NP-induced increase in lactate and 3-NT and decreased phosphocreatine and ATP levels (Matthews et al., 1998). Creatine is currently under phase III clinical trial for HD (clinicaltrials.gov).

The present study shows that striatal cells expressing full-length mHtt exhibit high ROS, at least partially produced by mitochondria, and that exposure to H_2O_2 or STS potentiate oxidative stress by increasing XO activity and lowering the antioxidant response, namely SOD2 and GRed, the later promoting GSSG levels. Moreover, cystamine and creatine protected against H_2O_2 or STS-induced ROS formation in HD striatal cells. Thus, a better understanding of the mechanisms involved in this neuroprotection may contribute to delay striatal cell degeneration in HD.

3.3 Insulin and IGF-1 reduce mitochondrial-driven oxidative stress in Huntington's disease knock-in striatal cells[108]

3.3.1 Summary

Oxidative stress and mitochondrial dysfunction have been described in HD, caused by expression of mHtt. We have previously shown that mHtt-induced enhanced glutathione redox cycle and that this antioxidant accumulates intracellularly due to decreased Mrp1 activity. We also have shown that despite enhanced antioxidant activities, HD knock-in striatal cells presented lower response capacity to exogenous stressors, and that creatine and cystamine treatment prevented ROS formation. However, neither creatine nor cystamine were able to revert the effects provoked by mHtt. Therefore, taken into account that IGF-1 was previously shown to protect HD cells, whereas insulin prevented neuronal oxidative stress, in this work we analysed the role of insulin/IGF-1 in striatal cells derived from HD knock-in mice against ROS production and related antioxidant and signaling pathways. Insulin and IGF-1 decreased mitochondrial-driven ROS formation induced by mHtt, without changing superoxide dismutase 2 activity or glutathione (GSH and GSSG) levels. Insulin and IGF-1 promoted Akt and Erk phosphorylation, respectively, and increased nuclear levels of phosphorylated Nrf2; however, this was not correlated with Nrf2 transcriptional activity or changes in mRNA levels of Nrf2 target genes. Insulin and IGF-1 treatment also ameliorated mitochondrial function in HD cells. In the case of insulin, this occurred in a PI-3K/Akt-dependent manner, concomitantly with reduced caspase-3 activation evoked by mHtt. Hence, insulin and IGF-1 improve mitochondrial function and reduce mitochondrial-driven ROS caused by mHtt, along with differential stimulation of Akt and Erk, in a process independent of Nrf2 transcriptional activity.

[108] Based on the following manuscript submission: Ribeiro, M.; Rosenstock, T. R.; Cunha-Oliveira, T.; Ferreira, I. L.; Oliveira, C. R.; Rego, A. C. Insulin and IGF-1 reduce mitochondrial-driven oxidative stress in Huntington's disease knock-in striatal cells. Free Radic Biol Med. 2014.

3.3.2 Introduction

Elevated levels of DNA oxidation were previously detected in blood plasma, serum and leukocytes from HD patients (Chen et al., 2007; Hersch et al., 2006; Tunez et al., 2011) and in striatum and cortex of HD *post-mortem* tissues (Browne et al., 1997; Polidori et al., 1999; Shirendeb et al., 2011). Moreover, DNA oxidation markers were detected in the striatum, urine and plasma of R6/2 HD mice (Bogdanov et al., 2001). Increased lipid peroxidation markers were also detected in HD patient's blood serum and plasma, correlating with disease severity (Chen et al., 2007; Stoy et al., 2005), in the striatum and cortex of *post-mortem* HD human brains (Browne et al., 1999; Lee et al., 2011), and in total brain and striatum of R6/2 mice co-localizing with mHtt inclusions (Lee et al., 2011; Tabrizi et al., 2000). Indeed, intracellular aggregates of mHtt exon-1 were directly linked to increased production of ROS, in a polyQ length-dependent manner, preceding cell death (Hands et al., 2011).

Impairment in antioxidant defenses also underlies oxidative stress in HD. In erythrocytes from HD patients, GPx and SOD1 activities were decreased (Chen et al., 2007). Moreover, proteomic analysis of HD *post-mortem* striatal and cortical brain samples revealed an induction of Prdx's 1, 2 and 6 and GPx 1 and 6, along with increased activities of SOD2 and catalase (Sorolla et al., 2008). More recently, we demonstrated increased ROS formation and deregulated glutathione redox cycle in HD striatal cells (Ribeiro et al., 2012), which presented lower response capacity to exogenous stressors (Ribeiro et al., 2013).

ROS formation in HD has been also attributed to impaired mitochondrial function. Evidences for mitochondrial dysfunction in HD include, among many other reports, decreased activity of mitochondrial complexes I-IV in caudate and putamen, and in muscle and platelets of HD patients (Ehrlich et al., 2012; Silva et al., 2013), reduced activities of complexes I, IV and V in progenitor and differentiated neuron-like ST*Hdh*$^{Q111/Q111}$ cells (Napoli et al., 2013), or mitochondrial membrane depolarization and decreased mitochondrial Ca^{2+} retention (Panov et al., 2002; Oliveira et al., 2006). ST*Hdh*$^{Q111/Q111}$ striatal cells also showed higher levels of mitochondrial ROS, mtDNA damage and a lower spare respiratory capacity compared to wild-type cells (Siddiqui et al., 2012). Importantly, treatment with a mitochondria-specific antioxidant improved function in isolated mitochondria from HdhQ150 knock-in mice and restored the mtDNA copy number *in vivo* to levels similar to the controls (Xun et al., 2012). These results

suggest oxidative stress-induced mitochondrial abnormalities upon expression of mHtt. Moreover, mHtt binding at the outer mitochondrial membrane was demonstrated in mitochondria from YAC72 transgenic mice, ST*Hdh*$^{Q111/Q111}$ striatal cells and in mouse liver mitochondria, inducing MPT pore opening (Panov et al., 2002; Choo et al., 2004).

Neuronal survival largely depends on growth factors through activation of kinases, namely Akt. IGF-1 levels are decreased in HD mutant striatal cells, in human HD striatal *post-mortem* tissues and in plasma of 11 week-old R6/2 mice (Pouladi et al., 2010). IGF-1-mediated Akt activation was previously described to be neuroprotective in HD and to reduce nuclear inclusions through phosphorylation of mHtt at Ser421 (Humbert et al., 2002); moreover, activation of Akt was described to reflect early striatal pro-survival response in HD knock-in HdhQ111 mice and ST*Hdh*$^{Q111/Q111}$ cells (Gines et al., 2003). Furthermore, we recently showed that intranasal administration IGF-1 in YAC128 HD mice activated Akt and concomitantly increased phosphorylation of mHtt on Ser421 (Lopes et al., 2013). We also showed that peripheral administration of IGF-1 protected against HD-associated impaired glucose tolerance, namely by enhancing blood insulin levels in R6/2 mice (Duarte et al., 2011), and that intranasal administration of IGF-1 enhanced IGF-1 cortical levels and improved motor activity and both peripheral and central metabolic abnormalities in YAC128 HD mice (Lopes et al., 2013). We also demonstrated that insulin (which shares structural and signaling properties with IGF-1) protects against oxidative stress in cortical neurons through Akt activation (Duarte et al., 2008). Interestingly, insulin was described to induce expression of GCLc through the transcription factor Nrf2 (Langston et al., 2008), directly interfering with cellular antioxidant profile in endothelial cells. Importantly, impaired Nrf2 signaling pathway was recently described in striatal ST*Hdh*$^{Q111/Q111}$ cells (Jin et al., 2013).

In this work we examine the role of insulin and IGF-1 on mitochondrial ROS generation and depolarization, and Nrf2 transcriptional activity related with antioxidant profile in HD knock-in striatal cells. We show that insulin and IGF-1 increase Akt and Erk activation and nuclear levels of phosphorylated Nrf2, as well as heighten mitochondrial function, precluding mitochondrial ROS formation induced by mHtt.

3.3.3 Results

3.3.3.1 **Insulin and IGF-1 prevent increased ROS generation in HD mutant striatal cells**

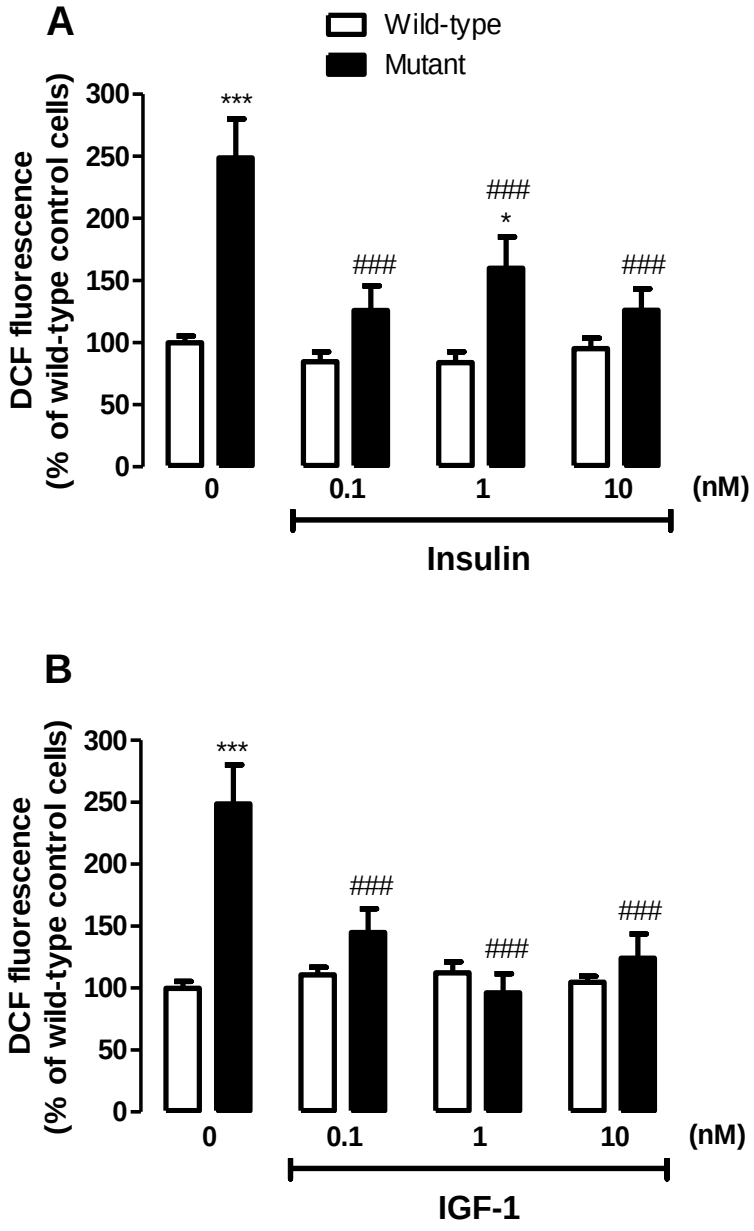
ROS levels in striatal cells were assessed with the fluorescent redox probe H_2DCFDA, which can be oxidized by ROS, including ONOO−, $^{\bullet}$OH or hypochlorous acid, among other oxidants, and redox-active metals such as iron (Kalyanaraman et al., 2012). A significant increase in DCF fluorescence was observed in HD knock-in striatal cells expressing full-length mHtt (ST*Hdh*$^{Q111/Q111}$ or mutant cells), when compared to ST*Hdh*$^{Q7/Q7}$ cells (wild-type cells) (Figure 3.3.1A,B), largely suggesting enhanced ROS production.

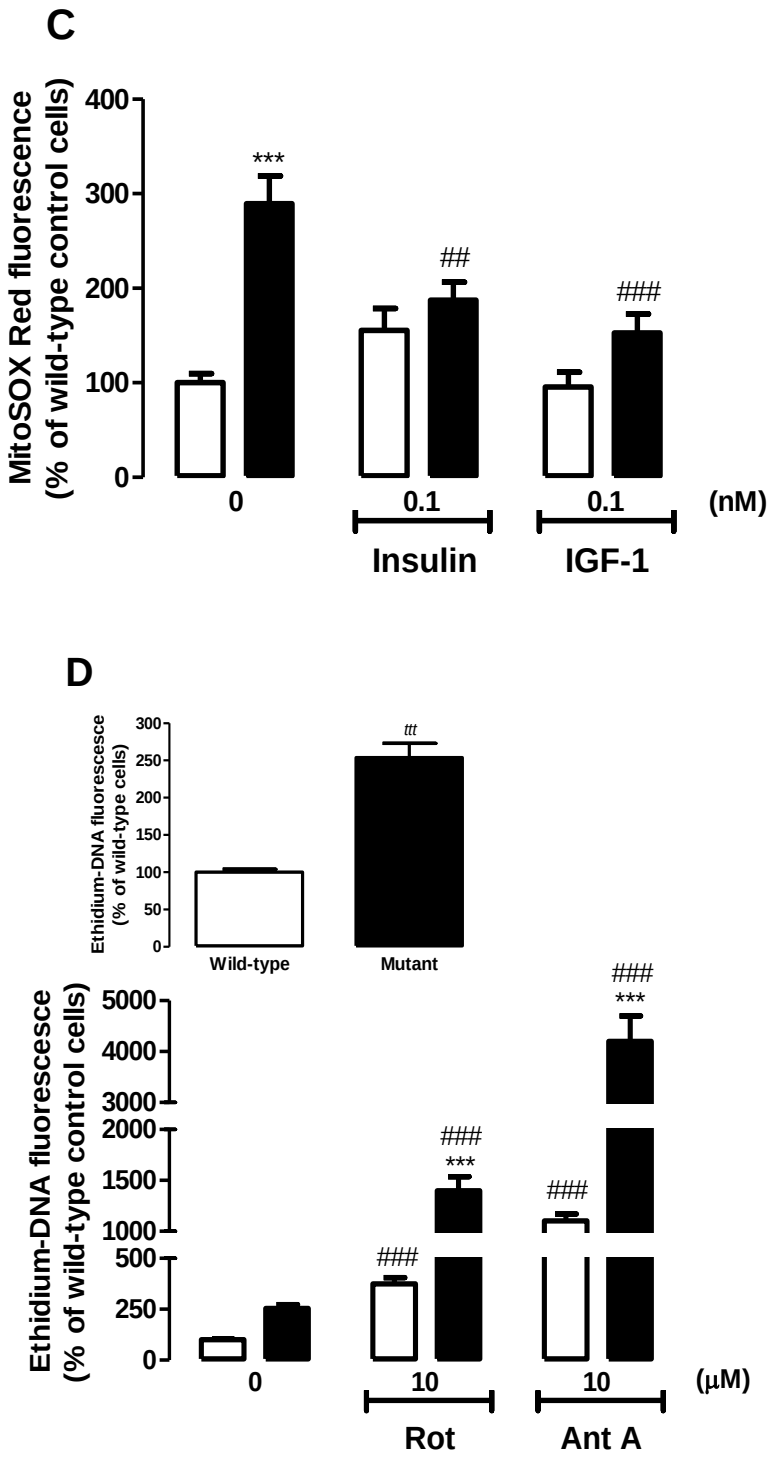
Since IGF-1 expression levels are largely decreased in mutant striatal cells (Pouladi et al., 2010), and both insulin (Duarte et al., 2005; 2006; 2008) and IGF-1 (Duarte et al., 2011; Humbert et al., 2002; Lopes et al., 2013) were neuroprotective following oxidative stress or expression of mHtt, respectively, we determined the effect of both insulin and IGF-1 on ROS formation in ST*Hdh*$^{Q111/Q111}$ cells. Due to structural and functional homology, insulin and IGF-1 can bind to (and activate) both IR and IGF1R, with insulin binding to the IR with higher affinity (< 1 nM) than IGF-1 (100–500-fold lower affinity), whereas IGF1R preferentially binds IGF-1 (< 1nM) as compared to insulin (100–500-fold lower affinity) (Rechler et al., 1980); therefore, striatal cells were exposed to insulin or IGF-1 in the low nM range (0.1-10 nM). Interestingly, both insulin and IGF-1 significantly precluded ROS generation in mutant cells (Figure 3.3.1A,B).

We also measured the levels of mitochondrial $O_2^{\bullet-}$ using MitoSOX Red, a TPP cation conjugated with hydroethidine, which may readily react with $O_2^{\bullet-}$ (Robinson et al., 2008), although it may also non-specifically oxidize (Kalyanaraman et al., 2012). Importantly, a significant increase in mitochondrial $O_2^{\bullet-}$ (Figure 3.3.1C) was observed in mutant cells, when compared to wild-type cells. Mitochondrial $O_2^{\bullet-}$ production in mutant cells was further revealed with DHE following exposure to rotenone (complex I inhibitor) or antimycin A (complex III inhibitor) (Figure 3.3.1D), thereby confirming increased susceptibility to mitochondrial ROS. When exposed to insulin or IGF-1, mutant cells presented a significant decrease in mitochondrial $O_2^{\bullet-}$ formation (Figure 3.3.1C).

These data show that both insulin and IGF-1 (at low nM) protect from mHtt-induced oxidative stress, by precluding ROS production and in particular mitochondrial $O_2^{\bullet-}$ formation.

Because caspase-3 activation was previously observed in HD striatal cells (Lim et al., 2008; Ribeiro et al., 2012; Rosenstock et al., 2011), we determined the effect of insulin and IGF-1 on caspase-3 activation in mutant *versus* wild-type cells. Insulin (0.1 nM) significantly protected against caspase-3 activation induced by expression of mHtt (Figure 3.3.1E).




C
MitoSOX Red fluorescence
(% of wild-type control cells)
400
300
200
100
0
***
##
###
0
0.1
0.1
(nM)
Insulin
IGF-1
D
Ethidium-DNA fluorescesce
(% of wild-type cells)
300
250
200
150
100
50
0
ttt
Wild-type
Mutant
Ethidium-DNA fluorescesce
(% of wild-type control cells)
5000
4000
3000
2000
1500
1000
500
250
0
###
***
###
###
***
0
10
10
(μM)
Rot
Ant A

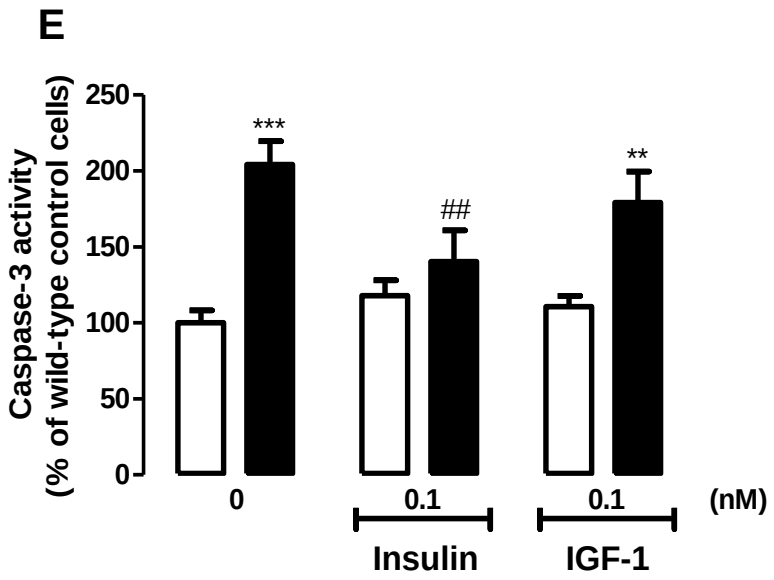
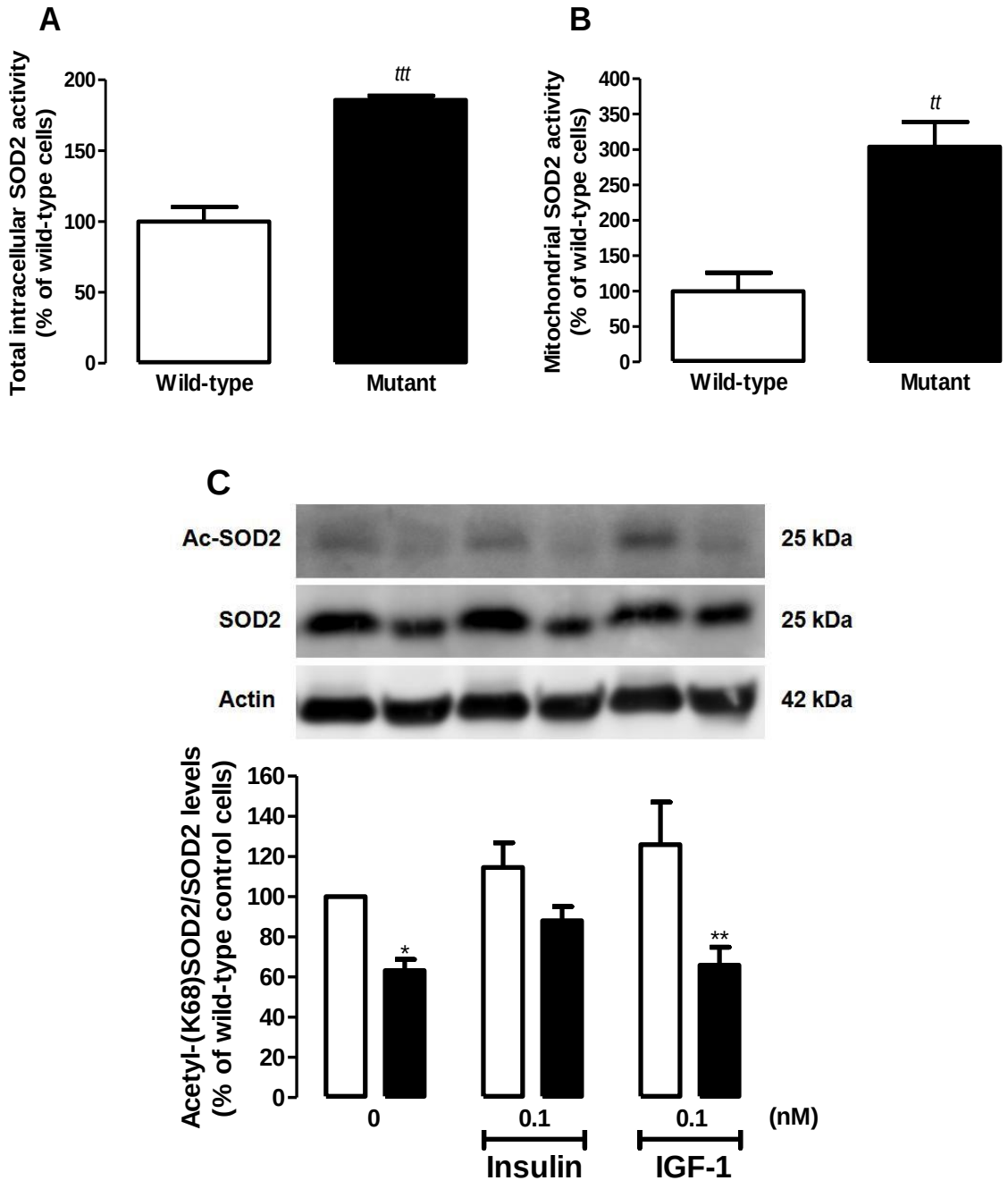


Figure 3.3.1 – Insulin and IGF-1 protects from ROS formation and caspase-3 activation in HD striatal cells. ROS formation, measured using (A,B) H_2DCFDA, (C) MitoSOX Red and (D) DHE, increases in mutant cells, compared to wild-type cells, whereas treatment with (A) insulin or (B) IGF-1 (0.1-10 nM) during 24h precludes ROS formation in mutant cells. (C) Mitochondrial and (D) total intracellular $O_2^{\bullet-}$ formation rises in mutant cells, which is prevented by (C) 0.1 nM insulin or IGF-1 (24h) and exacerbated by (D) mitochondrial complex I (rotenone) and III (antimycin A) inhibitors (10 µM, for 1h). (E) Caspase-3 activity is prevented by insulin treatment (0.1 nM) in mutant cells. Data are the mean ± S.E.M. of 3-6 independent experiments. Statistical analysis: $^{ttt}P<0.0001$ when comparing mutant *versus* wild-type cells by Student's *t*-test; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.0001$ by two-way ANOVA for multiple groups using Bonferroni as post-hoc test, when comparing mutant *versus* wild-type cells; or $^{\#\#}P<0.01$, $^{\#\#\#}P<0.0001$ by two-way ANOVA when comparing treated *versus* non-treated (control) cells.

3.3.3.2 **Full-length mHtt modifies SOD1/2, GCLc and glutathione levels – influence of insulin and IGF-1**

In order to examine whether higher ROS levels in mutant cells were due to alterations in antioxidant activities, we determined SOD1/2 activities and protein levels. A significant increase in SOD2 activity was observed in total cell extracts from mutant, compared to wild-type cells (Figure 3.3.2A), suggesting a compensatory mechanism against higher levels of $O_2^{\bullet-}$. Concordantly, SOD2 activity was increased in mitochondrial fractions from mutant cells (Figure 3.3.2B). Since SOD2 can be acetylated at Lys68 leading to a decrease in its activity (Chen et al., 2011), we measured the acetylation levels of SOD2 in striatal cells. In agreement with increased SOD2 activity, we detected a decrease in Ac-SOD2/SOD2 ratio in mutant cells (Figure 3.3.2C).

However, a decrease in SOD2 protein levels were also verified in mutant cells (Figure 3.3.2D). Although IGF-1 increased SOD2 protein levels in mutant cells (Figure 3.3.2D), neither insulin nor IGF-1 significantly changed SOD2 acetylation in mutant cells (Figure 3.3.2C). We also observed an increase in total (Figure 3.3.2E) and mitochondrial (Figure 3.3.2F) SOD1 activities, however no differences in SOD1 protein levels were detected in mutant *versus* wild-type cells (Figure 3.3.2G). These results indicate that increased ROS caused by expression of full-length mHtt largely correlate with increased SOD1 and 2 activities and that treatment with insulin or IGF-1 do not interfere with SOD2 activity.



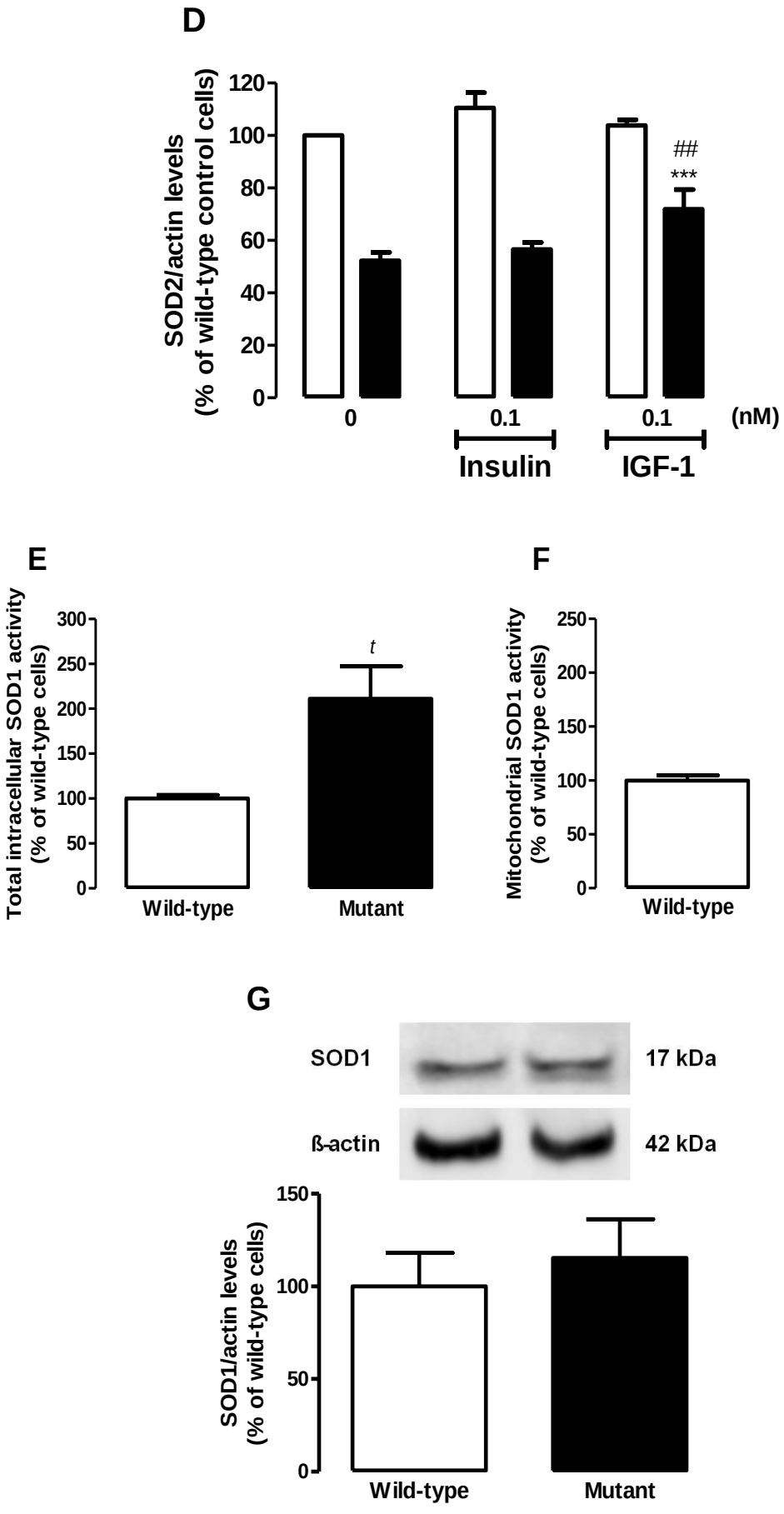
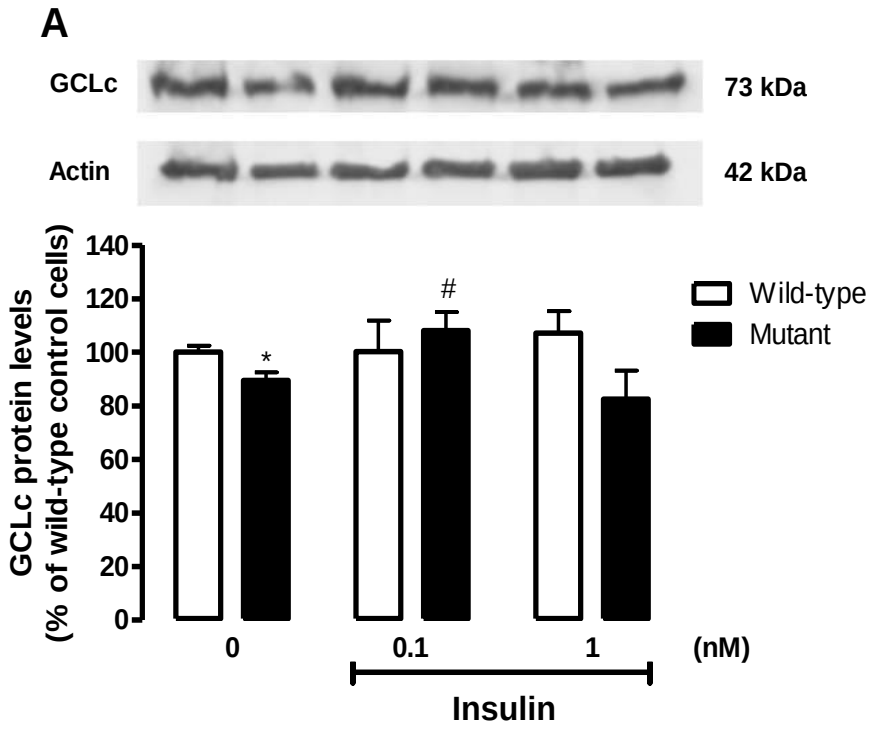
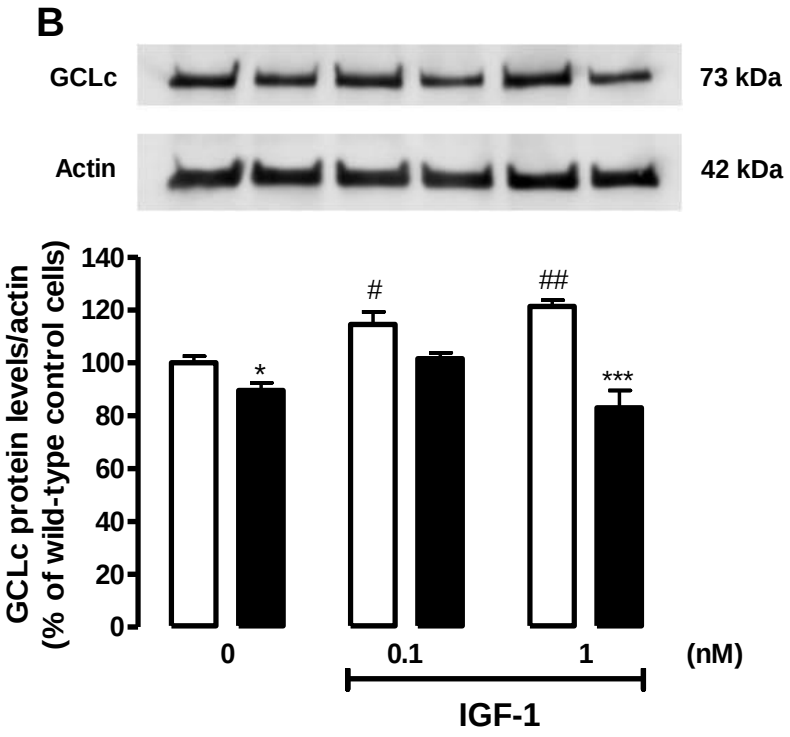

D
SOD2/actin levels
(% of wild-type control cells)
120
100
80
60
40
20
0
##
***
0
0.1
0.1
(nM)
Insulin
IGF-1
E
Total intracellular SOD1 activity
(% of wild-type cells)
300
250
200
150
100
50
0
t
Wild-type
Mutant
F
Mitochondrial SOD1 activity
(% of wild-type cells)
250
200
150
100
50
0
t
Wild-type
Mutant
G
SOD1
17 kDa
ß-actin
42 kDa
SOD1/actin levels
(% of wild-type cells)
150
100
50
0
Wild-type
Mutant

Figure 3.3.2 – Enhanced SOD1 and 2 activities in HD striatal cells. (A) Total and (B) mitochondrial SOD2 activities are increased, correlating with decreased (C) Ac(Lys68)SOD2/SOD2 ratio in mutant cells; (C) 0.1 nM insulin or IGF-1 treatment (24h) does not affect Ac(Lys68)SOD2/SOD2 ratio, but (D) 0.1 nM IGF-1 increases SOD2 protein levels in mutant cells. (E) Total and (F) mitochondrial SOD1 (Cu/Zn-SOD) activities increases significantly in mutant cells, but (G) SOD1 protein levels do not change in mutant, compared to wild-type cells. Data are the mean ± S.E.M. of 3-4 independent experiments. Statistical analysis: $^{t}P<0.05$, $^{tt}P<0.01$ and $^{ttt}P<0.0001$ by Student's *t*-test comparing mutant *versus* wild-type cells; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#\#}P<0.01$ by two-way ANOVA when comparing treated *versus* non-treated (control) cells.

We previously showed that the activity of enzymes of the glutathione redox cycle were enhanced, whereas GCL and GS activities and protein levels of GCLc were decreased in HD mutant striatal cells (Ribeiro et al., 2012). Therefore, in the present study, we determined the effect of insulin and IGF-1 on GCLc protein levels by western blotting. A significant decrease in GCLc levels were observed in mutant cells, when compared to wild-type cells (Figure 3.3.3A,B). Furthermore, treatment with 0.1 nM insulin increased GCLc levels in mutant cells, whereas IGF-1 (0.1-1 nM) increased GCLc levels in wild-type cells only, compared to non-treated conditions (Figure 3.3.3A,B).

We further demonstrated a rise in glutathione levels in mutant cells despite decreased GCL activity (Ribeiro et al., 2012). Therefore, we tested if insulin or IGF-1 could change GSH and GSSG levels. Despite an increase in GCLc levels in mutant cells induced by insulin, neither insulin nor IGF-1 altered GSH or GSSG levels in mutant cells (Figure 3.3.3C,D); nevertheless, insulin slightly increased GSH and GSSG levels in wild-type cells (Figure 3.3.3C,D). These data suggest that insulin ameliorate GCLc protein levels, although not affecting GSH or GSSG levels in cells expressing mHtt.


A
GCLc
73 kDa
Actin
42 kDa
GCLc protein levels
(% of wild-type control cells)
140
120
100
80
60
40
20
0
*
#
Wild-type
Mutant
0
0.1
1
(nM)
Insulin


B
GCLc
73 kDa
Actin
42 kDa
GCLc protein levels/actin
(% of wild-type control cells)
140
120
100
80
60
40
20
0
*
#
##
***
0
0.1
1
(nM)
IGF-1

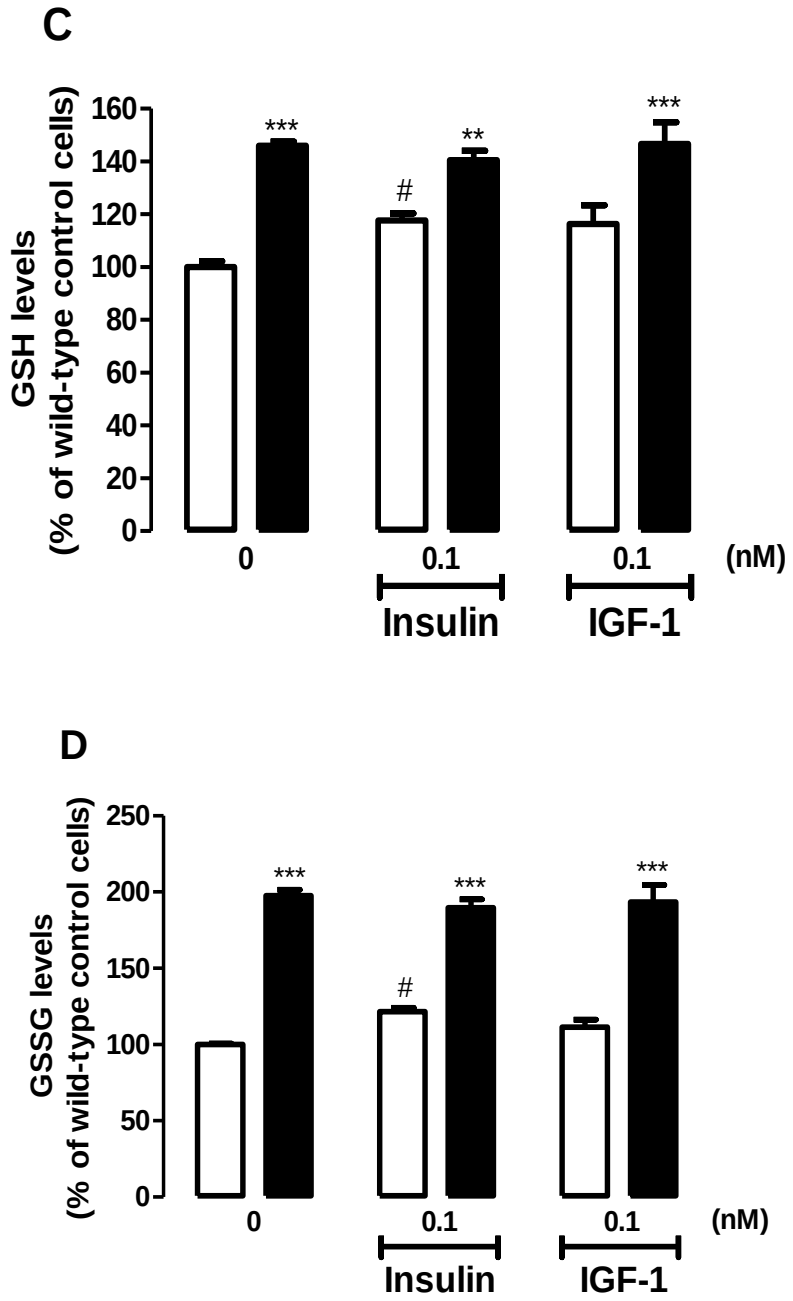
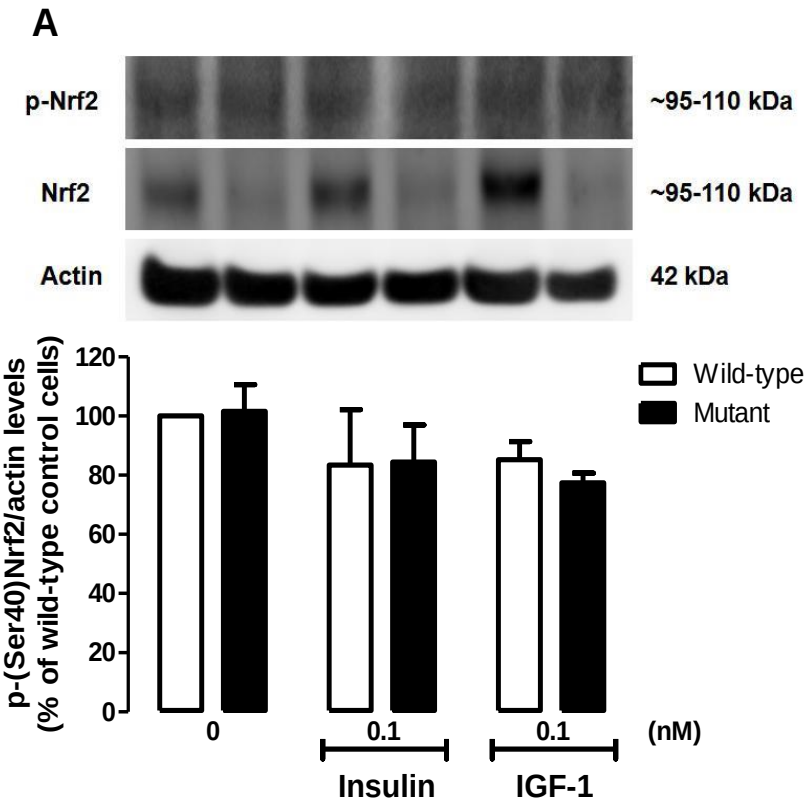
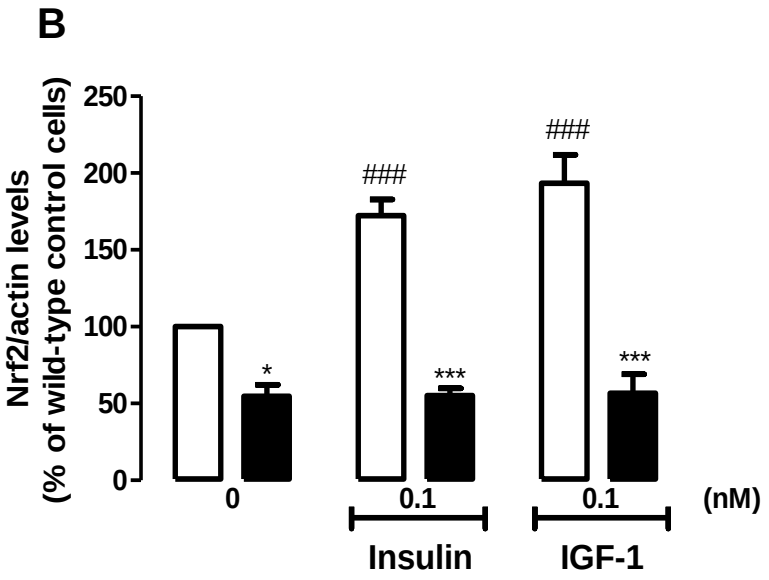


Figure 3.3.3 – Insulin increases GCLc protein levels in mutant cells. (A,B) GCLc protein levels decrease but (C) GSH and (D) GSSG levels increase in mutant compared to wild-type cells. 0.1 nM insulin (24h-treatment) rises (A) GCLc protein levels in mutant cells and (C) GSH and (D) GSSG levels in wild-type cells. 0.1 and 1 nM IGF-1 treatment (24 h) increase (B) GCLc protein levels in wild-type cells, but not in mutant cells, and do not change (C) GSH or (D) GSSG levels in both striatal cells. Data are the mean ± S.E.M. of 4 independent experiments. Statistical analysis: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ by two-way ANOVA when comparing treated *versus* non-treated (control) cells.

3.3.3.3 **Insulin and IGF-1 enhance phosphorylated Nrf2 nuclear levels, but do not affect its transcriptional activity in HD striatal cells**

We have previously shown that despite enhanced antioxidant activities, HD knock-in striatal cells presented lower response capacity to H_2O_2 or STS, which might be due to decreased activation of antioxidant enzyme transcription (Ribeiro et al., 2013). In order to evaluate whether antioxidant response was induced by insulin and/or IGF-1, we analysed the levels of the transcription factor Nrf2, which is responsible for the transcription of many antioxidant enzymes, including SOD1 and GCLc, and thus is directly implicated in cellular detoxifying systems (Langston et al., 2008; Nakaso et al., 2003). Human Nrf2 contains 605 amino acids with a predicted relative molecular mass (M_r) of 66 kDa (57 kDa in mice). However, it was shown that transcription and translation of the full-length Nrf2 cDNA produces a band at 66 kDa and a higher M_r band at approximately 96 kDa, likely due to the abundance of acidic residues in Nrf2 (Moi et al., 1994). Lau and collaborators (2013) have recently reported that the biologically relevant M_r of Nrf2 ranges from ~95-110 kDa. Therefore, in our experiments we quantified the Nrf2 M_r band between ~95-110 kDa. Phosphorylated p-(Ser40)-Nrf2 in total cell fractions did not differ significantly between both cell types (wild-type and mutant) (Figure 3.3.4A). However, under basal conditions, cellular Nrf2 levels decreased significantly in mutant cells (Figure 3.3.4B). Moreover, in nuclear-enriched fractions derived from mutant cells, p-Nrf2 levels decreased (Figure 3.3.4C), relatively to wild-type cells. Decreased nuclear p-Nrf2 by full-length mHtt suggests reduced efficiency of the antioxidant defense system through inhibition of gene transcription, most likely promoting cellular ROS generation (Figure 3.3.1). Indeed, Nrf2 transcriptional activity decreased significantly in mutant cells when compared to wild-type cells (Figure 3.3.4D). Incubation with 0.1 nM insulin or IGF-1 in wild-type cells significantly increased cellular Nrf2 levels (Figure 3.3.4B) and insulin further enhanced Nrf2 activity (Figure 3.3.4D). Moreover, in mutant cells nuclear p-Nrf2 levels were rescued by insulin or IGF-1 (Figure 3.3.4C). Despite this, insulin did not change Nrf2 transcriptional activity in mutant striatal cells (Figure 3.3.4D).


A
p-Nrf2
~95-110 kDa
Nrf2
~95-110 kDa
Actin
42 kDa
p-(Ser40)Nrf2/actin levels
(% of wild-type control cells)
120
100
80
60
40
20
0
Wild-type
Mutant
0
0.1
0.1
(nM)
Insulin
IGF-1


B
Nrf2/actin levels
(% of wild-type control cells)
250
200
150
100
50
0
*
###
***
###
***
0
0.1
0.1
(nM)
Insulin
IGF-1

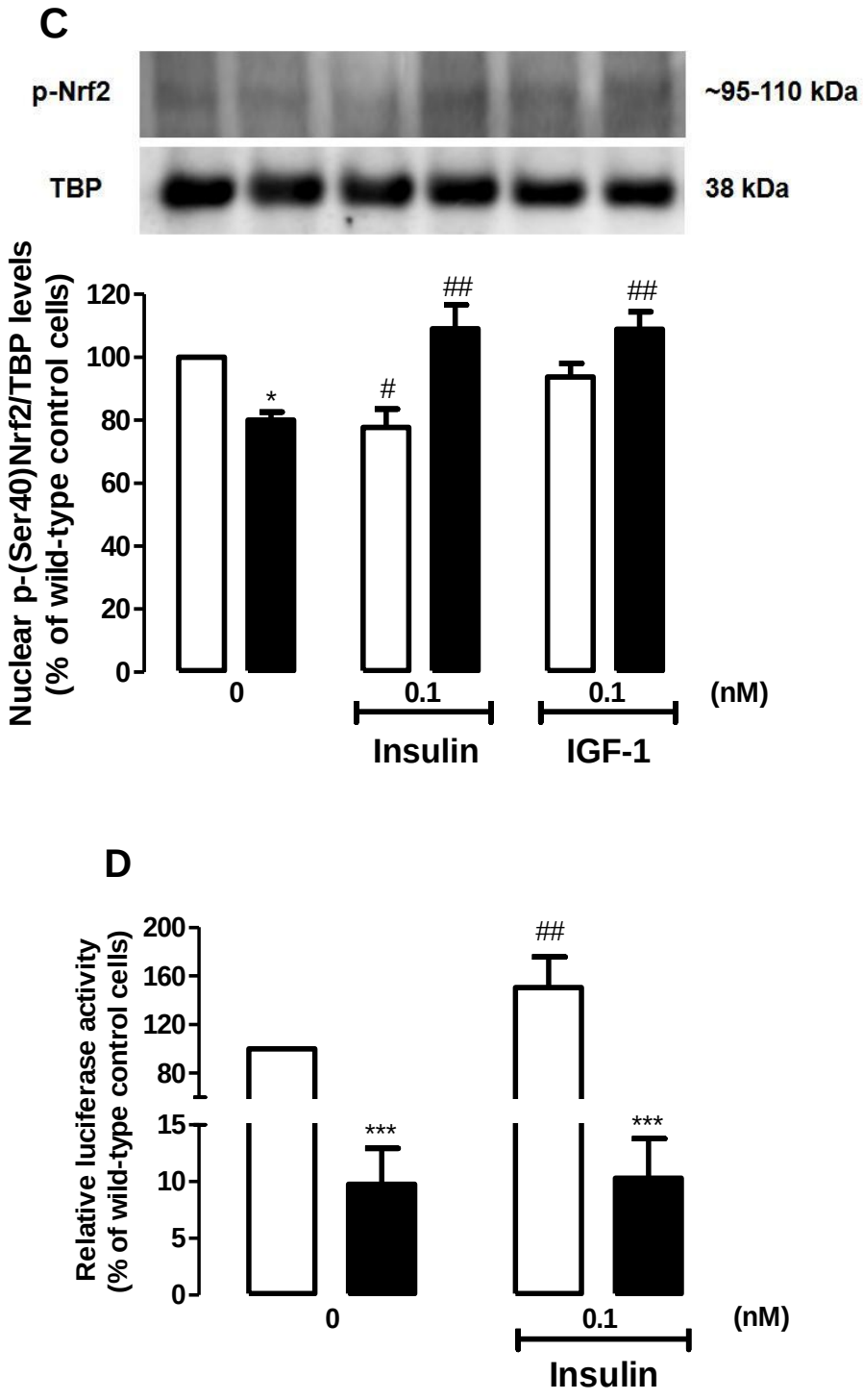
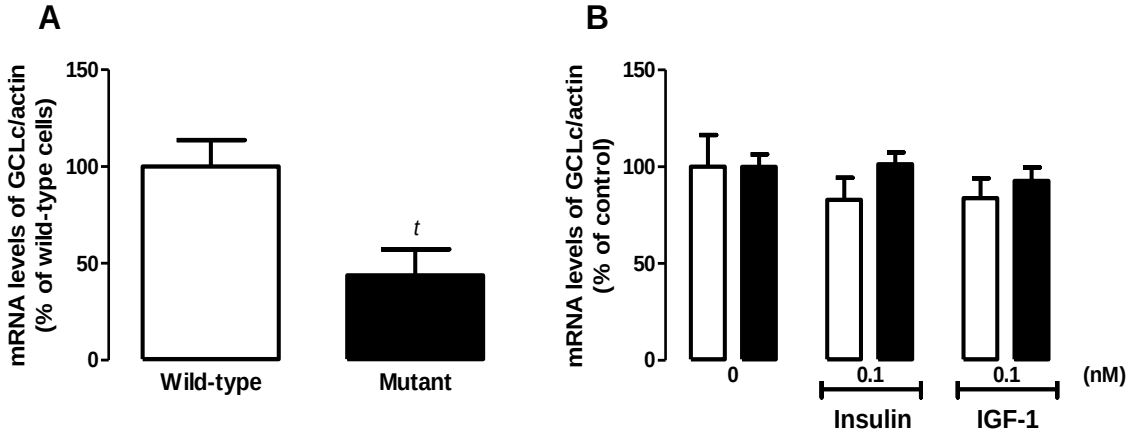


Figure 3.3.4 – Insulin and IGF-1 increase nuclear levels of phosphorylated Nrf2 in mutant cells. Striatal cells were treated with 0.1 nM insulin or IGF-1 during 24h for determination of cellular (A) p-(Ser40)Nrf2 and (B) Nrf2 levels and nuclear (C) p-(Ser40)Nrf2 levels and (D) Nrf2/ARE transcriptional activity by the luciferase reporter assay. (A) Cellular levels of p-Nrf2 are not changed, but decreased (B) cellular Nrf2, (C) nuclear p-(Ser40)Nrf2 levels and (D) Nrf2 transcriptional activity are observed in mutant, compared to wild-type cells. 0.1 nM insulin increases (B) total Nrf2 levels and (D) transcriptional activity, but decreases (C) nuclear p-(Ser40)Nrf2 levels in wild-type cells; insulin increases (C) nuclear p-(Ser40)Nrf2 levels in mutant

cells only. 0.1 nM IGF-1 increases (C) nuclear p-(Ser40)Nrf2 levels in mutant cells, but does not alter cellular (A) p-(Ser40)Nrf2 or (B) Nrf2 levels in both striatal cells. Results are the mean ± S.E.M. of 4-7 independent experiments. Statistical analysis: $^{*}P<0.05$ and $^{***}P<0.0001$ by two-way ANOVA when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.0001$ by two-way ANOVA when comparing treated *versus* non-treated (control) cells.

p-Nrf2 is known to translocate to the nucleus where it activates the transcription of genes involved in antioxidant defense, including GCLc, NQO1, HO-1, among others (Nakaso et al., 2003). In order to further examine the importance of Nrf2 in regulating the oxidant status in mutant cells, we determined the levels of mRNA of some Nrf2 targets, namely GCLc, NQO1 and HO-1 under basal conditions and after exposure to insulin and IGF-1 (Figure 3.3.5). In agreement with the decrease in nuclear p-Nrf2, Nrf2 transcriptional activity and GCLc protein levels in untreated mutant cells, we observed a significant reduction in GCLc mRNA levels in HD knock-in cells, when compared to wild-type cells (Figure 3.3.5A). Nevertheless, no significant changes were observed upon treatment with 0.1 nM insulin or IGF-1 (Figure 3.3.5B), in accordance with the inefficiency of insulin to promote Nrf2 transcriptional activity (Figure 3.3.4D). No significant changes in NQO1 and HO-1 mRNA levels were detected in untreated mutant *versus* wild-type cells or after exposure to insulin or IGF-1 either (Figure 3.3.5C,D,E,F).

Our data indicate that although insulin and IGF-1 induced translocation of p-Nrf2 to the nucleus in mutant cells, its activity and the transcription of related proteins was not changed, suggesting that insulin- and IGF-1-induced protection against ROS formation in HD striatal cells is independent of Nrf2-regulated antioxidant response.



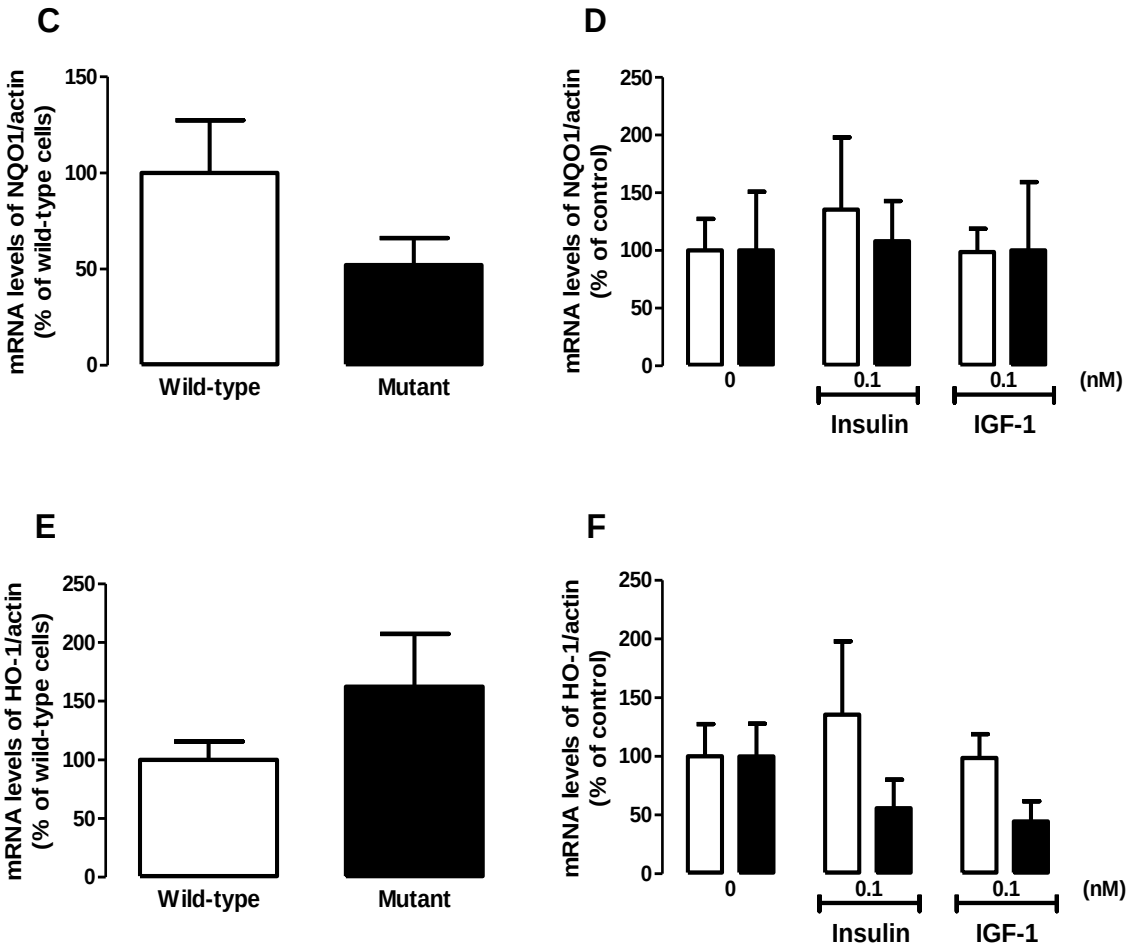


Figure 3.3.5 – Decreased mRNA levels of GCLc in mutant cells. 0.1 nM insulin or IGF-1 treatment during 24h was tested for analysis of mRNA levels of (A,B) GCLc, (C,D) NQO1, and (E,F) HO-1 in striatal cells. Decreased (A) GCLc without changes in (C) NQO1 or (E) HO-1 mRNA levels are verified in mutant *versus* wild-type cells. 0.1 nM insulin and IGF-1 (24h) do not significantly affect (B) GCLc, (D) NQO1 or (F) HO-1 mRNA levels in both striatal cells. Data are expressed as the mean ± S.E.M. of 3-7 independent experiments. Statistical analysis: $^{t}P<0.05$ by Student's *t*-test comparing mutant *versus* wild-type cells.

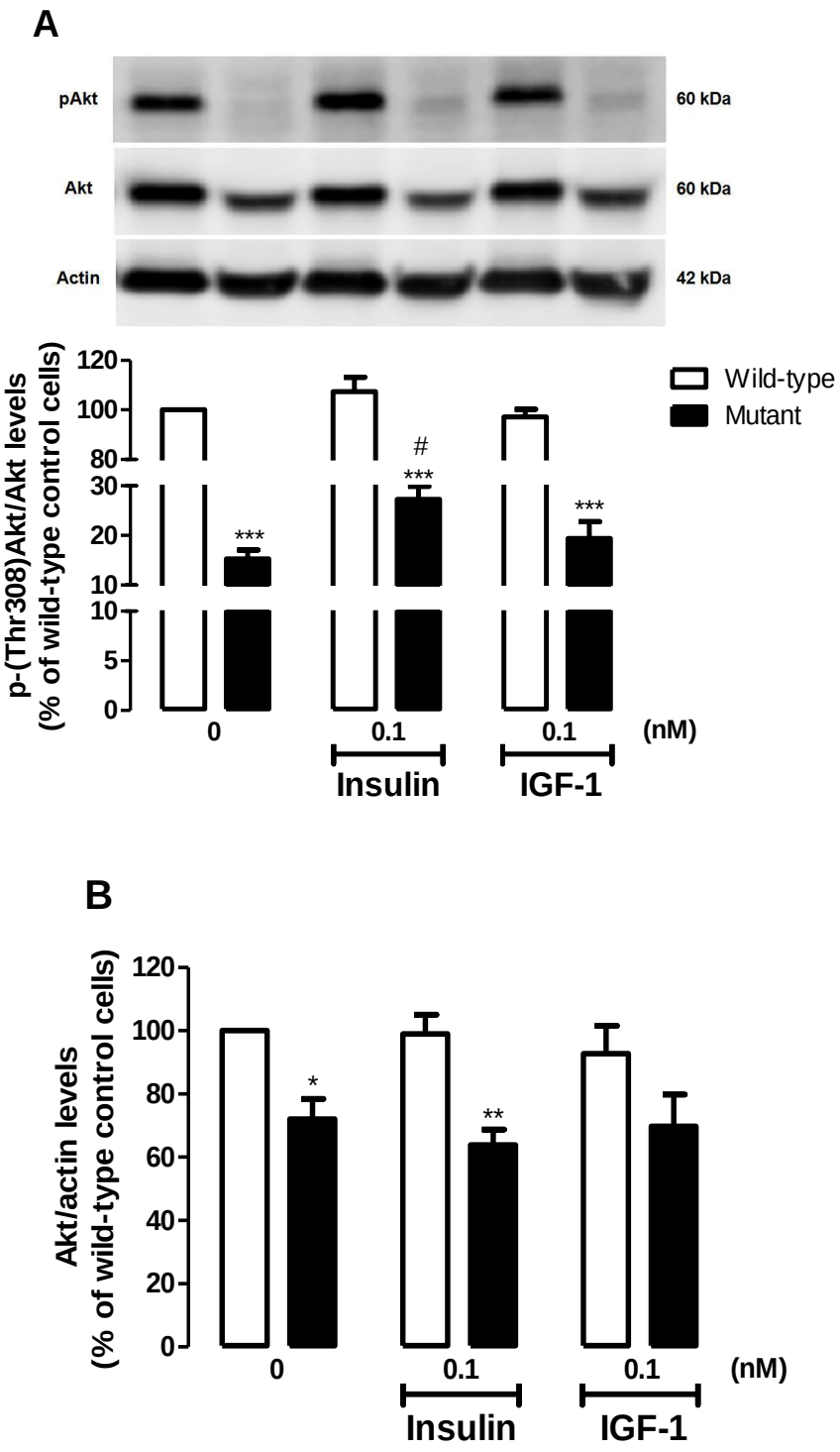
3.3.3.4 **Insulin prevent mitochondrial depolarization via PI-3K/Akt pathway**

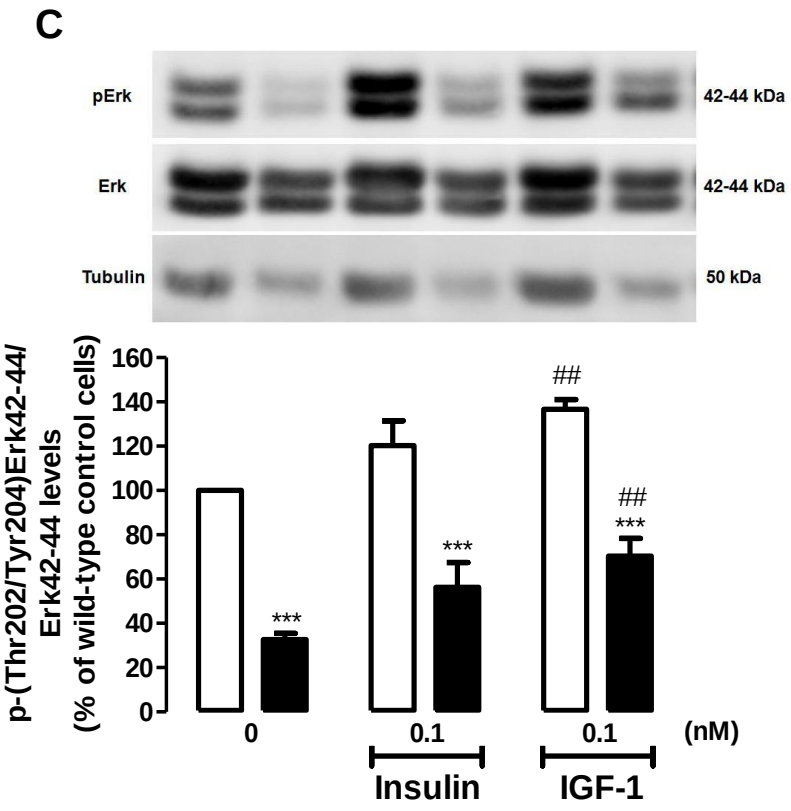
Because insulin and IGF-1 are able stimulate intracellular signaling pathways, we next investigated an upstream pathway of cell transcription and survival. We measured total and phosphorylated levels of Akt, and Erk42/44 in the absence or presence of 0.1 nM insulin or IGF-1 (Figure 3.3.6). In the present work we analysed p-(Thr308)Akt, phosphorylated by PDPK1, since it was described to better correlate with Akt protein kinase activity (Vincent et al., 2011).

Expression of mHtt *per se* significantly decreased the ratio of phosphorylated p-(Thr308)Akt/total Akt, total Akt/actin and p-(Thr202/Tyr204)Erk(42/44)/total Erk(42/44) (Figure 3.3.6A,B,C), largely suggesting decreased activation of both PI-3K/Akt and MEK/Erk signaling pathways. On the other hand, Erk(42/44) levels were only slightly affected in mutant cells (see representative blot in Figure 3.3.6C), as the increase in Erk(42/44)/tubulin levels (Figure 3.3.6D) were largely due to the decrease in tubulin levels in mutant cells, compared to wild-type cells; notably, tubulin was used as a loading control for normalizing Erk(42/44) protein levels because Erk and actin have similar M_r. Exposure to 0.1 nM insulin, but not 0.1 nM IGF-1, significantly increased Akt phosphorylation (p-(Thr308)Akt/Akt), whereas total Akt levels did not change with either insulin or IGF-1 stimuli in mutant cells (Figure 3.3.6A,B). On the other hand, 0.1 nM IGF-1 significantly increased p-Erk(42/44)/Erk(42/44) in both striatal cells, whereas 0.1 nM insulin did not significantly affect p-Erk(42/44)/Erk(42/44) ratio (Figure 3.3.6C). These results suggest that insulin and IGF-1 may differentially regulate pro-survival responses in mutant striatal cells by activating Akt and Erk, respectively.

Taking into account that insulin and IGF-1 protected from mitochondrial $O_2^{\bullet-}$ formation induced by expression of mHtt (Figure 3.3.1), we next tested whether insulin and IGF-1 influenced mitochondrial function. $\Delta\Psi m$, measured using $TMRM^+$ after complete depolarization with FCCP plus oligomycin, was decreased by almost 50% in mutant cells when compared to wild-type cells (Figure 3.3.6E), revealing higher mitochondrial depolarization induced by mHtt. Insulin (0.1 nM) significantly increased $\Delta\Psi m$ and IGF-1 ameliorated the changes observed in mutant cells (Figure 3.3.6E). These data showed that insulin and IGF-1 improve mitochondrial function, which appears to be in accordance with the decrease in mitochondrial ROS formation observed in HD striatal cells (Figure 3.3.1C).

Because insulin induced the activation of Akt and significantly decreased mitochondrial depolarization, compared to untreated mutant cells, we further tested if the PI-3K/Akt pathway was involved in improving mitochondrial function of mutant cells, by testing the effect of LY294002 (25 μM), a highly selective inhibitor of PI-3K (Vlahos et al., 1994). We observed that insulin-evoked increase in $\Delta\Psi m$ was completely abrogated by LY294002 (Figure 3.3.6F), whereas non-significant tendencies were observed in the case of IGF-1. Thus, insulin largely improves mitochondrial function through the PI-3K/Akt pathway in HD mutant cells.


A
pAkt
60 kDa
Akt
60 kDa
Actin
42 kDa
p-(Thr308)Akt/Akt levels
(% of wild-type control cells)
120
100
80
30
20
10
10
5
0
Wild-type
Mutant
#
***
***
***
0
0.1
0.1
(nM)
Insulin
IGF-1
B
Akt/actin levels
(% of wild-type control cells)
120
100
80
60
40
20
0
*
**
0
0.1
0.1
(nM)
Insulin
IGF-1


C
pErk
42-44 kDa
Erk
42-44 kDa
Tubulin
50 kDa
p-(Thr202/Tyr204)Erk42-44/
Erk42-44 levels
(% of wild-type control cells)
160
140
120
100
80
60
40
20
0
***
***
##
##
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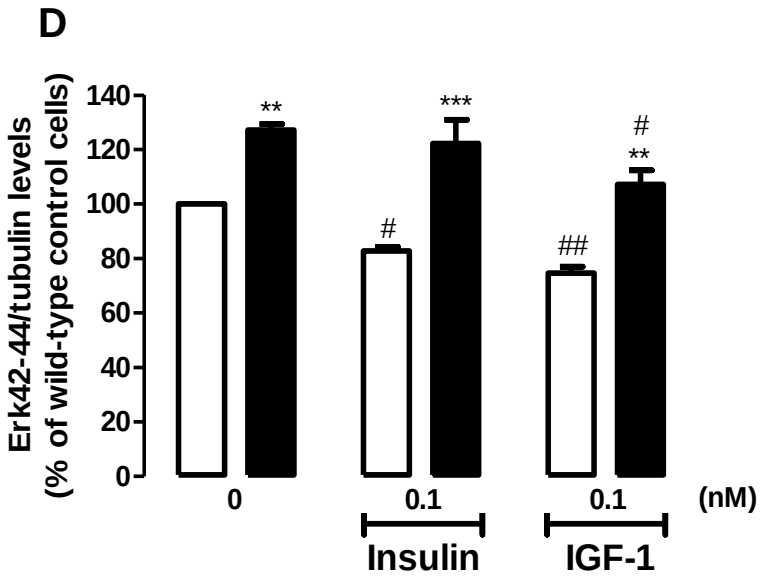
0
0.1
0.1
(nM)
Insulin
IGF-1


D
Erk42-44/tubulin levels
(% of wild-type control cells)
140
120
100
80
60
40
20
0
**
***
#
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##
0
0.1
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(nM)
Insulin
IGF-1

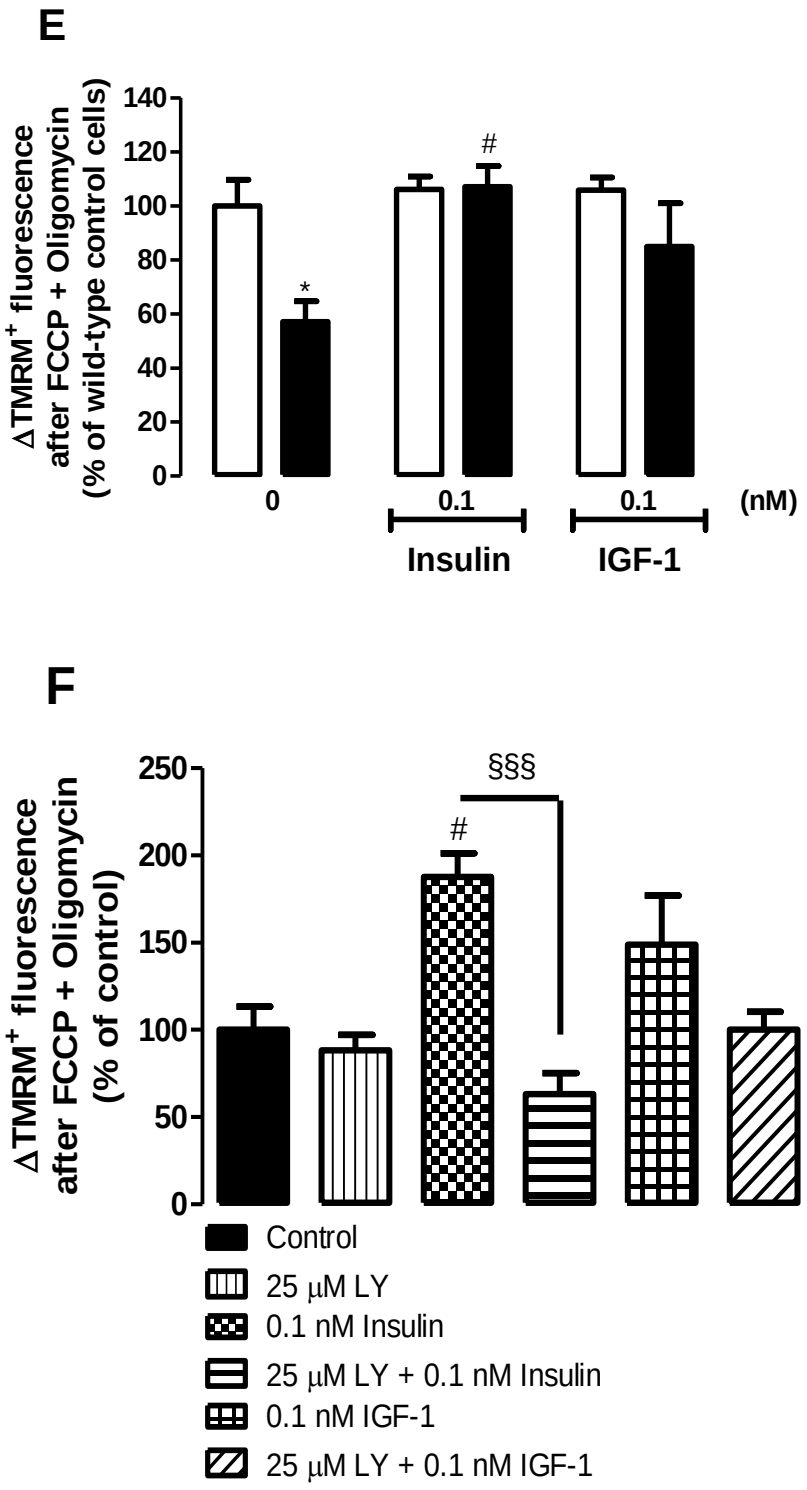


Figure 3.3.6 – Insulin and IGF-1 promote Akt and Erk activation, and insulin increases mitochondrial transmembrane potential (ΔΨm) in HD striatal cells. Striatal cells were treated with 0.1 nM insulin or IGF-1 during 24h for determination of (A) p-(Thr308)Akt/Akt, (B) Akt/actin, (C) p-(Thr202/Tyr204)Erk1/2, and (D) Erk/tubulin ratio and analysis of mitochondrial transmembrane potential (ΔΨm) with $TMRM^+$ (E,F). Decreased (A) p-(Thr308)Akt/Akt, (B) Akt/actin, and (C) p-(Thr202/Tyr204)Erk1/2, increased (D) Erk/tubulin and decreased (E) mitochondrial transmembrane potential (ΔΨm) are verified in mutant cells. 0.1 nM insulin

significantly increases (A) p-(Thr308)Akt/Akt ratio and (E,F) mitochondrial transmembrane potential (ΔΨm) in mutant cells, and decreases (D) Erk/tubulin ratio in wild-type cells, without changing (B) Akt/actin, and (C) p-(Thr202/Tyr204)Erk1/2 ratio in both striatal cells. IGF-1 (0.1 nM) increases (C) p-(Thr202/Tyr204)Erk1/2, and decreases (D) Erk/tubulin ratio, without altering (A) p-(Thr308)Akt/Akt or (B) Akt/actin ratio in both striatal cells; (E,F) mitochondrial transmembrane potential (ΔΨm) is ameliorated by IGF-1 in both striatal cells. Exposure to 25 μM LY294002, a PI-3K/Akt inhibitor, 30 min before insulin or IGF-1 treatment during 24h significantly blocks elevated (D) mitochondrial transmembrane potential (ΔΨm) induced by 0.1 nM insulin in mutant cells. Data are the mean ± S.E.M. of 4 independent experiments. Statistical analysis was performed by one-way or two-way ANOVA for multiple groups using Bonferroni as post-test: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.0001$ when comparing mutant *versus* wild-type cells; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ when comparing treated *versus* non-treated (control) cells; $^{\S\S\S}P<0.0001$ when comparing insulin *versus* LY294002 plus insulin treatment in mutant striatal cells.

3.3.4 Discussion

In this study we show that treatment of striatal cells expressing full-length mHtt with insulin and IGF-1 stimulate Akt and Erk pathways, respectively, which may promote phosphorylated Nrf2 in the nucleus, a transcription factor that acts at the ARE regulating the expression of detoxifying and antioxidant genes. Nevertheless, insulin-mediated rise in GCLc protein levels was not accompanied by enhanced Nrf2 transcriptional activity, GCLc mRNA or glutathione levels in mutant cells. Importantly, insulin and IGF-1 decreased mitochondrial-driven ROS formation and mitochondrial depolarization induced by mHtt. In the case of insulin, rescued mitochondrial function was dependent on PI-3K/Akt pathway, which may further underlie protection against mHtt-evoked caspase-3 activity.

In HD striatal cells we showed increased mitochondrial generation of ROS, accompanied by increased activities of SOD1/2, which was confirmed by reduced SOD2 acetylation. An increase in SOD activity was previously observed in R6/1 HD mice at 19 weeks of age, although older mice exhibited decreased SOD activity (Santamaria et al., 2001). Reduced activity of SOD1 was observed in cortex and cerebellum of *post-mortem* human brain tissues (Browne et al., 1997) and in erythrocytes (Chen et al., 2007) of HD patients. Interestingly, insulin- and IGF-1-induced protection against ROS formation in HD striatal cells was independent of changes in SOD2 activity. In a previous study we demonstrated increased ROS levels and a decrease in GCLc activity, despite increased glutathione levels in HD striatal cells, which occurred as a result of a lower capacity of Mrp1 to export GSH to the extracellular space (Ribeiro et al., 2012). While insulin

moderately enhanced GCLc protein levels in mutant striatal cells, no changes in GCLc mRNA were observed and glutathione levels remained unchanged, largely suggesting that ROS protection induced by insulin is also independent on glutathione antioxidant response. Moreover, the transcription of other Nrf2-target genes, such as HO-1 and NQO1, was not affected either. Surprisingly, this occurred despite insulin and IGF-1-induced translocation of phosphorylated Nrf2 to the nucleus in mutant cells. In contrast to van Roon-Mom and collaborators (2008), we did not detect activation of Nrf2-responsive genes upon expression of mHtt under non-stimulated conditions. Indeed, p-Nrf2 nuclear levels were decreased in HD knock-in striatal cells, underlying reduced ARE transcriptional activity and thus decreased mRNA and protein levels of GCLc. In agreement with our data, the striatum of the NLS-N171-82Q transgenic mice showed reduced levels of Nrf2 (Chaturvedi et al., 2010). Moreover, neurons derived from Nrf2 KO mice were greatly sensitive to mitochondrial complex II inhibitors (Calkins et al., 2005), used to model mitochondrial dysfunction in HD. In the present study, although insulin/IGF-1 could act as indirect antioxidants in HD cells *via* increased translocation of phosphorylated Nrf2 to the nucleus, the peptide was not able to affect ARE transcriptional activity. Acetylation of Nrf2 by CBP was previously described to increase Nrf2 promoter-specific DNA binding (Sun et al., 2009). Therefore, we hypothesize that insulin or IGF-1 may not be able to increase Nrf2 acetylation and promote its activity and/or may not efficiently bind to ARE due to the presence of mHtt in the nucleus.

We previously showed that HD striatal cells exhibit features of intrinsic apoptosis (Rosenstock et al., 2011). Enhanced caspase-3 activation has been linked to increased mitochondrial ROS formation. Indeed, we showed that there is a close interplay between endogenous ROS generation and caspase-3 activation in apoptotic neurons (Ferreira et al., 2010; Gil et al., 2003). Interestingly, although both insulin and IGF-1 prevented ROS generation in mutant cells, only insulin was able to significantly prevent caspase-3 activation in cells expressing mHtt. This may account for by differential activation of pro-survival signaling pathways in knock-in HD striatal cells exposed to insulin or IGF-1. In a previous study we demonstrated that insulin (at higher concentrations, potentially acting on IR and IGF1R) can act as an antioxidant in cortical neurons, under conditions involving Akt activation and GSK3-β inhibition (Duarte et al., 2005; 2008). In unstimulated HD knock-in striatal cells showed reduced activation of MEK/Erk1/2 and PI-3K/Akt signaling pathways, which were stimulated by and IGF-1 and insulin, respectively. Erk was previously described to protect against mHtt toxicity (Apostol et al.,

2006); thus, decreased Erk activation in mutant cells may contribute to increase mHtt cytotoxicity. In agreement with our work, ST*Hdh*$^{Q111/Q111}$ cells displayed significant decreased p-Erk/Erk (Sarantos et al., 2012). Decreased p-Erk levels were also observed in the striatum of 8-week old R6/2 HD mice (Fusco et al., 2012). Reduced Akt was previously reported in HD patients, appearing as a shorter inactive caspase-3-cleaved form (Colin et al., 2005; Humbert et al., 2002). Corroborating our study, p-(Ser473)Akt/Akt levels were significant decreased in ST*Hdh*$^{Q111/Q111}$ cells (Maglione et al., 2010), in HEK293 cells expressing mHtt with 68 CAG repeats (Nagata et al., 2011) and in HD patient's lymphoblasts and lymphocytes (Colin et al., 2005). Akt activation is an early pro-survival striatal response in knock-in HdhQ111 mice and ST*Hdh*$^{Q111/Q111}$ cells (Gines et al., 2033); importantly, activation of IGF-1/Akt pathway caused Htt phosphorylation at Ser421, decreasing mHtt nuclear inclusions and mHtt toxicity (Humbert et al., 2002), further regulating anterograde and retrograde transport defects in HD cortical neurons (Zala et al., 2008). Another study demonstrated that p-(Ser473)Akt was unchanged in YAC128 and in R6/2 HD mice, but the levels of p-(Ser421)Htt were decreased in the striatum of YAC128 HD mice and in cells expressing mHtt (Warby et al., 2005). In contrast, IRS-2, which activates PI-3K/Akt and mTOR cascade, promoted mitochondrial dysfunction and oxidative stress in R6/2 mice (Sadagurski et al., 2011). In this respect, plasma IGF-1 was correlated with cognitive decline in HD patients (Saleh et al., 2010). However, reduced IGF-1 mRNA was demonstrated in striatum and skin fibroblasts of HD patients and HD knock-in striatal cells (Pouladi et al., 2010). Moreover, IRS-2 was reported to be necessary for the elimination of mutant exon1htt aggregates. IRS-2 knockdown inhibited aggregate clearance, while IRS-2 activation induced by insulin, IGF-1 and interleukin-4 enhanced exon1htt clearance in a dose-dependent manner (Yamamoto et al., 2006). Additionally, we previously showed that IGF-1 rescued peripheral metabolic abnormalities linked to diabetes in R6/2 mice (Duarte et al., 2011), and recently, we also showed that intranasal administration of recombinant human IGF-1 (rhIGF-1) for 2 weeks, in order to promote IGF-1 delivery to the brain, enhanced IGF-1 cortical levels and improved motor activity and both peripheral and central metabolic abnormalities in YAC128 HD mice. Moreover, rhIGF-1 administration in YAC128 HD mice activated Akt and concomitantly increased phosphorylation of mHtt on Ser421, suggesting that intranasal administration of rhIGF-1 ameliorates HD-associated glucose metabolic brain abnormalities and mice phenotype (Lopes et al., 2013). Moreover, insulin stimulated neuronal glucose metabolism, restoring phosphocreatine and ATP levels upon

oxidative stress in primary cortical neurons (Duarte et al., 2006). These data are consistent with the stimulation of Akt and Erk signaling in insulin and IGF-1-treated HD striatal cells.

Mitochondria of HD striatal cells were recently described to be altered and more vulnerable to oxidative stress (Jin et al., 2013). Indeed, elevated mitochondrial $O_2^{\bullet-}$ generation in HD knock-in striatal cells has been linked to mitochondrial dysfunction (Milakovic and Johnson, 2005; Quintanilla et al., 2008). In HD striatal neurons, ROS formation may be a consequence of mitochondrial dysfunction through defects of mitochondrial respiratory complexes (Lim et al., 2008), which occur very early in disease progression, as recently shown by us (Silva et al., 2013). Indeed, the striatum is highly susceptible to dysfunction of mitochondrial oxidative phosphorylation (Pickrell et al., 2011). ROS formation in HD can further damage mitochondria, by dissipating the $\Delta\Psi m$, dysregulating ATP production and leading to oxidation of mtDNA (Acevedo-Torres et al., 2009; Polidori et al., 1999; Siddiqui et al., 2012), which is highly vulnerable to oxidative damage due to its proximity to the respiratory chain, limited repair mechanisms, few non-coding sequences and lack of histones (Richter et al., 1988). We have previously shown that $O_2^{\bullet-}$ formation also rises in HD human cybrids following exposure to 3-NP or STS (Ferreira et al., 2010). In this work, insulin and IGF-1 decreased mitochondrial $O_2^{\bullet-}$ formation in mutant cells. Interestingly, we also showed that insulin and IGF-1 improve mitochondrial function, evaluated through increased $\Delta\Psi m$, which was largely PI-3K/Akt-dependent in cells exposed to insulin. Interestingly, it was previously described that Akt can accumulate in mitochondria in its active state (Bijur and Jope, 2003) and this accumulation is dependent on the activity of heat shock protein 90 (Hsp90) (Barksdale and Bijur, 2009). Although the role of Akt in mitochondria is not well understood, active mitochondrial Akt has been shown to be neuroprotective (Mookherjee et al., 2007). In hepatocytes lacking PTEN which is an endogenous inhibitor of PI-3K/Akt pathway, activation of PI-3K/Akt pathway by IGF-1 induced anaerobic glycolysis and mitochondrial respiration (Li et al., 2013). Activated Akt is translocated to mitochondria, where it phosphorylates α and β subunits of ATP synthase leading to its activation. Furthermore, Akt translocation to mitochondria was associated with increased expression and activity of complex I (Li et al., 2013). Therefore, a better knowledge about the role of Akt in mitochondria of HD striatal cells might be essential to describe the role of insulin on mitochondrial function.

Chapter 4 – Conclusions and Future Perspectives

In the first part of our work we showed that striatal cells expressing full-lenght mHtt exhibit raised antioxidant defense profile through increased activity of the glutathione redox cycle and related metabolic enzymes, namely GST and γ-GT, despite decreased GSH synthesis. Interestingly, our work gives evidence that decreased expression and activity of Mrp1 results in decreased release of glutathione, leading to its intracellular accumulation (Figure 4.1). Although this is apparently insufficient to prevent oxidative stress and apoptotic features, it may result from an attempt of HD striatal cells that do not exhibit massive cell death or visible mHtt aggregates to counterbalance initial generation of ROS evoked by mHtt.

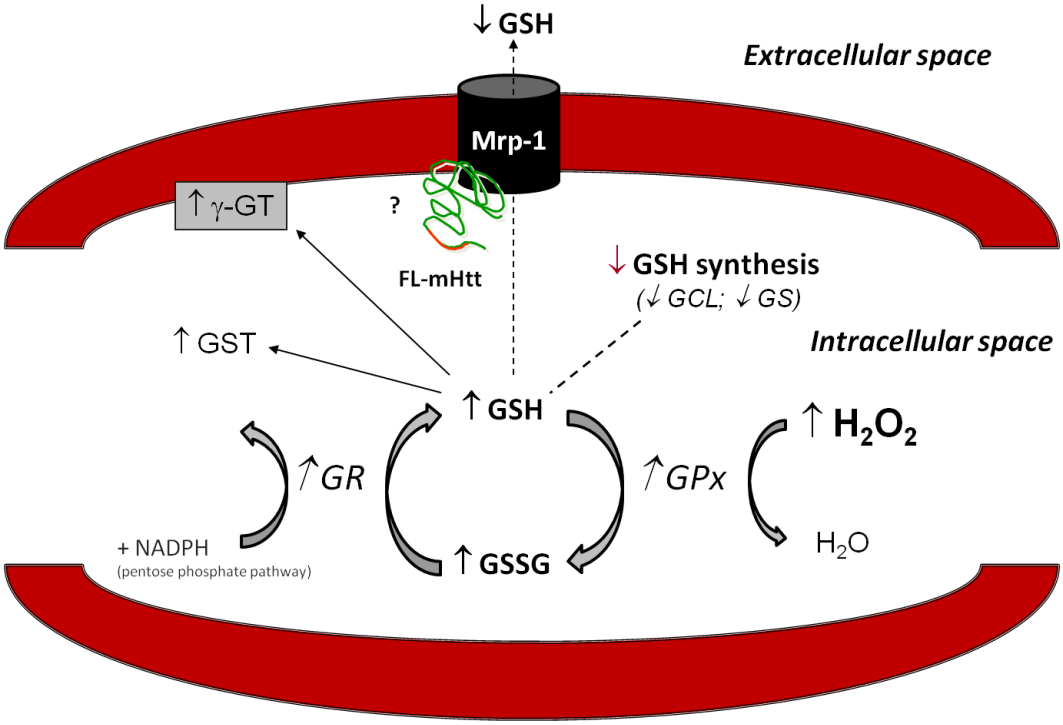


Figure 4.1 – Increased glutathione redox cycle in HD knock-in striatal cells. Despite decreased activities of glutamate-cysteine ligase (GCL) and glutathione synthetase (GS), both involved in the synthesis of glutathione, the levels of reduced (GSH) and oxidized (GSSG) forms of glutathione are significant increased in HD knock-in striatal cells, concomitantly with increased reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) levels, and increased activities of glutathione peroxidase (GPx), reductase (GRed), S-transferase (GST), γ-glutamyl transpeptidase (γ-GT), and glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) which are essential to produce NADPH, a cofactor used by GRed in the regeneration of GSH.

In the second part of our work we showed that striatal cells expressing full-length mHtt exhibit high ROS formation, at least partially produced by mitochondria, and that

exposure to H_2O_2 or STS potentiate oxidative stress by increasing XO activity and lowering the antioxidant response, namely SOD2 and GRed, the later promoting increased GSSG levels. Moreover, cystamine and creatine protected against H_2O_2 or STS-induced ROS formation in HD striatal cells (Figure 4.2). Thus, a better understanding of the mechanisms involved in this neuroprotection may contribute to delay striatal cell degeneration in HD.

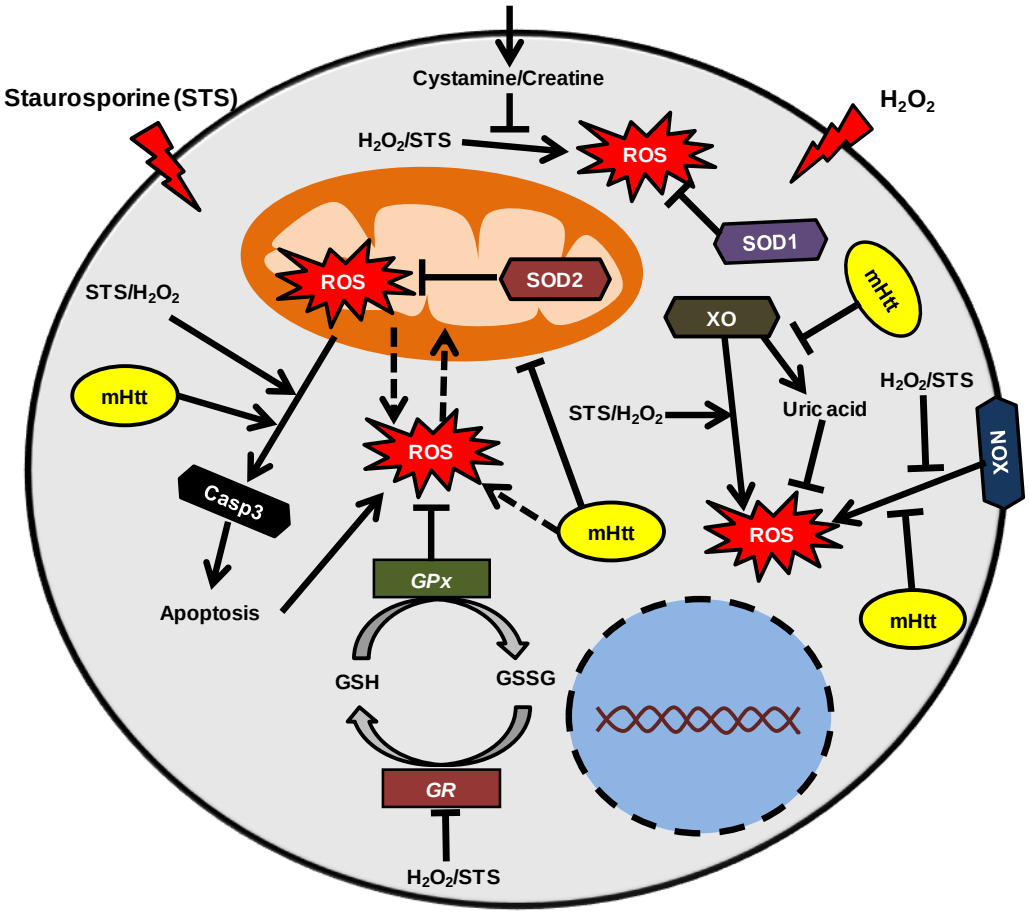
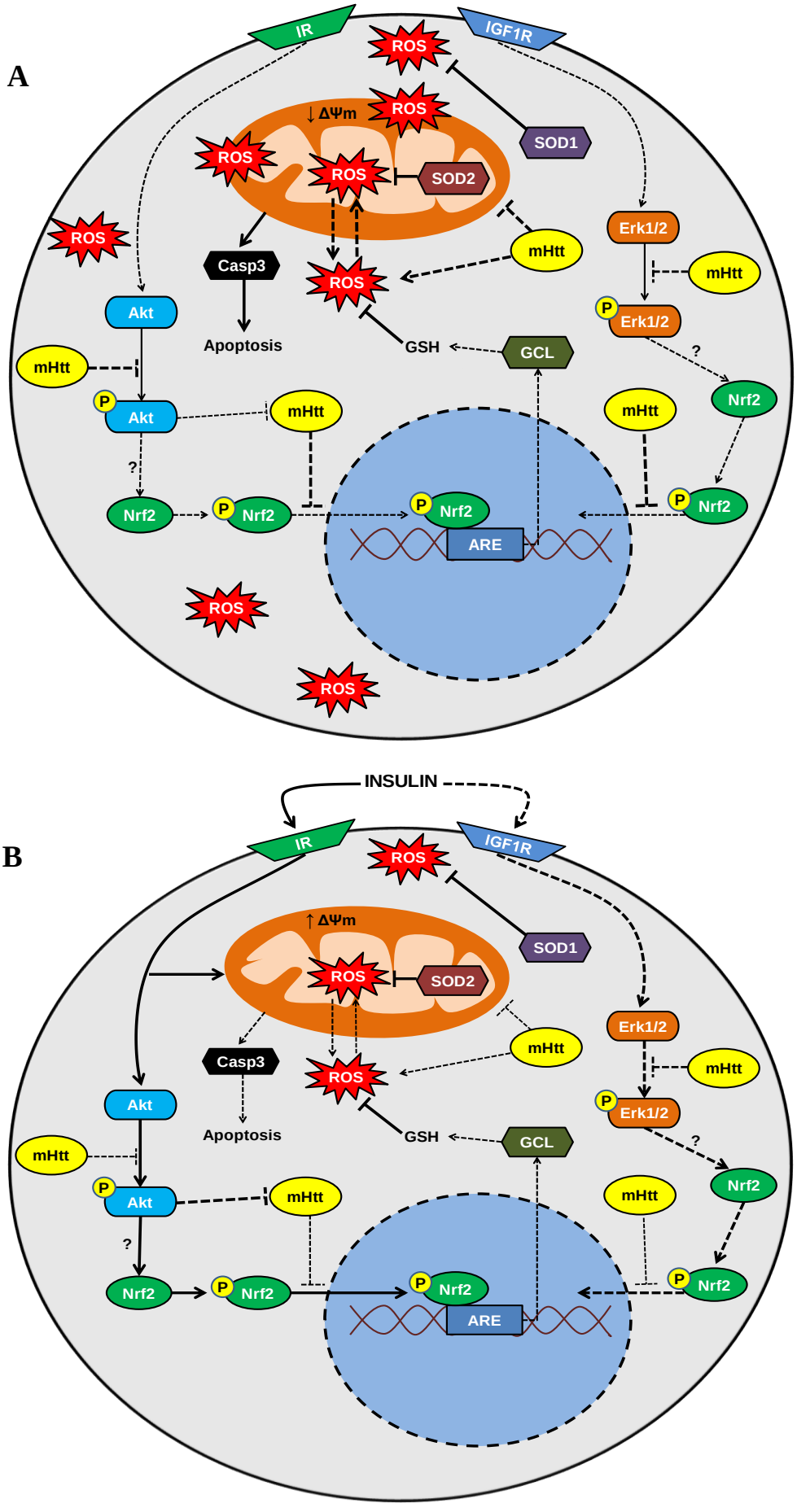


Figure 4.2 – Increased vulnerability of striatal cells expressing mHtt to exogenous stressors and the protective effect of cystamine and creatine. Exposure to noxious stimuli, staurosporine (STS) and hydrogen peroxide (H_2O_2), resulted in the exacerbation of mutant huntingtin (mHtt)-induced reactive oxygen species (ROS) formation and caspase-3 (Casp3) activation. STS and H_2O_2 exposure in mutant cells also decreased glutathione reductase (GRed) activity and increased the levels of the oxidized form of glutathione (GSSG) with minor changes in glutathione peroxidase (GPx), superoxide dismutase (SOD) 1 and 2 activities and in the levels of the reduced form of glutathione (GSH), indicating lower capacity of striatal cells expressing mHtt to reduce oxidative damage provoked by exogenous stress. mHtt expression also induced decreased activities of NADPH oxidase (NOX) and xanthine oxidase (XO) which were differentially affected by H_2O_2 or STS exposure. H_2O_2 and STS treatments exacerbated mHtt-induced decreased NOX activity, whereas XO activity was enhanced by the exogenous stressors. Creatine and cystamine treatment did not affect caspase-3 activity, but decreased ROS formation after exposure to H_2O_2 and STS.

In the third and last part of our work, we showed that expression of full-length mHtt promotes mitochondrial-driven oxidative stress, associated to compensatory enhanced SOD1/2 activities. Insulin and IGF-1 (at low nM) activated intracellular signaling pathways, Akt and Erk, respectively, and nuclear phosphorylated Nrf2 levels, but not its transcriptional activity. Insulin and IGF-1 further decreased mitochondrial ROS production and decreased mitochondrial depolarization in HD striatal cells. Insulin also induced cytoprotection through decreased caspase-3 activation, which might be due to its positive effect on mitochondria occurring through the PI-3K/Akt pathway (Figure 4.3). This work shows differential cellular effects exerted by insulin and IGF-1 under low nM concentrations, suggesting that insulin and/or IGF-1-mediated intracellular signaling pathways may constitute important therapeutic targets for pharmacological intervention as a way to ameliorate mitochondrial function and decrease oxidative stress and apoptotic features in HD.


A
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IGF1R
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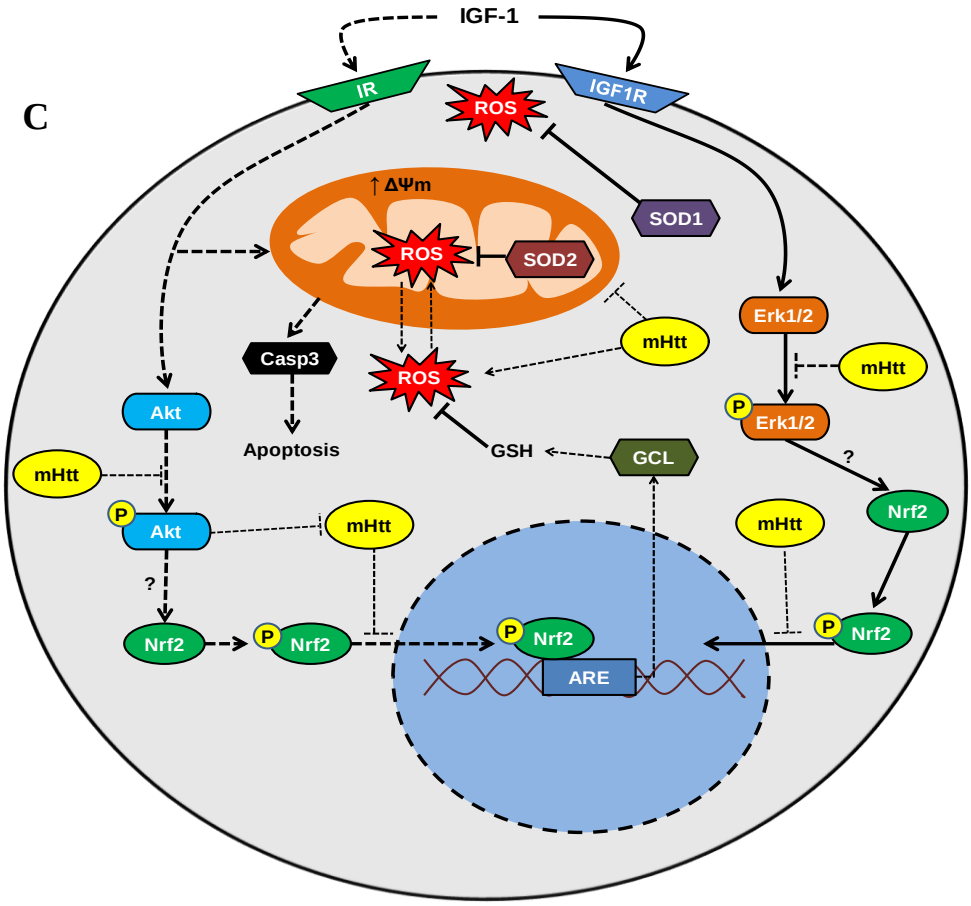

Figure 4.3 – Insulin and IGF-1 induces protection against mitochondrial-driven oxidative stress in HD knock-in striatal cells. (A) Under basal conditions, mutant huntingtin (mHtt) induces decreased phosphorylation and activation of protein kinase B (Akt) and extracellular-signal-regulated kinase 1/2 (Erk1/2), which can be both activated via stimulation of insulin receptor (IR) and/or insulin-like growth factor 1 (IGF-1) receptor (IGF1R). Also, Nrf2 phosphorylated levels, which can be dependent on Akt or Erk1/2 activities, are decreased in the nucleus of HD striatal cells, which correlated with decreased Nrf2-antioxidant response element (ARE) activity and decreased expression of the Nrf2-ARE target genes glutamate-cysteine ligase (GCL) (involved in the synthesis of the reduced form of glutathione (GSH)) and superoxide dismutase (SOD) 2, but not SOD1. Despite decreased Nrf2 activation and GCL and SOD2 expression, GSH levels and SOD1 and 2 activities are increased, but this is not sufficient to decrease mitochondrial reactive oxygen species (ROS) formation, to ameliorate the changes in mitochondrial membrane potential (ΔΨm), or to reduce caspase-3 (Casp3) activation upon expression of mHtt. (B) Insulin treatment induces Akt activation, increases nuclear phosphorylated levels of Nrf2 and total levels of GCL, reduces mitochondrial ROS formation, increases mitochondrial membrane potential (ΔΨm), and reduces caspase-3 activity, without changing GSH or GSSG levels and SOD2 activity in HD striatal cells. (C) IGF-1 treatment induces Erk1/2 activation, increases nuclear phosphorylated levels of Nrf2 and total levels of SOD1, reduces mitochondrial ROS formation, without changing GSH and GSSG levels, or SOD2 and caspase-3 activities.

In summary, in this work we demonstrate that striatal cells expressing mHtt exhibit enhanced ROS formation mainly *via* mitochondria, altered activities and levels of antioxidant defense systems, and decreased antioxidant response to exogenous stressors which may be correlated with impaired Nrf2 activation. Despite mHtt-induced decrease in Nrf2/ARE activation, HO-1, NQO1, and G6PD mRNA, and SOD1 protein levels were not changed; conversely, GCLc and Mrp1 mRNA, and also GCL and SOD2 protein levels, which expression is also regulated by Nrf2, were decreased following expression of mHtt. Therefore, it would be interesting to test the specificity of Nrf2 for these genes in the striatal cells and in the context of mHtt expression and evaluate whether other transcription factors may be involved in regulating the expression of these proteins in HD cells or tissues derived from HD transgenic mice (e.g. YAC128 or R6/2 mice). On the other hand, SOD2 activity was increased despite decreased protein levels, as revealed by a decrease in SOD2 acetylation at Lys68 (K68), which is a target residue for the mitochondrial NAD-dependent deacetylase SIRT3 (e.g. Chen et al., 2011; Qiu et al., 2010). Indeed, SIRT3 protein levels were reduced in ST*Hdh*$^{Q111/Q111}$ cells (Fu et al., 2012), which may contribute to decrease SOD2 acetylation and thus increase SOD2 activity. Moreover, G6PD and SOD1 activities were increased in mutant cells; however, no changes were observed in mRNA or protein levels, respectively. It is known that Src tyrosine kinase phosphorylates G6PD, enhancing its activity (Pan et al., 2009). Therefore, analysis of Src levels and activity (by following its phosphorylation) would be relevant upon expression of mHtt, and measurement of G6PD phosphorylated levels and activity after both silencing or overexpressing Src would reveal how G6PD activity might be regulated in HD cells. Relatively to SOD1, it was previously described that SOD1 can be phosphorylated at Thr-2 and -58, and at Ser-59 (by unknown kinase(s)); however it was not shown how phosphorylation may affect SOD1 activity. SOD1 is also glutathionylated at Cys111, which promotes SOD1 dimer dissociation, resulting in its decreased activity (Wilcox et al., 2009). Thus, it would be interesting to measure SOD1 glutathionylated and phosphorylated levels upon expression of mHtt. Moreover, we observed reduced activities of GCL (and also GS) and Mrp1, which are involved in the control of the glutathione system, oppositely contributing for a decrease in GSH synthesis or an increase in intracellular accumulation of GSH, respectively, in mutant striatal cells. Indeed, increased GSH levels seemed to boost both γ-GT and GST activities, although cellular detoxification associated with the formation of GSH conjugates with toxic

products might be compromised due to decreased Mrp1, potentially accumulating toxins intracellularly. Another feature that involves Mrp1 is the decrease in extracellular GSH levels, which might result in lower extracellular antioxidant capacity to decrease damage at the external part of cell membrane and also decrease the uptake of L-cysteine (which is essential for GSH biosynthesis) by neighboring cells. Therefore, and in order to determine whether mHtt affects cysteine uptake in striatal cells, it would be relevant to measure extracellular L-cysteine levels and uptake, and also the activities and levels of the excitatory amino acid carrier 1 (EAAC1) (mediates the cysteine uptake in cells) and cystine/glutamate antiporter (Xc-) (mediates the oxidized form of cysteine (cystine) uptake in cells), which can be both regulated by Nrf2. As a result, the overall cell defense appears to be largely compromised by mHtt expression. In fact, exposure to stress stimuli, such as H_2O_2 and STS, interfered with an adequate response of the antioxidant systems, potentiating ROS formation and cell death in striatal cells expressing mHtt.

We also observed that cell survival signaling pathways, namely Akt and Erk, were decreased by mHtt, which might contribute for a decrease in Nrf2 activation and consequent decreased transcription of target genes. However, the mechanism that leads to Akt and Erk inactivation still needs to be investigated. In this respect, IR and IGF1R expression levels and activities, as well as PI-3K, and PDPK1 activities could be affected in mutant striatal cells. Moreover, both Akt and Erk were described to be targets for oxidative or nitrosative modifications, leading to protein kinase inactivation (Durgadoss et al., 2012; Feng et al., 2013); thus, we might verify if mHtt expression induces oxidative changes in both proteins. In the present study we showed that insulin and IGF-1 induced the activation of Akt and Erk, respectively, and increased nuclear phosphorylated Nrf2; however, this was not accompanied by increased activity of Nrf2/ARE, nor by a consequent rise in mRNA levels of target genes. Nevertheless, insulin and IGF-1 protected against ROS formation, mitochondrial dysfunction and cell death. Indeed, inhibition of PI-3K/Akt pathway blocked insulin-induced protection against mitochondrial depolarization; nonetheless the specific mechanism(s) involved in such protection still need to be investigated. Therefore, it would be interesting to analyse the levels of total and phosphorylated Akt induced by insulin in isolated mitochondrial fractions. Recently, Akt was described to phosphorylate subunits of ATP synthase, increasing its activity (Li et al., 2013), thus analysis of phosphorylated levels of Akt-target subunits of ATP synthase, in the presence or absence of insulin and also in the presence of a specific Akt inhibitor would help to complement this study. Moreover, it

was previously reported that Akt phosphorylates mHtt on Ser421, decreasing its toxicity (Humbert et al., 2002), and that mHtt interacts with mitochondria, leading to the formation of mitochondrial mHtt aggregates, increasing mitochondrial fission, MPT pore opening and contributing for the occurrence of intrinsic apoptotic cell death pathway, and impaired vesicular and mitochondrial fast axonal trafficking (see Chapter 1.2.3.3). Therefore, determining whether phosphorylation of mHtt Ser421 could affect its direct interaction with mitochondria and consequent mitochondrial destabilization and function, by mutating Ser421 to Asp (mimicking the phosphorylated residue) or Ala (to mimick the non-phosphorylated form) on mHtt would rather complement our study.

Additionally, we observed protection of striatal cells expressing mHtt following treatment with GSHee, creatine or cystamine. GSHee and also insulin treatment were able to revert the effects of mHtt expression *per se* on ROS formation and caspase-3 activity, whereas creatine and cystamine offered protection against ROS formation when the cells were exposed to stress stimuli only. IGF-1 treatment also offered protection against ROS formation, which may be due to its capacity to increase Erk1/2 activation and SOD2 protein levels. However, in previous studies higher plasma IGF-1 levels were shown to correlate with cognitive decline in HD patients (Saleh et al., 2010). In another study plasma IGF-1 levels were shown to be reduced in HD patients compared with controls (Mochel et al., 2007), and IGF-1 mRNA levels were reduced in the striatum an in skin fibroblasts from HD patients (Pouladi et al., 2010); moreover, plasma IGF-1 levels correlated with decreased body weight in R6/2 HD mice (Pouladi et al., 2010). Importantly, we previously showed that IGF-1 rescued peripheral metabolic abnormalities linked to diabetes in R6/2 mice (Duarte et al., 2011), and recently, we showed that intranasal administration of recombinant human IGF-1 (rhIGF-1) improved motor activity and both peripheral and central metabolic abnormalities in YAC128 HD mice (Lopes et al., 2013). Therefore, treatment with IGF-1 at low doses may be highly relevant and protective to the striatum.

Considering that the model used in our work represents initial stages of HD, our results indicate that oxidative stress might be an early feature of the disease. Indeed, this may constitute an important biomarker of disease progression that can be tested by using YAC128 HD transgenic mice at different ages, namely at presymptomatic *versus* symptomatic stages, and follow striatal specific alteration of proteins investigated in this study.

In conclusion, our results defined new targets and strategies for investigation of mHtt-induced striatal neurodegeneration demonstrating that insulin or IGF-1 treatment might be benefic against striatal cytotoxicity induced by mHtt. Therapeutics targets include the overexpression of Mrp-1 to regulate glutathione levels, the selective activation of Nrf2 to protect against mHtt-induced oxidative damage and the stimulation of insulin/IGF-1 intracellular signaling pathways, which may ameliorate mitochondrial function and decrease oxidative damage in HD. Taking into account present and previous studies, it would be relevant to conduct further studies that might support evaluating the tolerability and efficacy of intranasal insulin or IGF-1 treatments in HD patients. While intravenous application of insulin is a highly invasive technique that leads to hypoglycemia, which itself has detrimental effects on brain function (Freiherr et al., 2013), clinical trials have demonstrated that intranasal insulin improves both memory performance and metabolic integrity of the brain in patients suffering from Alzheimer's disease or its prodrome, mild cognitive impairment. Additionally, creatine and cystamine have been tested in HD clinical trials and may be used as preventive treatments (clinicaltrials.gov; http://www.raptorpharma.com) by precluding oxidative stress.

Chapter 5 – References

Acevedo-Torres, K., Berrios, L., Rosario, N., Dufault, V., Skatchkov, S., Eaton, M. J., Torres-Ramos, C. A. and Ayala-Torres, S. (2009). Mitochondrial DNA damage is a hallmark of chemically induced and the R6/2 transgenic model of Huntington's disease. *DNA repair* **8**(1), 126-36, 10.1016/j.dnarep.2008.09.004.

Aidt, F. H., Nielsen, S. M., Kanters, J., Pesta, D., Nielsen, T. T., Norremolle, A., Hasholt, L., Christiansen, M. and Hagen, C. M. (2013). Dysfunctional mitochondrial respiration in the striatum of the Huntington's disease transgenic R6/2 mouse model. *PLoS currents* **5**, 10.1371/currents.hd.d8917b4862929772c5a2f2a34ef1c201.

Alam, J. and Cook, J. L. (2003). Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. *Current pharmaceutical design* **9**(30), 2499-511.

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M. and Cook, J. L. (1999). Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *The Journal of biological chemistry* **274**(37), 26071-8.

Albin, R. L. (1995). Selective neurodegeneration in Huntington's disease. *Annals of neurology* **38**(6), 835-6, 10.1002/ana.410380602.

Albin, R. L., Reiner, A., Anderson, K. D., Dure, L. S. t., Handelin, B., Balfour, R., Whetsell, W. O., Jr., Penney, J. B. and Young, A. B. (1992). Preferential loss of striato-external pallidal projection neurons in presymptomatic Huntington's disease. *Annals of neurology* **31**(4), 425-30, 10.1002/ana.410310412.

Albin, R. L., Reiner, A., Anderson, K. D., Penney, J. B. and Young, A. B. (1990). Striatal and nigral neuron subpopulations in rigid Huntington's disease: implications for the functional anatomy of chorea and rigidity-akinesia. *Annals of neurology* **27**(4), 357-65, 10.1002/ana.410270403.

Alcauter-Solorzano, S., Pasaye-Alcaraz, E. H., Alvarado-Alanis, P., Fermin-Delgado, R. O., Alonso-Vilatela, M. E., Salgado-Lujambio, P. and Barrios, F. A. (2010). [Hydrogen magnetic resonance quantitative spectroscopy at 3 T in symptomatic and asymptomatic Huntington's disease patients]. *Revista de neurologia* **51**(4), 208-12. First published on Espectroscopia cuantitativa de hidrogeno por resonancia magnetica de 3 T en pacientes sintomaticos y asintomaticoscon enfermedad de Huntington.

Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal* **15**(23), 6541-51.

Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D., Ashworth, A. and Bownes, M. (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Current biology : CB* **7**(10), 776-89.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current biology : CB* **7**(4), 261-9.

Andrade, M. A. and Bork, P. (1995). HEAT repeats in the Huntington's disease protein. *Nature genetics* **11**(2), 115-6, 10.1038/ng1095-115.

Andreassen, O. A., Dedeoglu, A., Ferrante, R. J., Jenkins, B. G., Ferrante, K. L., Thomas, M., Friedlich, A., Browne, S. E., Schilling, G., Borchelt, D. R., Hersch, S. M., Ross, C. A. and Beal, M. F. (2001). Creatine increase survival and delays motor symptoms in a transgenic animal model of Huntington's disease. *Neurobiology of disease* **8**(3), 479-91.

Andreassen, O. A., Dedeoglu, A., Stanojevic, V., Hughes, D. B., Browne, S. E., Leech, C. A., Ferrante, R. J., Habener, J. F., Beal, M. F. and Thomas, M. K. (2002). Huntington's disease of the endocrine pancreas: insulin deficiency and diabetes mellitus due to impaired insulin gene expression. *Neurobiology of disease* **11**(3), 410-24.

Andreassen, O. A., Ferrante, R. J., Dedeoglu, A., Albers, D. W., Klivenyi, P., Carlson, E. J., Epstein, C. J. and Beal, M. F. (2001). Mice with a partial deficiency of manganese superoxide dismutase show increased vulnerability to the mitochondrial toxins malonate, 3-nitropropionic acid, and MPTP. *Experimental neurology* **167**(1), 189-95, 10.1006/exnr.2000.7525.

Andreassen, O. A., Ferrante, R. J., Dedeoglu, A. and Beal, M. F. (2001). Lipoic acid improves survival in transgenic mouse models of Huntington's disease. *Neuroreport* **12**(15), 3371-3.

Andrews, T. C. and Brooks, D. J. (1998). Advances in the understanding of early Huntington's disease using the functional imaging techniques of PET and SPET. *Molecular medicine today* **4**(12), 532-9.

Antonawich, F. J., Fiore-Marasa, S. M. and Parker, C. P. (2002). Modulation of apoptotic regulatory proteins and early activation of cytochrome C following systemic 3-nitropropionic acid administration. *Brain research bulletin* **57**(5), 647-9.

Antonini, A., Leenders, K. L., Spiegel, R., Meier, D., Vontobel, P., Weigell-Weber, M., Sanchez-Pernaute, R., de Yebenez, J. G., Boesiger, P., Weindl, A. and Maguire, R. P. (1996). Striatal glucose metabolism and dopamine D2 receptor binding in asymptomatic gene carriers and patients with Huntington's disease. *Brain : a journal of neurology* **119 (Pt 6)**, 2085-95.

Apopa, P. L., He, X. and Ma, Q. (2008). Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. *Journal of biochemical and molecular toxicology* **22**(1), 63-76, 10.1002/jbt.20212.

Apostol, B. L., Illes, K., Pallos, J., Bodai, L., Wu, J., Strand, A., Schweitzer, E. S., Olson, J. M., Kazantsev, A., Marsh, J. L. and Thompson, L. M. (2006). Mutant huntingtin alters MAPK signaling pathways in PC12 and striatal cells: ERK1/2 protects against mutant huntingtin-associated toxicity. *Human molecular genetics* **15**(2), 273-85, 10.1093/hmg/ddi443.

Appelt, D. M., Kopen, G. C., Boyne, L. J. and Balin, B. J. (1996). Localization of transglutaminase in hippocampal neurons: implications for Alzheimer's disease. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **44**(12), 1421-7.

Arenas, J., Campos, Y., Ribacoba, R., Martin, M. A., Rubio, J. C., Ablanedo, P. and Cabello, A. (1998). Complex I defect in muscle from patients with Huntington's disease. *Annals of neurology* **43**(3), 397-400, 10.1002/ana.410430321.

Arrasate, M., Mitra, S., Schweitzer, E. S., Segal, M. R. and Finkbeiner, S. (2004). Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature* **431**(7010), 805-10, 10.1038/nature02998.

Arrowsmith, C. H., Bountra, C., Fish, P. V., Lee, K. and Schapira, M. (2012). Epigenetic protein families: a new frontier for drug discovery. *Nature reviews. Drug discovery* **11**(5), 384-400, 10.1038/nrd3674.

Atwal, R. S., Desmond, C. R., Caron, N., Maiuri, T., Xia, J., Sipione, S. and Truant, R. (2011). Kinase inhibitors modulate huntingtin cell localization and toxicity. *Nature chemical biology* **7**(7), 453-60, 10.1038/nchembio.582.

Aylward, E. H. (2007). Change in MRI striatal volumes as a biomarker in preclinical Huntington's disease. *Brain research bulletin* **72**(2-3), 152-8, 10.1016/j.brainresbull.2006.10.028.

Aylward, E. H., Codori, A. M., Rosenblatt, A., Sherr, M., Brandt, J., Stine, O. C., Barta, P. E., Pearlson, G. D. and Ross, C. A. (2000). Rate of caudate atrophy in presymptomatic and symptomatic stages of Huntington's disease. *Movement disorders : official journal of the Movement Disorder Society* **15**(3), 552-60.

Aziz, N. A., van der Burg, J. M., Landwehrmeyer, G. B., Brundin, P., Stijnen, T., Group, E. S. and Roos, R. A. (2008). Weight loss in Huntington disease increases with higher CAG repeat number. *Neurology* **71**(19), 1506-13, 10.1212/01.wnl.0000334276.09729.0e.

Bae, B. I., Hara, M. R., Cascio, M. B., Wellington, C. L., Hayden, M. R., Ross, C. A., Ha, H. C., Li, X. J., Snyder, S. H. and Sawa, A. (2006). Mutant huntingtin: nuclear translocation and cytotoxicity mediated by GAPDH. *Proceedings of the National Academy of Sciences of the United States of America* **103**(9), 3405-9, 10.1073/pnas.0511316103.

Bae, B. I., Xu, H., Igarashi, S., Fujimuro, M., Agrawal, N., Taya, Y., Hayward, S. D., Moran, T. H., Montell, C., Ross, C. A., Snyder, S. H. and Sawa, A. (2005). p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. *Neuron* **47**(1), 29-41, 10.1016/j.neuron.2005.06.005.

Bae, S. H., Sung, S. H., Oh, S. Y., Lim, J. M., Lee, S. K., Park, Y. N., Lee, H. E., Kang, D. and Rhee, S. G. (2013). Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. *Cell metabolism* **17**(1), 73-84, 10.1016/j.cmet.2012.12.002.

Bae, S. H., Woo, H. A., Sung, S. H., Lee, H. E., Lee, S. K., Kil, I. S. and Rhee, S. G. (2009). Induction of sulfiredoxin via an Nrf2-dependent pathway and hyperoxidation of peroxiredoxin III in the lungs of mice exposed to hyperoxia. *Antioxidants & redox signaling* **11**(5), 937-48, 10.1089/ARS.2008.2325.

Bailey, C. D. and Johnson, G. V. (2004). Developmental regulation of tissue transglutaminase in the mouse forebrain. *Journal of neurochemistry* **91**(6), 1369-79, 10.1111/j.1471-4159.2004.02825.x.

Bailey, C. D. and Johnson, G. V. (2005). Tissue transglutaminase contributes to disease progression in the R6/2 Huntington's disease mouse model via aggregate-independent mechanisms. *Journal of neurochemistry* **92**(1), 83-92, 10.1111/j.1471-4159.2004.02839.x.

Bailey, C. D. and Johnson, G. V. (2006). The protective effects of cystamine in the R6/2 Huntington's disease mouse involve mechanisms other than the inhibition of tissue transglutaminase. *Neurobiology of aging* **27**(6), 871-9, 10.1016/j.neurobiolaging.2005.04.001.

Balu, M., Sangeetha, P., Haripriya, D. and Panneerselvam, C. (2005). Rejuvenation of antioxidant system in central nervous system of aged rats by grape seed extract. *Neuroscience letters* **383**(3), 295-300, 10.1016/j.neulet.2005.04.042.

Banks, W. A. (2004). The source of cerebral insulin. *European journal of pharmacology* **490**(1-3), 5-12, 10.1016/j.ejphar.2004.02.040.

Banning, A., Deubel, S., Kluth, D., Zhou, Z. and Brigelius-Flohe, R. (2005). The GI-GPx gene is a target for Nrf2. *Molecular and cellular biology* **25**(12), 4914-23, 10.1128/MCB.25.12.4914-4923.2005.

Banoei, M. M., Houshmand, M., Panahi, M. S., Shariati, P., Rostami, M., Manshadi, M. D. and Majidizadeh, T. (2007). Huntington's disease and mitochondrial DNA deletions: event or regular mechanism for mutant huntingtin protein and CAG repeats expansion?! *Cellular and molecular neurobiology* **27**(7), 867-75, 10.1007/s10571-007-9206-5.

Barksdale, K. A. and Bijur, G. N. (2009). The basal flux of Akt in the mitochondria is mediated by heat shock protein 90. *Journal of neurochemistry* **108**(5), 1289-99, 10.1111/j.1471-4159.2009.05878.x.

Basso, M., Berlin, J., Xia, L., Sleiman, S. F., Ko, B., Haskew-Layton, R., Kim, E., Antonyak, M. A., Cerione, R. A., Iismaa, S. E., Willis, D., Cho, S. and Ratan, R. R. (2012). Transglutaminase inhibition protects against oxidative stress-induced neuronal death downstream of pathological ERK activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**(19), 6561-9, 10.1523/JNEUROSCI.3353-11.2012.

Bayram-Weston, Z., Jones, L., Dunnett, S. B. and Brooks, S. P. (2012). Light and electron microscopic characterization of the evolution of cellular pathology in the R6/1 Huntington's disease transgenic mice. *Brain research bulletin* **88**(2-3), 104-12, 10.1016/j.brainresbull.2011.07.009.

Beal, M. F. (1992). Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Annals of neurology* **31**(2), 119-30, 10.1002/ana.410310202.

Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J. and Martin, J. B. (1986). Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* **321**(6066), 168-71, 10.1038/321168a0.

Beal, M. F., Matson, W. R., Storey, E., Milbury, P., Ryan, E. A., Ogawa, T. and Bird, E. D. (1992). Kynurenic acid concentrations are reduced in Huntington's disease cerebral cortex. *Journal of the neurological sciences* **108**(1), 80-7.

Benarroch, E. E. (2012). Insulin-like growth factors in the brain and their potential clinical implications. *Neurology* **79**(21), 2148-53, 10.1212/WNL.0b013e3182752eef.

Benchoua, A., Trioulier, Y., Zala, D., Gaillard, M. C., Lefort, N., Dufour, N., Saudou, F., Elalouf, J. M., Hirsch, E., Hantraye, P., Deglon, N. and Brouillet, E. (2006). Involvement of mitochondrial complex II defects in neuronal death produced by N-terminus fragment of mutated huntingtin. *Molecular biology of the cell* **17**(4), 1652-63, 10.1091/mbc.E05-07-0607.

Berardelli, A., Noth, J., Thompson, P. D., Bollen, E. L., Curra, A., Deuschl, G., van Dijk, J. G., Topper, R., Schwarz, M. and Roos, R. A. (1999). Pathophysiology of chorea and bradykinesia in Huntington's disease. *Movement disorders : official journal of the Movement Disorder Society* **14**(3), 398-403.

Berg, J. M., Tymoczko, J. L., Stryer, L. (2002). Biochemistry. 5th edition. New York: W H Freeman. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21154/

Berlett, B. S. and Stadtman, E. R. (1997). Protein oxidation in aging, disease, and oxidative stress. *The Journal of biological chemistry* **272**(33), 20313-6.

Bernhardt, R. (1996). Cytochrome P450: structure, function, and generation of reactive oxygen species. *Reviews of physiology, biochemistry and pharmacology* **127**, 137-221.

Bettencourt, C. and Lima, M. (2011). Machado-Joseph Disease: from first descriptions to new perspectives. *Orphanet journal of rare diseases* **6**, 35, 10.1186/1750-1172-6-35.

Bijur, G. N. and Jope, R. S. (2003). Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. *Journal of neurochemistry* **87**(6), 1427-35.

Bindokas, V. P., Jordan, J., Lee, C. C. and Miller, R. J. (1996). Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **16**(4), 1324-36.

Bizat, N., Hermel, J. M., Boyer, F., Jacquard, C., Creminon, C., Ouary, S., Escartin, C., Hantraye, P., Kajewski, S. and Brouillet, E. (2003). Calpain is a major cell death effector in selective striatal degeneration induced in vivo by 3-nitropropionate: implications for Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**(12), 5020-30.

Bjorkqvist, M., Wild, E. J., Thiele, J., Silvestroni, A., Andre, R., Lahiri, N., Raibon, E., Lee, R. V., Benn, C. L., Soulet, D., Magnusson, A., Woodman, B., Landles, C., Pouladi, M. A., Hayden, M. R., Khalili-Shirazi, A., Lowdell, M. W., Brundin, P., Bates, G. P., Leavitt, B. R., Moller, T. and Tabrizi, S. J. (2008). A novel pathogenic pathway of immune activation detectable before clinical

onset in Huntington's disease. *The Journal of experimental medicine* **205**(8), 1869-77, 10.1084/jem.20080178.

Bogdanov, M. B., Andreassen, O. A., Dedeoglu, A., Ferrante, R. J. and Beal, M. F. (2001). Increased oxidative damage to DNA in a transgenic mouse model of Huntington's disease. *Journal of neurochemistry* **79**(6), 1246-9.

Borrell-Pages, M., Canals, J. M., Cordelieres, F. P., Parker, J. A., Pineda, J. R., Grange, G., Bryson, E. A., Guillermier, M., Hirsch, E., Hantraye, P., Cheetham, M. E., Neri, C., Alberch, J., Brouillet, E., Saudou, F. and Humbert, S. (2006). Cystamine and cysteamine increase brain levels of BDNF in Huntington disease via HSJ1b and transglutaminase. *The Journal of clinical investigation* **116**(5), 1410-24, 10.1172/JCI27607.

Borrell-Pages, M., Zala, D., Humbert, S. and Saudou, F. (2006). Huntington's disease: from huntingtin function and dysfunction to therapeutic strategies. *Cellular and molecular life sciences : CMLS* **63**(22), 2642-60, 10.1007/s00018-006-6242-0.

Bousquet, M., Gibrat, C., Ouellet, M., Rouillard, C., Calon, F. and Cicchetti, F. (2010). Cystamine metabolism and brain transport properties: clinical implications for neurodegenerative diseases. *Journal of neurochemistry* **114**(6), 1651-8, 10.1111/j.1471-4159.2010.06874.x.

Brand, M. D. (2010). The sites and topology of mitochondrial superoxide production. *Experimental gerontology* **45**(7-8), 466-72, 10.1016/j.exger.2010.01.003.

Brennan, W. A., Jr., Bird, E. D. and Aprille, J. R. (1985). Regional mitochondrial respiratory activity in Huntington's disease brain. *Journal of neurochemistry* **44**(6), 1948-50.

Brignull, H. R., Morley, J. F., Garcia, S. M. and Morimoto, R. I. (2006). Modeling polyglutamine pathogenesis in C. elegans. *Methods in enzymology* **412**, 256-82, 10.1016/S0076-6879(06)12016-9.

Brouillet, E., Conde, F., Beal, M. F. and Hantraye, P. (1999). Replicating Huntington's disease phenotype in experimental animals. *Progress in neurobiology* **59**(5), 427-68.

Brouillet, E. and Hantraye, P. (1995). Effects of chronic MPTP and 3-nitropropionic acid in nonhuman primates. *Current opinion in neurology* **8**(6), 469-73.

Brouillet, E., Hantraye, P., Ferrante, R. J., Dolan, R., Leroy-Willig, A., Kowall, N. W. and Beal, M. F. (1995). Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proceedings of the National Academy of Sciences of the United States of America* **92**(15), 7105-9.

Brouillet, E., Jacquard, C., Bizat, N. and Blum, D. (2005). 3-Nitropropionic acid: a mitochondrial toxin to uncover physiopathological mechanisms underlying striatal degeneration in Huntington's disease. *Journal of neurochemistry* **95**(6), 1521-40, 10.1111/j.1471-4159.2005.03515.x.

Browne, S. E., Bowling, A. C., MacGarvey, U., Baik, M. J., Berger, S. C., Muqit, M. M., Bird, E. D. and Beal, M. F. (1997). Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Annals of neurology* **41**(5), 646-53, 10.1002/ana.410410514.

Browne, S. E., Ferrante, R. J. and Beal, M. F. (1999). Oxidative stress in Huntington's disease. *Brain pathology* **9**(1), 147-63.

Bunn, L. M., Marsden, J. F., Giunti, P. and Day, B. L. (2013). Stance instability in spinocerebellar ataxia type 6. *Movement disorders : official journal of the Movement Disorder Society* **28**(4), 510-6, 10.1002/mds.25163.

Burke, J. R., Enghild, J. J., Martin, M. E., Jou, Y. S., Myers, R. M., Roses, A. D., Vance, J. M. and Strittmatter, W. J. (1996). Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH. *Nature medicine* **2**(3), 347-50.

Burns, J. M., Donnelly, J. E., Anderson, H. S., Mayo, M. S., Spencer-Gardner, L., Thomas, G., Cronk, B. B., Haddad, Z., Klima, D., Hansen, D. and Brooks, W. M. (2007). Peripheral insulin and brain structure in early Alzheimer disease. *Neurology* **69**(11), 1094-104, 10.1212/01.wnl.0000276952.91704.af.

Butterworth, J., Yates, C. M. and Reynolds, G. P. (1985). Distribution of phosphate-activated glutaminase, succinic dehydrogenase, pyruvate dehydrogenase and gamma-glutamyl transpeptidase in post-mortem brain from Huntington's disease and agonal cases. *Journal of the neurological sciences* **67**(2), 161-71.

Cai, J. and Jones, D. P. (1998). Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. *The Journal of biological chemistry* **273**(19), 11401-4.

Cai, Z. and Yan, L. J. (2013). Protein Oxidative Modifications: Beneficial Roles in Disease and Health. *Journal of biochemical and pharmacological research* **1**(1), 15-26.

Caine, E. D. and Shoulson, I. (1983). Psychiatric syndromes in Huntington's disease. *The American journal of psychiatry* **140**(6), 728-33.

Calkins, M. J., Jakel, R. J., Johnson, D. A., Chan, K., Kan, Y. W. and Johnson, J. A. (2005). Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. *Proceedings of the National Academy of Sciences of the United States of America* **102**(1), 244-9, 10.1073/pnas.0408487101.

Calkins, M. J., Townsend, J. A., Johnson, D. A. and Johnson, J. A. (2010). Cystamine protects from 3-nitropropionic acid lesioning via induction of nf-e2 related factor 2 mediated transcription. *Experimental neurology* **224**(1), 307-17, 10.1016/j.expneurol.2010.04.008.

Camp, N. D., James, R. G., Dawson, D. W., Yan, F., Davison, J. M., Houck, S. A., Tang, X., Zheng, N., Major, M. B. and Moon, R. T. (2012). Wilms tumor gene on X chromosome (WTX) inhibits degradation of NRF2 protein through competitive binding to KEAP1 protein. *The Journal of biological chemistry* **287**(9), 6539-50, 10.1074/jbc.M111.316471.

Cardona-Gomez, G. P., Mendez, P., DonCarlos, L. L., Azcoitia, I. and Garcia-Segura, L. M. (2001). Interactions of estrogens and insulin-like growth factor-I in the brain: implications for neuroprotection. *Brain research. Brain research reviews* **37**(1-3), 320-34.

Cattaneo, E., Zuccato, C. and Tartari, M. (2005). Normal huntingtin function: an alternative approach to Huntington's disease. *Nature reviews. Neuroscience* **6**(12), 919-30, 10.1038/nrn1806.

Chanas, S. A., Jiang, Q., McMahon, M., McWalter, G. K., McLellan, L. I., Elcombe, C. R., Henderson, C. J., Wolf, C. R., Moffat, G. J., Itoh, K., Yamamoto, M. and Hayes, J. D. (2002). Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *The Biochemical journal* **365**(Pt 2), 405-16, 10.1042/BJ20020320.

Chang, D. T., Rintoul, G. L., Pandipati, S. and Reynolds, I. J. (2006). Mutant huntingtin aggregates impair mitochondrial movement and trafficking in cortical neurons. *Neurobiology of disease* **22**(2), 388-400, 10.1016/j.nbd.2005.12.007.

Chaturvedi, R. K., Adhihetty, P., Shukla, S., Hennessy, T., Calingasan, N., Yang, L., Starkov, A., Kiaei, M., Cannella, M., Sassone, J., Ciammola, A., Squitieri, F. and Beal, M. F. (2009). Impaired PGC-1alpha function in muscle in Huntington's disease. *Human molecular genetics* **18**(16), 3048-65, 10.1093/hmg/ddp243.

Chaturvedi, R. K., Calingasan, N. Y., Yang, L., Hennessey, T., Johri, A. and Beal, M. F. (2010). Impairment of PGC-1alpha expression, neuropathology and hepatic steatosis in a transgenic mouse model of Huntington's disease following chronic energy deprivation. *Human molecular genetics* **19**(16), 3190-205, 10.1093/hmg/ddq229.

Chen, C. M., Wu, Y. R., Cheng, M. L., Liu, J. L., Lee, Y. M., Lee, P. W., Soong, B. W. and Chiu, D. T. (2007). Increased oxidative damage and mitochondrial abnormalities in the peripheral blood of Huntington's disease patients. *Biochemical and biophysical research communications* **359**(2), 335-40, 10.1016/j.bbrc.2007.05.093.

Chen, H. and Chan, D. C. (2005). Emerging functions of mammalian mitochondrial fusion and fission. *Human molecular genetics* **14 Spec No. 2**, R283-9, 10.1093/hmg/ddi270.

Chen, H. and Chan, D. C. (2010). Physiological functions of mitochondrial fusion. *Annals of the New York Academy of Sciences* **1201**, 21-5, 10.1111/j.1749-6632.2010.05615.x.

Chen, W., Sun, Z., Wang, X. J., Jiang, T., Huang, Z., Fang, D. and Zhang, D. D. (2009). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. *Molecular cell* **34**(6), 663-73, 10.1016/j.molcel.2009.04.029.

Chen, Y., Zhang, J., Lin, Y., Lei, Q., Guan, K. L., Zhao, S. and Xiong, Y. (2011). Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO reports* **12**(6), 534-41, 10.1038/embor.2011.65.

Chen, Z. H., Saito, Y., Yoshida, Y., Sekine, A., Noguchi, N. and Niki, E. (2005). 4-Hydroxynonenal induces adaptive response and enhances PC12 cell tolerance primarily through induction of thioredoxin reductase 1 via activation of Nrf2. *The Journal of biological chemistry* **280**(51), 41921-7, 10.1074/jbc.M508556200.

Chen-Plotkin, A. S., Sadri-Vakili, G., Yohrling, G. J., Braveman, M. W., Benn, C. L., Glajch, K. E., DiRocco, D. P., Farrell, L. A., Krainc, D., Gines, S., MacDonald, M. E. and Cha, J. H. (2006). Decreased association of the transcription factor Sp1 with genes downregulated in Huntington's disease. *Neurobiology of disease* **22**(2), 233-41, 10.1016/j.nbd.2005.11.001.

Chiu, C. T., Liu, G., Leeds, P. and Chuang, D. M. (2011). Combined treatment with the mood stabilizers lithium and valproate produces multiple beneficial effects in transgenic mouse models of Huntington's disease. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* **36**(12), 2406-21, 10.1038/npp.2011.128.

Cho, H. Y., Jedlicka, A. E., Reddy, S. P., Kensler, T. W., Yamamoto, M., Zhang, L. Y. and Kleeberger, S. R. (2002). Role of NRF2 in protection against hyperoxic lung injury in mice. *American journal of respiratory cell and molecular biology* **26**(2), 175-82, 10.1165/ajrcmb.26.2.4501.

Choi, Y. J., Kim, S. I., Lee, J. W., Kwon, Y. S., Lee, H. J., Kim, S. S. and Chun, W. (2012). Suppression of aggregate formation of mutant huntingtin potentiates CREB-binding protein sequestration and apoptotic cell death. *Molecular and cellular neurosciences* **49**(2), 127-37, 10.1016/j.mcn.2011.11.003.

Chong, Z. Z., Li, F. and Maiese, K. (2005). Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury. *Histology and histopathology* **20**(1), 299-315.

Chong, Z. Z., Shang, Y. C., Wang, S. and Maiese, K. (2012). A Critical Kinase Cascade in Neurological Disorders: PI 3-K, Akt, and mTOR. *Future neurology* **7**(6), 733-748.

Choo, Y. S., Johnson, G. V., MacDonald, M., Detloff, P. J. and Lesort, M. (2004). Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. *Human molecular genetics* **13**(14), 1407-20, 10.1093/hmg/ddh162.

Choo, Y. S., Mao, Z., Johnson, G. V. and Lesort, M. (2005). Increased glutathione levels in cortical and striatal mitochondria of the R6/2 Huntington's disease mouse model. *Neuroscience letters* **386**(1), 63-8, 10.1016/j.neulet.2005.05.065.

Chowdhry, S., Zhang, Y., McMahon, M., Sutherland, C., Cuadrado, A. and Hayes, J. D. (2013). Nrf2 is controlled by two distinct beta-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* **32**(32), 3765-81, 10.1038/onc.2012.388.

Chowdhury, I., Mo, Y., Gao, L., Kazi, A., Fisher, A. B. and Feinstein, S. I. (2009). Oxidant stress stimulates expression of the human peroxiredoxin 6 gene by a transcriptional mechanism involving an antioxidant response element. *Free radical biology & medicine* **46**(2), 146-53, 10.1016/j.freeradbiomed.2008.09.027.

Ciarmiello, A., Cannella, M., Lastoria, S., Simonelli, M., Frati, L., Rubinsztein, D. C. and Squitieri, F. (2006). Brain white-matter volume loss and glucose hypometabolism precede the clinical symptoms of Huntington's disease. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **47**(2), 215-22.

Cisbani, G. and Cicchetti, F. (2012). An in vitro perspective on the molecular mechanisms underlying mutant huntingtin protein toxicity. *Cell death & disease* **3**, e382, 10.1038/cddis.2012.121.

Clarke, C. E., Lowry, M. and Quarrell, O. W. (1998). No change in striatal glutamate in Huntington's disease measured by proton magnetic resonance spectroscopy. *Parkinsonism & related disorders* **4**(3), 123-7.

Cohen-Armon, M. (2007). PARP-1 activation in the ERK signaling pathway. *Trends in pharmacological sciences* **28**(11), 556-60, 10.1016/j.tips.2007.08.005.

Colin, E., Regulier, E., Perrin, V., Durr, A., Brice, A., Aebischer, P., Deglon, N., Humbert, S. and Saudou, F. (2005). Akt is altered in an animal model of Huntington's disease and in patients. *The European journal of neuroscience* **21**(6), 1478-88, 10.1111/j.1460-9568.2005.03985.x.

Colle, D., Hartwig, J. M., Soares, F. A. and Farina, M. (2012). Probucol modulates oxidative stress and excitotoxicity in Huntington's disease models in vitro. *Brain research bulletin* **87**(4-5), 397-405, 10.1016/j.brainresbull.2012.01.003.

Colomer Gould, V. F. (2012). Mouse models of spinocerebellar ataxia type 3 (Machado-Joseph disease). *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* **9**(2), 285-96, 10.1007/s13311-012-0117-x.

Comporti, M., Signorini, C., Arezzini, B., Vecchio, D., Monaco, B. and Gardi, C. (2008). F2-isoprostanes are not just markers of oxidative stress. *Free radical biology & medicine* **44**(3), 247-56, 10.1016/j.freeradbiomed.2007.10.004.

Cong, S. Y., Pepers, B. A., Evert, B. O., Rubinsztein, D. C., Roos, R. A., van Ommen, G. J. and Dorsman, J. C. (2005). Mutant huntingtin represses CBP, but not p300, by binding and protein degradation. *Molecular and cellular neurosciences* **30**(4), 560-71.

Cooper, A. J., Sheu, K. F., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D. and Blass, J. P. (1997). Polyglutamine domains are substrates of tissue transglutaminase: does transglutaminase play a role in expanded CAG/poly-Q neurodegenerative diseases? *Journal of neurochemistry* **69**(1), 431-4.

Cooper, A. J., Sheu, K. F., Burke, J. R., Strittmatter, W. J. and Blass, J. P. (1998). Glyceraldehyde 3-phosphate dehydrogenase abnormality in metabolically stressed Huntington disease fibroblasts. *Developmental neuroscience* **20**(4-5), 462-8.

Cooper, A. J., Sheu, K. R., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D. and Blass, J. P. (1997). Transglutaminase-catalyzed inactivation of glyceraldehyde 3-phosphate dehydrogenase

and alpha-ketoglutarate dehydrogenase complex by polyglutamine domains of pathological length. *Proceedings of the National Academy of Sciences of the United States of America* **94**(23), 12604-9.

Copple, I. M., Lister, A., Obeng, A. D., Kitteringham, N. R., Jenkins, R. E., Layfield, R., Foster, B. J., Goldring, C. E. and Park, B. K. (2010). Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. *The Journal of biological chemistry* **285**(22), 16782-8, 10.1074/jbc.M109.096545.

Cornett, J., Cao, F., Wang, C. E., Ross, C. A., Bates, G. P., Li, S. H. and Li, X. J. (2005). Polyglutamine expansion of huntingtin impairs its nuclear export. *Nature genetics* **37**(2), 198-204, 10.1038/ng1503.

Costa Mdo, C. and Paulson, H. L. (2012). Toward understanding Machado-Joseph disease. *Progress in neurobiology* **97**(2), 239-57, 10.1016/j.pneurobio.2011.11.006.

Costa, V., Giacomello, M., Hudec, R., Lopreiato, R., Ermak, G., Lim, D., Malorni, W., Davies, K. J., Carafoli, E. and Scorrano, L. (2010). Mitochondrial fission and cristae disruption increase the response of cell models of Huntington's disease to apoptotic stimuli. *EMBO molecular medicine* **2**(12), 490-503, 10.1002/emmm.201000102.

Cowan, C. M. and Raymond, L. A. (2006). Selective neuronal degeneration in Huntington's disease. *Current topics in developmental biology* **75**, 25-71, 10.1016/S0070-2153(06)75002-5.

Craig, K., Keers, S. M., Archibald, K., Curtis, A. and Chinnery, P. F. (2004). Molecular epidemiology of spinocerebellar ataxia type 6. *Annals of neurology* **55**(5), 752-5, 10.1002/ana.20110.

Crocker, S. F., Costain, W. J. and Robertson, H. A. (2006). DNA microarray analysis of striatal gene expression in symptomatic transgenic Huntington's mice (R6/2) reveals neuroinflammation and insulin associations. *Brain research* **1088**(1), 176-86, 10.1016/j.brainres.2006.02.102.

Cruz-Aguado, R., Turner, L. F., Diaz, C. M. and Pinero, J. (2000). Nerve growth factor and striatal glutathione metabolism in a rat model of Huntington's disease. *Restorative neurology and neuroscience* **17**(4), 217-221.

Cui, L., Jeong, H., Borovecki, F., Parkhurst, C. N., Tanese, N. and Krainc, D. (2006). Transcriptional repression of PGC-1alpha by mutant huntingtin leads to mitochondrial dysfunction and neurodegeneration. *Cell* **127**(1), 59-69, 10.1016/j.cell.2006.09.015.

Cullinan, S. B., Zhang, D., Hannink, M., Arvisais, E., Kaufman, R. J. and Diehl, J. A. (2003). Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Molecular and cellular biology* **23**(20), 7198-209.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D. and Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clinical chemistry* **52**(4), 601-23, 10.1373/clinchem.2005.061408.

Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., Colombo, R., Rossi, R. and Milzani, A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass spectrometry reviews* **24**(1), 55-99, 10.1002/mas.20006.

Dautry, C., Conde, F., Brouillet, E., Mittoux, V., Beal, M. F., Bloch, G. and Hantraye, P. (1999). Serial 1H-NMR spectroscopy study of metabolic impairment in primates chronically treated with the succinate dehydrogenase inhibitor 3-nitropropionic acid. *Neurobiology of disease* **6**(4), 259-68, 10.1006/nbdi.1999.0244.

Davies, S. W., Turmaine, M., Cozens, B. A., DiFiglia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanker, E. E., Mangiarini, L. and Bates, G. P. (1997). Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell* **90**(3), 537-48.

Dawbarn, D., De Quidt, M. E. and Emson, P. C. (1985). Survival of basal ganglia neuropeptide Y-somatostatin neurones in Huntington's disease. *Brain research* **340**(2), 251-60.

de la Monte, S., Derdak, Z. and Wands, J. R. (2012). Alcohol, insulin resistance and the liver-brain axis. *Journal of gastroenterology and hepatology* **27 Suppl 2**, 33-41, 10.1111/j.1440-1746.2011.07023.x.

Dedeoglu, A., Kubilus, J. K., Jeitner, T. M., Matson, S. A., Bogdanov, M., Kowall, N. W., Matson, W. R., Cooper, A. J., Ratan, R. R., Beal, M. F., Hersch, S. M. and Ferrante, R. J. (2002). Therapeutic effects of cystamine in a murine model of Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**(20), 8942-50.

Dedeoglu, A., Kubilus, J. K., Yang, L., Ferrante, K. L., Hersch, S. M., Beal, M. F. and Ferrante, R. J. (2003). Creatine therapy provides neuroprotection after onset of clinical symptoms in Huntington's disease transgenic mice. *Journal of neurochemistry* **85**(6), 1359-67.

del Hoyo, P., Garcia-Redondo, A., de Bustos, F., Molina, J. A., Sayed, Y., Alonso-Navarro, H., Caballero, L., Arenas, J. and Jimenez-Jimenez, F. J. (2006). Oxidative stress in skin fibroblasts cultures of patients with Huntington's disease. *Neurochemical research* **31**(9), 1103-9, 10.1007/s11064-006-9110-2.

del Toro, D., Xifro, X., Pol, A., Humbert, S., Saudou, F., Canals, J. M. and Alberch, J. (2010). Altered cholesterol homeostasis contributes to enhanced excitotoxicity in Huntington's disease. *Journal of neurochemistry* **115**(1), 153-67, 10.1111/j.1471-4159.2010.06912.x.

Dhar, S. S., Ongwijitwat, S. and Wong-Riley, M. T. (2008). Nuclear respiratory factor 1 regulates all ten nuclear-encoded subunits of cytochrome c oxidase in neurons. *The Journal of biological chemistry* **283**(6), 3120-9, 10.1074/jbc.M707587200.

DiFiglia, M. (2002). Huntingtin fragments that aggregate go their separate ways. *Molecular cell* **10**(2), 224-5.

DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J. P., Carraway, R., Reeves, S. A. and et al. (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron* **14**(5), 1075-81.

DiFiglia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P. and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* **277**(5334), 1990-3.

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M. and Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* **99**(18), 11908-13, 10.1073/pnas.172398899.

Dong, G., Callegari, E. A., Gloeckner, C. J., Ueffing, M. and Wang, H. (2012). Prothymosin-alpha interacts with mutant huntingtin and suppresses its cytotoxicity in cell culture. *The Journal of biological chemistry* **287**(2), 1279-89, 10.1074/jbc.M111.294280.

Dong, X. X., Wang, Y. and Qin, Z. H. (2009). Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta pharmacologica Sinica* **30**(4), 379-87, 10.1038/aps.2009.24.

Dorner, J. L., Miller, B. R., Barton, S. J., Brock, T. J. and Rebec, G. V. (2007). Sex differences in behavior and striatal ascorbate release in the 140 CAG knock-in mouse model of Huntington's disease. *Behavioural brain research* **178**(1), 90-7, 10.1016/j.bbr.2006.12.004.

Dorner, J. L., Miller, B. R., Klein, E. L., Murphy-Nakhnikian, A., Andrews, R. L., Barton, S. J. and Rebec, G. V. (2009). Corticostriatal dysfunction underlies diminished striatal ascorbate release in the R6/2 mouse model of Huntington's disease. *Brain research* **1290**, 111-20, 10.1016/j.brainres.2009.07.019.

Dowling, R. J., Topisirovic, I., Fonseca, B. D. and Sonenberg, N. (2010). Dissecting the role of mTOR: lessons from mTOR inhibitors. *Biochimica et biophysica acta* **1804**(3), 433-9, 10.1016/j.bbapap.2009.12.001.

Dragatsis, I., Efstratiadis, A. and Zeitlin, S. (1998). Mouse mutant embryos lacking huntingtin are rescued from lethality by wild-type extraembryonic tissues. *Development* **125**(8), 1529-39.

Dragatsis, I., Levine, M. S. and Zeitlin, S. (2000). Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nature genetics* **26**(3), 300-6, 10.1038/81593.

Driver-Dunckley, E., Caviness, J. N. (2007). Huntington's disease. In Schapira AHV. Neurology and Clinical Neuroscience. Mosby Elsevier. pp. 879–85.

Du, K. and Montminy, M. (1998). CREB is a regulatory target for the protein kinase Akt/PKB. *The Journal of biological chemistry* **273**(49), 32377-9.

Duan, W., Guo, Z., Jiang, H., Ware, M., Li, X. J. and Mattson, M. P. (2003). Dietary restriction normalizes glucose metabolism and BDNF levels, slows disease progression, and increases survival in huntingtin mutant mice. *Proceedings of the National Academy of Sciences of the United States of America* **100**(5), 2911-6, 10.1073/pnas.0536856100.

Duarte, A. I., Petit, G. H., Ranganathan, S., Li, J. Y., Oliveira, C. R., Brundin, P., Bjorkqvist, M. and Rego, A. C. (2011). IGF-1 protects against diabetic features in an in vivo model of Huntington's disease. *Experimental neurology* **231**(2), 314-9, 10.1016/j.expneurol.2011.06.016.

Duarte, A. I., Proenca, T., Oliveira, C. R., Santos, M. S. and Rego, A. C. (2006). Insulin restores metabolic function in cultured cortical neurons subjected to oxidative stress. *Diabetes* **55**(10), 2863-70, 10.2337/db06-0030.

Duarte, A. I., Santos, M. S., Oliveira, C. R. and Rego, A. C. (2005). Insulin neuroprotection against oxidative stress in cortical neurons--involvement of uric acid and glutathione antioxidant defenses. *Free radical biology & medicine* **39**(7), 876-89, 10.1016/j.freeradbiomed.2005.05.002.

Duarte, A. I., Santos, P., Oliveira, C. R., Santos, M. S. and Rego, A. C. (2008). Insulin neuroprotection against oxidative stress is mediated by Akt and GSK-3beta signaling pathways and changes in protein expression. *Biochimica et biophysica acta* **1783**(6), 994-1002, 10.1016/j.bbamcr.2008.02.016.

Duff, K., Paulsen, J. S., Beglinger, L. J., Langbehn, D. R., Stout, J. C. and Predict, H. D. I. o. t. H. S. G. (2007). Psychiatric symptoms in Huntington's disease before diagnosis: the predict-HD study. *Biological psychiatry* **62**(12), 1341-6, 10.1016/j.biopsych.2006.11.034.

Dunah, A. W., Jeong, H., Griffin, A., Kim, Y. M., Standaert, D. G., Hersch, S. M., Mouradian, M. M., Young, A. B., Tanese, N. and Krainc, D. (2002). Sp1 and TAFII130 transcriptional activity disrupted in early Huntington's disease. *Science* **296**(5576), 2238-43, 10.1126/science.1072613.

Durgadoss, L., Nidadavolu, P., Valli, R. K., Saeed, U., Mishra, M., Seth, P. and Ravindranath, V. (2012). Redox modification of Akt mediated by the dopaminergic neurotoxin MPTP, in mouse midbrain, leads to down-regulation of pAkt. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **26**(4), 1473-83, 10.1096/fj.11-194100.

Duyao, M. P., Auerbach, A. B., Ryan, A., Persichetti, F., Barnes, G. T., McNeil, S. M., Ge, P., Vonsattel, J. P., Gusella, J. F., Joyner, A. L. and et al. (1995). Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science* **269**(5222), 407-10.

Ehrlich, M. E. (2012). Huntington's disease and the striatal medium spiny neuron: cell-autonomous and non-cell-autonomous mechanisms of disease. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* **9**(2), 270-84, 10.1007/s13311-012-0112-2.

Ehrnhoefer, D. E., Skotte, N. H., Ladha, S., Nguyen, Y. T., Qiu, X., Deng, Y., Huynh, K. T., Engemann, S., Nielsen, S. M., Becanovic, K., Leavitt, B. R., Hasholt, L. and Hayden, M. R. (2014). p53 increases caspase-6 expression and activation in muscle tissue expressing mutant huntingtin. *Human molecular genetics* **23**(3), 717-29, 10.1093/hmg/ddt458.

Elgass, K., Pakay, J., Ryan, M. T. and Palmer, C. S. (2013). Recent advances into the understanding of mitochondrial fission. *Biochimica et biophysica acta* **1833**(1), 150-61, 10.1016/j.bbamcr.2012.05.002.

Ellrichmann, G., Petrasch-Parwez, E., Lee, D. H., Reick, C., Arning, L., Saft, C., Gold, R. and Linker, R. A. (2011). Efficacy of fumaric acid esters in the R6/2 and YAC128 models of Huntington's disease. *PloS one* **6**(1), e16172, 10.1371/journal.pone.0016172.

Erttmann, S. F., Bast, A., Seidel, J., Breitbach, K., Walther, R. and Steinmetz, I. (2011). PGD2 and PGE2 regulate gene expression of Prx 6 in primary macrophages via Nrf2. *Free radical biology & medicine* **51**(3), 626-40, 10.1016/j.freeradbiomed.2011.05.022.

Escartin, C., Won, S. J., Malgorn, C., Auregan, G., Berman, A. E., Chen, P. C., Deglon, N., Johnson, J. A., Suh, S. W. and Swanson, R. A. (2011). Nuclear factor erythroid 2-related factor 2 facilitates neuronal glutathione synthesis by upregulating neuronal excitatory amino acid transporter 3 expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**(20), 7392-401, 10.1523/JNEUROSCI.6577-10.2011.

Esterbauer, H., Schaur, R. J. and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free radical biology & medicine* **11**(1), 81-128.

Estrada-Sanchez, A. M., Montiel, T. and Massieu, L. (2010). Glycolysis inhibition decreases the levels of glutamate transporters and enhances glutamate neurotoxicity in the R6/2 Huntington's disease mice. *Neurochemical research* **35**(8), 1156-63, 10.1007/s11064-010-0168-5.

Evans, M. D., Dizdaroglu, M. and Cooke, M. S. (2004). Oxidative DNA damage and disease: induction, repair and significance. *Mutation research* **567**(1), 1-61, 10.1016/j.mrrev.2003.11.001.

Evans, S. J., Douglas, I., Rawlins, M. D., Wexler, N. S., Tabrizi, S. J. and Smeeth, L. (2013). Prevalence of adult Huntington's disease in the UK based on diagnoses recorded in general practice records. *Journal of neurology, neurosurgery, and psychiatry* **84**(10), 1156-60, 10.1136/jnnp-2012-304636.

Fan, M. M., Zhang, H., Hayden, M. R., Pelech, S. L. and Raymond, L. A. (2008). Protective up-regulation of CK2 by mutant huntingtin in cells co-expressing NMDA receptors. *Journal of neurochemistry* **104**(3), 790-805, 10.1111/j.1471-4159.2007.05016.x.

Feng, J., Park, J., Cron, P., Hess, D. and Hemmings, B. A. (2004). Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *The Journal of biological chemistry* **279**(39), 41189-96, 10.1074/jbc.M406731200.

Feng, X., Sun, T., Bei, Y., Ding, S., Zheng, W., Lu, Y. and Shen, P. (2013). S-nitrosylation of ERK inhibits ERK phosphorylation and induces apoptosis. *Scientific reports* **3**, 1814, 10.1038/srep01814.

Feng, Z., Jin, S., Zupnick, A., Hoh, J., de Stanchina, E., Lowe, S., Prives, C. and Levine, A. J. (2006). p53 tumor suppressor protein regulates the levels of huntingtin gene expression. *Oncogene* **25**(1), 1-7, 10.1038/sj.onc.1209021.

Fernandez, A. M. and Torres-Aleman, I. (2012). The many faces of insulin-like peptide signalling in the brain. *Nature reviews. Neuroscience* **13**(4), 225-39, 10.1038/nrn3209.

Ferrante, R. J., Andreassen, O. A., Jenkins, B. G., Dedeoglu, A., Kuemmerle, S., Kubilus, J. K., Kaddurah-Daouk, R., Hersch, S. M. and Beal, M. F. (2000). Neuroprotective effects of creatine in a

transgenic mouse model of Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(12), 4389-97.

Ferrante, R. J., Beal, M. F., Kowall, N. W., Richardson, E. P., Jr. and Martin, J. B. (1987). Sparing of acetylcholinesterase-containing striatal neurons in Huntington's disease. *Brain research* **411**(1), 162-6.

Ferrante, R. J., Kowall, N. W., Beal, M. F., Martin, J. B., Bird, E. D. and Richardson, E. P., Jr. (1987). Morphologic and histochemical characteristics of a spared subset of striatal neurons in Huntington's disease. *Journal of neuropathology and experimental neurology* **46**(1), 12-27.

Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D. and Martin, J. B. (1985). Selective sparing of a class of striatal neurons in Huntington's disease. *Science* **230**(4725), 561-3.

Ferrante, R. J., Kowall, N. W., Cipolloni, P. B., Storey, E. and Beal, M. F. (1993). Excitotoxin lesions in primates as a model for Huntington's disease: histopathologic and neurochemical characterization. *Experimental neurology* **119**(1), 46-71, 10.1006/exnr.1993.1006.

Ferreira, I. L., Cunha-Oliveira, T., Nascimento, M. V., Ribeiro, M., Proenca, M. T., Januario, C., Oliveira, C. R. and Rego, A. C. (2011). Bioenergetic dysfunction in Huntington's disease human cybrids. *Experimental neurology* **231**(1), 127-34, 10.1016/j.expneurol.2011.05.024.

Ferreira, I. L., Nascimento, M. V., Ribeiro, M., Almeida, S., Cardoso, S. M., Grazina, M., Pratas, J., Santos, M. J., Januario, C., Oliveira, C. R. and Rego, A. C. (2010). Mitochondrial-dependent apoptosis in Huntington's disease human cybrids. *Experimental neurology* **222**(2), 243-55, 10.1016/j.expneurol.2010.01.002.

Fesus, L. and Piacentini, M. (2002). Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends in biochemical sciences* **27**(10), 534-9.

Fialkow, L., Wang, Y. and Downey, G. P. (2007). Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free radical biology & medicine* **42**(2), 153-64, 10.1016/j.freeradbiomed.2006.09.030.

Finsterer, J. (2009). Bulbar and spinal muscular atrophy (Kennedy's disease): a review. *European journal of neurology : the official journal of the European Federation of Neurological Societies* **16**(5), 556-61.

Firdaus, W. J., Wyttenbach, A., Giuliano, P., Kretz-Remy, C., Currie, R. W. and Arrigo, A. P. (2006). Huntingtin inclusion bodies are iron-dependent centers of oxidative events. *The FEBS journal* **273**(23), 5428-41, 10.1111/j.1742-4658.2006.05537.x.

Fleckenstein, A. E., Metzger, R. R., Beyeler, M. L., Gibb, J. W. and Hanson, G. R. (1997). Oxygen radicals diminish dopamine transporter function in rat striatum. *European journal of pharmacology* **334**(1), 111-4.

Folk, J. E. (1983). Mechanism and basis for specificity of transglutaminase-catalyzed epsilon-(gamma-glutamyl) lysine bond formation. *Advances in enzymology and related areas of molecular biology* **54**, 1-56.

Fourquet, S., Guerois, R., Biard, D. and Toledano, M. B. (2010). Activation of NRF2 by nitrosative agents and H2O2 involves KEAP1 disulfide formation. *The Journal of biological chemistry* **285**(11), 8463-71, 10.1074/jbc.M109.051714.

Fox, J. H., Barber, D. S., Singh, B., Zucker, B., Swindell, M. K., Norflus, F., Buzescu, R., Chopra, R., Ferrante, R. J., Kazantsev, A. and Hersch, S. M. (2004). Cystamine increases L-cysteine levels in Huntington's disease transgenic mouse brain and in a PC12 model of polyglutamine aggregation. *Journal of neurochemistry* **91**(2), 413-22, 10.1111/j.1471-4159.2004.02726.x.

Fox, J. H., Connor, T., Stiles, M., Kama, J., Lu, Z., Dorsey, K., Lieberman, G., Sapp, E., Cherny, R. A., Banks, M., Volitakis, I., DiFiglia, M., Berezovska, O., Bush, A. I. and Hersch, S. M. (2011). Cysteine oxidation within N-terminal mutant huntingtin promotes oligomerization and delays clearance of soluble protein. *The Journal of biological chemistry* **286**(20), 18320-30, 10.1074/jbc.M110.199448.

Fox, J. H., Kama, J. A., Lieberman, G., Chopra, R., Dorsey, K., Chopra, V., Volitakis, I., Cherny, R. A., Bush, A. I. and Hersch, S. (2007). Mechanisms of copper ion mediated Huntington's disease progression. *PloS one* **2**(3), e334, 10.1371/journal.pone.0000334.

Freiherr, J., Hallschmid, M., Frey, W. H., 2nd, Brunner, Y. F., Chapman, C. D., Holscher, C., Craft, S., De Felice, F. G. and Benedict, C. (2013). Intranasal insulin as a treatment for Alzheimer's disease: a review of basic research and clinical evidence. *CNS drugs* **27**(7), 505-14, 10.1007/s40263-013-0076-8.

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annual review of biochemistry* **64**, 97-112, 10.1146/annurev.bi.64.070195.000525.

Friedman, J. R., Lackner, L. L., West, M., DiBenedetto, J. R., Nunnari, J. and Voeltz, G. K. (2011). ER tubules mark sites of mitochondrial division. *Science* **334**(6054), 358-62, 10.1126/science.1207385.

Friedman, M. J., Wang, C. E., Li, X. J. and Li, S. (2008). Polyglutamine expansion reduces the association of TATA-binding protein with DNA and induces DNA binding-independent neurotoxicity. *The Journal of biological chemistry* **283**(13), 8283-90, 10.1074/jbc.M709674200.

Fu, J., Jin, J., Cichewicz, R. H., Hageman, S. A., Ellis, T. K., Xiang, L., Peng, Q., Jiang, M., Arbez, N., Hotaling, K., Ross, C. A. and Duan, W. (2012). trans-(-)-epsilon-Viniferin increases mitochondrial sirtuin 3 (SIRT3), activates AMP-activated protein kinase (AMPK), and protects cells in models of Huntington Disease. *The Journal of biological chemistry* **287**(29), 24460-72, 10.1074/jbc.M112.382226.

Fujioka, S., Sundal, C. and Wszolek, Z. K. (2013). Autosomal dominant cerebellar ataxia type III: a review of the phenotypic and genotypic characteristics. *Orphanet journal of rare diseases* **8**, 14, 10.1186/1750-1172-8-14.

Fukui, H. and Moraes, C. T. (2007). Extended polyglutamine repeats trigger a feedback loop involving the mitochondrial complex III, the proteasome and huntingtin aggregates. *Human molecular genetics* **16**(7), 783-97, 10.1093/hmg/ddm023.

Fusco, F. R., Anzilotti, S., Giampa, C., Dato, C., Laurenti, D., Leuti, A., Colucci D'Amato, L., Perrone, L., Bernardi, G. and Melone, M. A. (2012). Changes in the expression of extracellular regulated kinase (ERK 1/2) in the R6/2 mouse model of Huntington's disease after phosphodiesterase IV inhibition. *Neurobiology of disease* **46**(1), 225-33, 10.1016/j.nbd.2012.01.011.

Gafni, J. and Ellerby, L. M. (2002). Calpain activation in Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**(12), 4842-9.

Gafni, J., Hermel, E., Young, J. E., Wellington, C. L., Hayden, M. R. and Ellerby, L. M. (2004). Inhibition of calpain cleavage of huntingtin reduces toxicity: accumulation of calpain/caspase fragments in the nucleus. *The Journal of biological chemistry* **279**(19), 20211-20, 10.1074/jbc.M401267200.

Gagnon, J. F., Petit, D., Latreille, V. and Montplaisir, J. (2008). Neurobiology of sleep disturbances in neurodegenerative disorders. *Current pharmaceutical design* **14**(32), 3430-45.

Gamberino, W. C. and Brennan, W. A., Jr. (1994). Glucose transporter isoform expression in Huntington's disease brain. *Journal of neurochemistry* **63**(4), 1392-7.

Gao, H. M., Zhou, H. and Hong, J. S. (2012). NADPH oxidases: novel therapeutic targets for neurodegenerative diseases. *Trends in pharmacological sciences* **33**(6), 295-303, 10.1016/j.tips.2012.03.008.

Gao, R., Matsuura, T., Coolbaugh, M., Zuhlke, C., Nakamura, K., Rasmussen, A., Siciliano, M. J., Ashizawa, T. and Lin, X. (2008). Instability of expanded CAG/CAA repeats in spinocerebellar ataxia type 17. *European journal of human genetics : EJHG* **16**(2), 215-22, 10.1038/sj.ejhg.5201954.

Garden, G. (1998) [Updated 2012 Dec 20]. Spinocerebellar Ataxia Type 7. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1256/

Garden, G. A. and La Spada, A. R. (2008). Molecular pathogenesis and cellular pathology of spinocerebellar ataxia type 7 neurodegeneration. *Cerebellum* **7**(2), 138-49, 10.1007/s12311-008-0027-y.

Garseth, M., Sonnewald, U., White, L. R., Rod, M., Zwart, J. A., Nygaard, O. and Aasly, J. (2000). Proton magnetic resonance spectroscopy of cerebrospinal fluid in neurodegenerative disease: indication of glial energy impairment in Huntington chorea, but not Parkinson disease. *Journal of neuroscience research* **60**(6), 779-82.

Gauthier, L. R., Charrin, B. C., Borrell-Pages, M., Dompierre, J. P., Rangone, H., Cordelieres, F. P., De Mey, J., MacDonald, M. E., Lessmann, V., Humbert, S. and Saudou, F. (2004). Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell* **118**(1), 127-38, 10.1016/j.cell.2004.06.018.

Ghasemi, R., Dargahi, L., Haeri, A., Moosavi, M., Mohamed, Z. and Ahmadiani, A. (2013). Brain insulin dysregulation: implication for neurological and neuropsychiatric disorders. *Molecular neurobiology* **47**(3), 1045-65, 10.1007/s12035-013-8404-z.

Ghasemi, R., Haeri, A., Dargahi, L., Mohamed, Z. and Ahmadiani, A. (2013). Insulin in the brain: sources, localization and functions. *Molecular neurobiology* **47**(1), 145-71, 10.1007/s12035-012-8339-9.

Ghose, J., Sinha, M., Das, E., Jana, N. R. and Bhattacharyya, N. P. (2011). Regulation of miR-146a by RelA/NFkB and p53 in STHdh(Q111)/Hdh(Q111) cells, a cell model of Huntington's disease. *PloS one* **6**(8), e23837, 10.1371/journal.pone.0023837.

Gianfriddo, M., Melani, A., Turchi, D., Giovannini, M. G. and Pedata, F. (2004). Adenosine and glutamate extracellular concentrations and mitogen-activated protein kinases in the striatum of Huntington transgenic mice. Selective antagonism of adenosine A2A receptors reduces transmitter outflow. *Neurobiology of disease* **17**(1), 77-88, 10.1016/j.nbd.2004.05.008.

Gibrat, C. and Cicchetti, F. (2011). Potential of cystamine and cysteamine in the treatment of neurodegenerative diseases. *Progress in neuro-psychopharmacology & biological psychiatry* **35**(2), 380-9, 10.1016/j.pnpbp.2010.11.023.

Gil, J., Almeida, S., Oliveira, C. R. and Rego, A. C. (2003). Cytosolic and mitochondrial ROS in staurosporine-induced retinal cell apoptosis. *Free radical biology & medicine* **35**(11), 1500-14.

Gil, J. M. and Rego, A. C. (2008). Mechanisms of neurodegeneration in Huntington's disease. *The European journal of neuroscience* **27**(11), 2803-20, 10.1111/j.1460-9568.2008.06310.x.

Gines, S., Ivanova, E., Seong, I. S., Saura, C. A. and MacDonald, M. E. (2003). Enhanced Akt signaling is an early pro-survival response that reflects N-methyl-D-aspartate receptor activation in Huntington's disease knock-in striatal cells. *The Journal of biological chemistry* **278**(50), 50514-22, 10.1074/jbc.M309348200.

Gines, S., Seong, I. S., Fossale, E., Ivanova, E., Trettel, F., Gusella, J. F., Wheeler, V. C., Persichetti, F. and MacDonald, M. E. (2003). Specific progressive cAMP reduction implicates energy deficit in presymptomatic Huntington's disease knock-in mice. *Human molecular genetics* **12**(5), 497-508.

Gleyzer, N., Vercauteren, K. and Scarpulla, R. C. (2005). Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. *Molecular and cellular biology* **25**(4), 1354-66, 10.1128/MCB.25.4.1354-1366.2005.

Godin, J. D., Poizat, G., Hickey, M. A., Maschat, F. and Humbert, S. (2010). Mutant huntingtin-impaired degradation of beta-catenin causes neurotoxicity in Huntington's disease. *The EMBO journal* **29**(14), 2433-45, 10.1038/emboj.2010.117.

Goffredo, D., Rigamonti, D., Tartari, M., De Micheli, A., Verderio, C., Matteoli, M., Zuccato, C. and Cattaneo, E. (2002). Calcium-dependent cleavage of endogenous wild-type huntingtin in primary cortical neurons. *The Journal of biological chemistry* **277**(42), 39594-8, 10.1074/jbc.C200353200.

Goldberg, Y. P., Nicholson, D. W., Rasper, D. M., Kalchman, M. A., Koide, H. B., Graham, R. K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N. A., Vaillancourt, J. P. and Hayden, M. R. (1996). Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nature genetics* **13**(4), 442-9, 10.1038/ng0896-442.

Gomez, C. M. (1998) [Updated 2013]. Spinocerebellar Ataxia Type 6. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1140/

Gorrini, C., Baniasadi, P. S., Harris, I. S., Silvester, J., Inoue, S., Snow, B., Joshi, P. A., Wakeham, A., Molyneux, S. D., Martin, B., Bouwman, P., Cescon, D. W., Elia, A. J., Winterton-Perks, Z., Cruickshank, J., Brenner, D., Tseng, A., Musgrave, M., Berman, H. K., Khokha, R., Jonkers, J., Mak, T. W. and Gauthier, M. L. (2013). BRCA1 interacts with Nrf2 to regulate antioxidant signaling and cell survival. *The Journal of experimental medicine* **210**(8), 1529-44, 10.1084/jem.20121337.

Goswami, A., Dikshit, P., Mishra, A., Mulherkar, S., Nukina, N. and Jana, N. R. (2006). Oxidative stress promotes mutant huntingtin aggregation and mutant huntingtin-dependent cell death by mimicking proteasomal malfunction. *Biochemical and biophysical research communications* **342**(1), 184-90, 10.1016/j.bbrc.2006.01.136.

Graham, R. K., Slow, E. J., Deng, Y., Bissada, N., Lu, G., Pearson, J., Shehadeh, J., Leavitt, B. R., Raymond, L. A. and Hayden, M. R. (2006). Levels of mutant huntingtin influence the phenotypic severity of Huntington disease in YAC128 mouse models. *Neurobiology of disease* **21**(2), 444-55, 10.1016/j.nbd.2005.08.007.

Gray, M., Shirasaki, D. I., Cepeda, C., Andre, V. M., Wilburn, B., Lu, X. H., Tao, J., Yamazaki, I., Li, S. H., Sun, Y. E., Li, X. J., Levine, M. S. and Yang, X. W. (2008). Full-length human mutant huntingtin with a stable polyglutamine repeat can elicit progressive and selective neuropathogenesis in BACHD mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**(24), 6182-95, 10.1523/JNEUROSCI.0857-08.2008.

Greenberg, C. S., Birckbichler, P. J. and Rice, R. H. (1991). Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **5**(15), 3071-7.

Griffiths, H. R., Moller, L., Bartosz, G., Bast, A., Bertoni-Freddari, C., Collins, A., Cooke, M., Coolen, S., Haenen, G., Hoberg, A. M., Loft, S., Lunec, J., Olinski, R., Parry, J., Pompella, A., Poulsen, H.,

Verhagen, H. and Astley, S. B. (2002). Biomarkers. *Molecular aspects of medicine* **23**(1-3), 101-208.

Griparic, L. and van der Bliek, A. M. (2001). The many shapes of mitochondrial membranes. *Traffic* **2**(4), 235-44.

Grosso, H. and Mouradian, M. M. (2012). Transglutaminase 2: biology, relevance to neurodegenerative diseases and therapeutic implications. *Pharmacology & therapeutics* **133**(3), 392-410, 10.1016/j.pharmthera.2011.12.003.

Gu, M., Gash, M. T., Mann, V. M., Javoy-Agid, F., Cooper, J. M. and Schapira, A. H. (1996). Mitochondrial defect in Huntington's disease caudate nucleus. *Annals of neurology* **39**(3), 385-9, 10.1002/ana.410390317.

Gusella, J. F. and MacDonald, M. E. (2006). Huntington's disease: seeing the pathogenic process through a genetic lens. *Trends in biochemical sciences* **31**(9), 533-40, 10.1016/j.tibs.2006.06.009.

Gusella, J. F., Tanzi, R. E., Bader, P. I., Phelan, M. C., Stevenson, R., Hayden, M. R., Hofman, K. J., Faryniarz, A. G. and Gibbons, K. (1985). Deletion of Huntington's disease-linked G8 (D4S10) locus in Wolf-Hirschhorn syndrome. *Nature* **318**(6041), 75-8.

Gusella, J. F., Wexler, N. S., Conneally, P. M., Naylor, S. L., Anderson, M. A., Tanzi, R. E., Watkins, P. C., Ottina, K., Wallace, M. R., Sakaguchi, A. Y. and et al. (1983). A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* **306**(5940), 234-8.

Gutekunst, C. A., Levey, A. I., Heilman, C. J., Whaley, W. L., Yi, H., Nash, N. R., Rees, H. D., Madden, J. J. and Hersch, S. M. (1995). Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **92**(19), 8710-4.

Hackam, A. S., Singaraja, R., Wellington, C. L., Metzler, M., McCutcheon, K., Zhang, T., Kalchman, M. and Hayden, M. R. (1998). The influence of huntingtin protein size on nuclear localization and cellular toxicity. *The Journal of cell biology* **141**(5), 1097-105.

Hahn-Windgassen, A., Nogueira, V., Chen, C. C., Skeen, J. E., Sonenberg, N. and Hay, N. (2005). Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *The Journal of biological chemistry* **280**(37), 32081-9, 10.1074/jbc.M502876200.

Hales, K. G. (2004). The machinery of mitochondrial fusion, division, and distribution, and emerging connections to apoptosis. *Mitochondrion* **4**(4), 285-308, 10.1016/j.mito.2004.05.007.

Halliwell, B., and Gutteridge, J. M. C. (1999). Free Radicals in Biology and Medicine, 3rd edn. Oxford: Clarendon Press.

Hands, S., Sajjad, M. U., Newton, M. J. and Wyttenbach, A. (2011). In vitro and in vivo aggregation of a fragment of huntingtin protein directly causes free radical production. *The Journal of biological chemistry* **286**(52), 44512-20, 10.1074/jbc.M111.307587.

Handschin, C. and Spiegelman, B. M. (2006). Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocrine reviews* **27**(7), 728-35, 10.1210/er.2006-0037.

Hansson, O., Castilho, R. F., Korhonen, L., Lindholm, D., Bates, G. P. and Brundin, P. (2001). Partial resistance to malonate-induced striatal cell death in transgenic mouse models of Huntington's disease is dependent on age and CAG repeat length. *Journal of neurochemistry* **78**(4), 694-703.

Hansson, O., Guatteo, E., Mercuri, N. B., Bernardi, G., Li, X. J., Castilho, R. F. and Brundin, P. (2001). Resistance to NMDA toxicity correlates with appearance of nuclear inclusions, behavioural deficits and changes in calcium homeostasis in mice transgenic for exon 1 of the huntington gene. *The European journal of neuroscience* **14**(9), 1492-504.

Harjes, P. and Wanker, E. E. (2003). The hunt for huntingtin function: interaction partners tell many different stories. *Trends in biochemical sciences* **28**(8), 425-33, 10.1016/S0968-0004(03)00168-3.

Harrington, K. M., Kowall, N. M. (1991). Parvalbumin immunoreactive neurons resist degeneration in Huntington's disease striatum. *Journal of neuropathology and experimental neurology* **50**:309.

Harris, A. S., Denovan-Wright, E. M., Hamilton, L. C. and Robertson, H. A. (2001). Protein kinase C beta II mRNA levels decrease in the striatum and cortex of transgenic Huntington's disease mice. *Journal of psychiatry & neuroscience : JPN* **26**(2), 117-22.

Harrison, R. (2002). Structure and function of xanthine oxidoreductase: where are we now? *Free radical biology & medicine* **33**(6), 774-97.

Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y. and Saito, Y. (2003). A novel function of tissue-type transglutaminase: protein disulphide isomerase. *The Biochemical journal* **373**(Pt 3), 793-803, 10.1042/BJ20021084.

Hashimoto, T., Kitamura, N., Saito, N., Komure, O., Nishino, N. and Tanaka, C. (1992). The loss of beta II-protein kinase C in the striatum from patients with Huntington's disease. *Brain research* **585**(1-2), 303-6.

Hast, B. E., Goldfarb, D., Mulvaney, K. M., Hast, M. A., Siesser, P. F., Yan, F., Hayes, D. N. and Major, M. B. (2013). Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination. *Cancer research* **73**(7), 2199-210, 10.1158/0008-5472.CAN-12-4400.

Haun, F., Nakamura, T., Shiu, A. D., Cho, D. H., Tsunemi, T., Holland, E. A., La Spada, A. R. and Lipton, S. A. (2013). S-nitrosylation of dynamin-related protein 1 mediates mutant huntingtin-induced mitochondrial fragmentation and neuronal injury in Huntington's disease. *Antioxidants & redox signaling* **19**(11), 1173-84, 10.1089/ars.2012.4928.

Havel, L. S., Li, S. and Li, X. J. (2009). Nuclear accumulation of polyglutamine disease proteins and neuropathology. *Molecular brain* **2**, 21, 10.1186/1756-6606-2-21.

Havrankova, J., Roth, J. and Brownstein, M. (1978). Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* **272**(5656), 827-9.

Havrankova, J., Schmechel, D., Roth, J. and Brownstein, M. (1978). Identification of insulin in rat brain. *Proceedings of the National Academy of Sciences of the United States of America* **75**(11), 5737-41.

Hayashi, A., Suzuki, H., Itoh, K., Yamamoto, M. and Sugiyama, Y. (2003). Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. *Biochemical and biophysical research communications* **310**(3), 824-9.

He, X. and Ma, Q. (2009). NRF2 cysteine residues are critical for oxidant/electrophile-sensing, Kelch-like ECH-associated protein-1-dependent ubiquitination-proteasomal degradation, and transcription activation. *Molecular pharmacology* **76**(6), 1265-78, 10.1124/mol.109.058453.

Heales, S. J. and Bolanos, J. P. (2002). Impairment of brain mitochondrial function by reactive nitrogen species: the role of glutathione in dictating susceptibility. *Neurochemistry international* **40**(6), 469-74.

Heinrich, J. N., Kwak, S. P., Howland, D. S., Chen, J., Sturner, S., Sullivan, K., Lipinski, K., Cheng, K. Y., She, Y., Lo, F. and Ghavami, A. (2006). Disruption of ShcA signaling halts cell proliferation--characterization of ShcC residues that influence signaling pathways using yeast. *Cellular signalling* **18**(6), 795-806, 10.1016/j.cellsig.2005.07.004.

Heng, M. Y., Tallaksen-Greene, S. J., Detloff, P. J. and Albin, R. L. (2007). Longitudinal evaluation of the Hdh(CAG)150 knock-in murine model of Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**(34), 8989-98, 10.1523/JNEUROSCI.1830-07.2007.

Hermel, E., Gafni, J., Propp, S. S., Leavitt, B. R., Wellington, C. L., Young, J. E., Hackam, A. S., Logvinova, A. V., Peel, A. L., Chen, S. F., Hook, V., Singaraja, R., Krajewski, S., Goldsmith, P. C., Ellerby, H. M., Hayden, M. R., Bredesen, D. E. and Ellerby, L. M. (2004). Specific caspase interactions and amplification are involved in selective neuronal vulnerability in Huntington's disease. *Cell death and differentiation* **11**(4), 424-38, 10.1038/sj.cdd.4401358.

Herrera-Mundo, N. and Sitges, M. (2013). Vinpocetine and alpha-tocopherol prevent the increase in DA and oxidative stress induced by 3-NPA in striatum isolated nerve endings. *Journal of neurochemistry* **124**(2), 233-40, 10.1111/jnc.12082.

Hersch, S. M. and Ferrante, R. J. (2004). Translating therapies for Huntington's disease from genetic animal models to clinical trials. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* **1**(3), 298-306, 10.1602/neurorx.1.3.298.

Hersch, S. M., Gevorkian, S., Marder, K., Moskowitz, C., Feigin, A., Cox, M., Como, P., Zimmerman, C., Lin, M., Zhang, L., Ulug, A. M., Beal, M. F., Matson, W., Bogdanov, M., Ebbel, E., Zaleta, A., Kaneko, Y., Jenkins, B., Hevelone, N., Zhang, H., Yu, H., Schoenfeld, D., Ferrante, R. and Rosas, H. D. (2006). Creatine in Huntington disease is safe, tolerable, bioavailable in brain and reduces serum 8OH2'dG. *Neurology* **66**(2), 250-2, 10.1212/01.wnl.0000194318.74946.b6.

Hirsch, E. C., Graybiel, A. M., Hersh, L. B., Duyckaerts, C. and Agid, Y. (1989). Striosomes and extrastriosomal matrix contain different amounts of immunoreactive choline acetyltransferase in the human striatum. *Neuroscience letters* **96**(2), 145-50.

Hirth, F. (2010). Drosophila melanogaster in the study of human neurodegeneration. *CNS & neurological disorders drug targets* **9**(4), 504-23.

Hissin, P. J. and Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical biochemistry* **74**(1), 214-26.

Ho, A. K., Sahakian, B. J., Brown, R. G., Barker, R. A., Hodges, J. R., Ane, M. N., Snowden, J., Thompson, J., Esmonde, T., Gentry, R., Moore, J. W., Bodner, T. and Consortium, N.-H. (2003). Profile of cognitive progression in early Huntington's disease. *Neurology* **61**(12), 1702-6.

Hodgson, J. G., Agopyan, N., Gutekunst, C. A., Leavitt, B. R., LePiane, F., Singaraja, R., Smith, D. J., Bissada, N., McCutcheon, K., Nasir, J., Jamot, L., Li, X. J., Stevens, M. E., Rosemond, E., Roder, J. C., Phillips, A. G., Rubin, E. M., Hersch, S. M. and Hayden, M. R. (1999). A YAC mouse model

for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* **23**(1), 181-92.

Hodgson, J. G., Smith, D. J., McCutcheon, K., Koide, H. B., Nishiyama, K., Dinulos, M. B., Stevens, M. E., Bissada, N., Nasir, J., Kanazawa, I., Disteche, C. M., Rubin, E. M. and Hayden, M. R. (1996). Human huntingtin derived from YAC transgenes compensates for loss of murine huntingtin by rescue of the embryonic lethal phenotype. *Human molecular genetics* **5**(12), 1875-85.

Hoogeveen, A. T., Willemsen, R., Meyer, N., de Rooij, K. E., Roos, R. A., van Ommen, G. J. and Galjaard, H. (1993). Characterization and localization of the Huntington disease gene product. *Human molecular genetics* **2**(12), 2069-73.

Horton, L. C., Frosch, M. P., Vangel, M. G., Weigel-DiFranco, C., Berson, E. L. and Schmahmann, J. D. (2013). Spinocerebellar ataxia type 7: clinical course, phenotype-genotype correlations, and neuropathology. *Cerebellum* **12**(2), 176-93, 10.1007/s12311-012-0412-4.

Horton, T. M., Graham, B. H., Corral-Debrinski, M., Shoffner, J. M., Kaufman, A. E., Beal, M. F. and Wallace, D. C. (1995). Marked increase in mitochondrial DNA deletion levels in the cerebral cortex of Huntington's disease patients. *Neurology* **45**(10), 1879-83.

Hoyer, S. (2004). Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *European journal of pharmacology* **490**(1-3), 115-25, 10.1016/j.ejphar.2004.02.049.

Hu, Y., Szente, B., Kiely, J. M. and Gimbrone, M. A., Jr. (2001). Molecular events in transmembrane signaling via E-selectin. SHP2 association, adaptor protein complex formation and ERK1/2 activation. *The Journal of biological chemistry* **276**(51), 48549-53, 10.1074/jbc.M105513200.

Huang, C. C., Faber, P. W., Persichetti, F., Mittal, V., Vonsattel, J. P., MacDonald, M. E. and Gusella, J. F. (1998). Amyloid formation by mutant huntingtin: threshold, progressivity and recruitment of normal polyglutamine proteins. *Somatic cell and molecular genetics* **24**(4), 217-33.

Huang, H. C., Nguyen, T. and Pickett, C. B. (2002). Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *The Journal of biological chemistry* **277**(45), 42769-74, 10.1074/jbc.M206911200.

Huang, X. S., Chen, H. P., Yu, H. H., Yan, Y. F., Liao, Z. P. and Huang, Q. R. (2014). Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for hypoxic preconditioning-mediated delayed cardioprotection. *Molecular and cellular biochemistry* **385**(1-2), 33-41, 10.1007/s11010-013-1812-6.

Hult, S., Soylu, R., Bjorklund, T., Belgardt, B. F., Mauer, J., Bruning, J. C., Kirik, D. and Petersen, A. (2011). Mutant huntingtin causes metabolic imbalance by disruption of hypothalamic neurocircuits. *Cell metabolism* **13**(4), 428-39, 10.1016/j.cmet.2011.02.013.

Humbert, S., Bryson, E. A., Cordelieres, F. P., Connors, N. C., Datta, S. R., Finkbeiner, S., Greenberg, M. E. and Saudou, F. (2002). The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. *Developmental cell* **2**(6), 831-7.

Huntington, G. (1872). On chorea. *Medical and Surgical Reporter of Philadelphia* **26**(15), 317-21.

Huntington, G. (1910). Recollections of Huntington's chorea as I saw it at East Hampton, Long Island, during my boyhood. *The Journal of Nervous and Mental Disease* **37**, 255-7.

Huntington Study Group Pre, C. I., Hyson, H. C., Kieburtz, K., Shoulson, I., McDermott, M., Ravina, B., de Blieck, E. A., Cudkowicz, M. E., Ferrante, R. J., Como, P., Frank, S., Zimmerman, C., Cudkowicz, M. E., Ferrante, K., Newhall, K., Jennings, D., Kelsey, T., Walker, F., Hunt, V., Daigneault, S., Goldstein, M., Weber, J., Watts, A., Beal, M. F., Browne, S. E. and Metakis, L. J. (2010). Safety and tolerability of high-dosage coenzyme Q10 in Huntington's disease and healthy subjects. *Movement disorders : official journal of the Movement Disorder Society* **25**(12), 1924-8, 10.1002/mds.22408.

Im, J. Y., Lee, K. W., Woo, J. M., Junn, E. and Mouradian, M. M. (2012). DJ-1 induces thioredoxin 1 expression through the Nrf2 pathway. *Human molecular genetics* **21**(13), 3013-24, 10.1093/hmg/dds131.

Itoh, K., Tong, K. I. and Yamamoto, M. (2004). Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free radical biology & medicine* **36**(10), 1208-13, 10.1016/j.freeradbiomed.2004.02.075.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T. and Yamamoto, M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes to cells : devoted to molecular & cellular mechanisms* **8**(4), 379-91.

Jain, A. K. and Jaiswal, A. K. (2006). Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. *The Journal of biological chemistry* **281**(17), 12132-42, 10.1074/jbc.M511198200.

Jain, A. K., Mahajan, S. and Jaiswal, A. K. (2008). Phosphorylation and dephosphorylation of tyrosine 141 regulate stability and degradation of INrf2: a novel mechanism in Nrf2 activation. *The Journal of biological chemistry* **283**(25), 17712-20, 10.1074/jbc.M709854200.

Jeitner, T. M., Bogdanov, M. B., Matson, W. R., Daikhin, Y., Yudkoff, M., Folk, J. E., Steinman, L., Browne, S. E., Beal, M. F., Blass, J. P. and Cooper, A. J. (2001). N(epsilon)-(gamma-L-glutamyl)-L-lysine (GGEL) is increased in cerebrospinal fluid of patients with Huntington's disease. *Journal of neurochemistry* **79**(5), 1109-12.

Jenkins, B. G., Koroshetz, W. J., Beal, M. F. and Rosen, B. R. (1993). Evidence for impairment of energy metabolism in vivo in Huntington's disease using localized 1H NMR spectroscopy. *Neurology* **43**(12), 2689-95.

Jenkins, B. G., Rosas, H. D., Chen, Y. C., Makabe, T., Myers, R., MacDonald, M., Rosen, B. R., Beal, M. F. and Koroshetz, W. J. (1998). 1H NMR spectroscopy studies of Huntington's disease: correlations with CAG repeat numbers. *Neurology* **50**(5), 1357-65.

Jeon, G. S., Kim, K. Y., Hwang, Y. J., Jung, M. K., An, S., Ouchi, M., Ouchi, T., Kowall, N., Lee, J. and Ryu, H. (2012). Deregulation of BRCA1 leads to impaired spatiotemporal dynamics of gamma-H2AX and DNA damage responses in Huntington's disease. *Molecular neurobiology* **45**(3), 550-63, 10.1007/s12035-012-8274-9.

Jeong, H., Cohen, D. E., Cui, L., Supinski, A., Savas, J. N., Mazzulli, J. R., Yates, J. R., 3rd, Bordone, L., Guarente, L. and Krainc, D. (2012). Sirt1 mediates neuroprotection from mutant huntingtin by activation of the TORC1 and CREB transcriptional pathway. *Nature medicine* **18**(1), 159-65, 10.1038/nm.2559.

Jeyapaul, J. and Jaiswal, A. K. (2000). Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of gamma-glutamylcysteine synthetase heavy subunit gene. *Biochemical pharmacology* **59**(11), 1433-9.

Ji, L., Li, H., Gao, P., Shang, G., Zhang, D. D., Zhang, N. and Jiang, T. (2013). Nrf2 pathway regulates multidrug-resistance-associated protein 1 in small cell lung cancer. *PloS one* **8**(5), e63404, 10.1371/journal.pone.0063404.

Jiang, H., Nucifora, F. C., Jr., Ross, C. A. and DeFranco, D. B. (2003). Cell death triggered by polyglutamine-expanded huntingtin in a neuronal cell line is associated with degradation of CREB-binding protein. *Human molecular genetics* **12**(1), 1-12.

Jiang, H., Poirier, M. A., Liang, Y., Pei, Z., Weiskittel, C. E., Smith, W. W., DeFranco, D. B. and Ross, C. A. (2006). Depletion of CBP is directly linked with cellular toxicity caused by mutant huntingtin. *Neurobiology of disease* **23**(3), 543-51, 10.1016/j.nbd.2006.04.011.

Jiang, M., Wang, J., Fu, J., Du, L., Jeong, H., West, T., Xiang, L., Peng, Q., Hou, Z., Cai, H., Seredenina, T., Arbez, N., Zhu, S., Sommers, K., Qian, J., Zhang, J., Mori, S., Yang, X. W., Tamashiro, K. L., Aja, S., Moran, T. H., Luthi-Carter, R., Martin, B., Maudsley, S., Mattson, M. P., Cichewicz, R. H., Ross, C. A., Holtzman, D. M., Krainc, D. and Duan, W. (2012). Neuroprotective role of Sirt1 in mammalian models of Huntington's disease through activation of multiple Sirt1 targets. *Nature medicine* **18**(1), 153-8, 10.1038/nm.2558.

Jin, Y. N., Yu, Y. V., Gundemir, S., Jo, C., Cui, M., Tieu, K. and Johnson, G. V. (2013). Impaired mitochondrial dynamics and Nrf2 signaling contribute to compromised responses to oxidative stress in striatal cells expressing full-length mutant huntingtin. *PloS one* **8**(3), e57932, 10.1371/journal.pone.0057932.

Jin, Z., Gao, F., Flagg, T. and Deng, X. (2004). Nicotine induces multi-site phosphorylation of Bad in association with suppression of apoptosis. *The Journal of biological chemistry* **279**(22), 23837-44, 10.1074/jbc.M402566200.

Johnson, G. V., Cox, T. M., Lockhart, J. P., Zinnerman, M. D., Miller, M. L. and Powers, R. E. (1997). Transglutaminase activity is increased in Alzheimer's disease brain. *Brain research* **751**(2), 323-9.

Johri, A. and Beal, M. F. (2012). Antioxidants in Huntington's disease. *Biochimica et biophysica acta* **1822**(5), 664-74, 10.1016/j.bbadis.2011.11.014.

Jones, D. P. (2006). Redefining oxidative stress. *Antioxidants & redox signaling* **8**(9-10), 1865-79, 10.1089/ars.2006.8.1865.

Jones, D. P. (2008). Radical-free biology of oxidative stress. *American journal of physiology. Cell physiology* **295**(4), C849-68, 10.1152/ajpcell.00283.2008.

Josiassen, R. C., Curry, L. M. and Mancall, E. L. (1983). Development of neuropsychological deficits in Huntington's disease. *Archives of neurology* **40**(13), 791-6.

Ju, T. C., Chen, H. M., Lin, J. T., Chang, C. P., Chang, W. C., Kang, J. J., Sun, C. P., Tao, M. H., Tu, P. H., Chang, C., Dickson, D. W. and Chern, Y. (2011). Nuclear translocation of AMPK-alpha1 potentiates striatal neurodegeneration in Huntington's disease. *The Journal of cell biology* **194**(2), 209-27, 10.1083/jcb.201105010.

Kahlem, P., Green, H. and Djian, P. (1998). Transglutaminase action imitates Huntington's disease: selective polymerization of Huntingtin containing expanded polyglutamine. *Molecular cell* **1**(4), 595-601.

Kahlem, P., Terre, C., Green, H. and Djian, P. (1996). Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed cross-linking: relevance to diseases of the nervous system. *Proceedings of the National Academy of Sciences of the United States of America* **93**(25), 14580-5.

Kalyanaraman, B., Darley-Usmar, V., Davies, K. J., Dennery, P. A., Forman, H. J., Grisham, M. B., Mann, G. E., Moore, K., Roberts, L. J., 2nd and Ischiropoulos, H. (2012). Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free radical biology & medicine* **52**(1), 1-6, 10.1016/j.freeradbiomed.2011.09.030.

Karpuj, M. V., Becher, M. W., Springer, J. E., Chabas, D., Youssef, S., Pedotti, R., Mitchell, D. and Steinman, L. (2002). Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. *Nature medicine* **8**(2), 143-9, 10.1038/nm0202-143.

Karpuj, M. V., Garren, H., Slunt, H., Price, D. L., Gusella, J., Becher, M. W. and Steinman, L. (1999). Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei. *Proceedings of the National Academy of Sciences of the United States of America* **96**(13), 7388-93.

Kasai, H. (2002). Chemistry-based studies on oxidative DNA damage: formation, repair, and mutagenesis. *Free radical biology & medicine* **33**(4), 450-6.

Kaspar, J. W. and Jaiswal, A. K. (2010). An autoregulatory loop between Nrf2 and Cul3-Rbx1 controls their cellular abundance. *The Journal of biological chemistry* **285**(28), 21349-58, 10.1074/jbc.M110.121863.

Kaspar, J. W., Niture, S. K. and Jaiswal, A. K. (2012). Antioxidant-induced INrf2 (Keap1) tyrosine 85 phosphorylation controls the nuclear export and degradation of the INrf2-Cul3-Rbx1 complex to allow normal Nrf2 activation and repression. *Journal of cell science* **125**(Pt 4), 1027-38, 10.1242/jcs.097295.

Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A. and Yamamoto, M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes to cells : devoted to molecular & cellular mechanisms* **6**(10), 857-68.

Kawai, Y., Garduno, L., Theodore, M., Yang, J. and Arinze, I. J. (2011). Acetylation-deacetylation of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates its transcriptional activity and nucleocytoplasmic localization. *The Journal of biological chemistry* **286**(9), 7629-40, 10.1074/jbc.M110.208173.

Kees, F. (2013). Dimethyl fumarate : a Janus-faced substance? *Expert opinion on pharmacotherapy* **14**(11), 1559-67, 10.1517/14656566.2013.804912.

Kegel, K. B., Meloni, A. R., Yi, Y., Kim, Y. J., Doyle, E., Cuiffo, B. G., Sapp, E., Wang, Y., Qin, Z. H., Chen, J. D., Nevins, J. R., Aronin, N. and DiFiglia, M. (2002). Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. *The Journal of biological chemistry* **277**(9), 7466-76, 10.1074/jbc.M103946200.

Kegel, K. B., Sapp, E., Yoder, J., Cuiffo, B., Sobin, L., Kim, Y. J., Qin, Z. H., Hayden, M. R., Aronin, N., Scott, D. L., Isenberg, G., Goldmann, W. H. and DiFiglia, M. (2005). Huntingtin associates with acidic phospholipids at the plasma membrane. *The Journal of biological chemistry* **280**(43), 36464-73, 10.1074/jbc.M503672200.

Kelly, D. P. and Scarpulla, R. C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes & development* **18**(4), 357-68, 10.1101/gad.1177604.

Keum, Y. S. (2011). Regulation of the Keap1/Nrf2 system by chemopreventive sulforaphane: implications of posttranslational modifications. *Annals of the New York Academy of Sciences* **1229**, 184-9, 10.1111/j.1749-6632.2011.06092.x.

Kiechle, T., Dedeoglu, A., Kubilus, J., Kowall, N. W., Beal, M. F., Friedlander, R. M., Hersch, S. M. and Ferrante, R. J. (2002). Cytochrome C and caspase-9 expression in Huntington's disease. *Neuromolecular medicine* **1**(3), 183-95, 10.1385/NMM:1:3:183.

Killoran, A. and Biglan, K. M. (2012). 8-OHdG: its (limited) potential as a biomarker for Huntington's disease. *Biomarkers in medicine* **6**(6), 777-80, 10.2217/bmm.12.84.

Kim, A. H., Khursigara, G., Sun, X., Franke, T. F. and Chao, M. V. (2001). Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Molecular and cellular biology* **21**(3), 893-901, 10.1128/MCB.21.3.893-901.2001.

Kim, J., Amante, D. J., Moody, J. P., Edgerly, C. K., Bordiuk, O. L., Smith, K., Matson, S. A., Matson, W. R., Scherzer, C. R., Rosas, H. D., Hersch, S. M. and Ferrante, R. J. (2010). Reduced creatine kinase as a central and peripheral biomarker in Huntington's disease. *Biochimica et biophysica acta* **1802**(7-8), 673-81, 10.1016/j.bbadis.2010.05.001.

Kim, J., Moody, J. P., Edgerly, C. K., Bordiuk, O. L., Cormier, K., Smith, K., Beal, M. F. and Ferrante, R. J. (2010). Mitochondrial loss, dysfunction and altered dynamics in Huntington's disease. *Human molecular genetics* **19**(20), 3919-35, 10.1093/hmg/ddq306.

Kim, J. H., Yu, S., Chen, J. D. and Kong, A. N. (2013). The nuclear cofactor RAC3/AIB1/SRC-3 enhances Nrf2 signaling by interacting with transactivation domains. *Oncogene* **32**(4), 514-27, 10.1038/onc.2012.59.

Kim, S. H., Thomas, C. A., Andre, V. M., Cummings, D. M., Cepeda, C., Levine, M. S. and Ehrlich, M. E. (2011). Forebrain striatal-specific expression of mutant huntingtin protein in vivo induces cell-autonomous age-dependent alterations in sensitivity to excitotoxicity and mitochondrial function. *ASN neuro* **3**(3), e00060, 10.1042/AN20110009.

Kim, Y. C., Yamaguchi, Y., Kondo, N., Masutani, H. and Yodoi, J. (2003). Thioredoxin-dependent redox regulation of the antioxidant responsive element (ARE) in electrophile response. *Oncogene* **22**(12), 1860-5, 10.1038/sj.onc.1206369.

Kim, Y. J., Ahn, J. Y., Liang, P., Ip, C., Zhang, Y. and Park, Y. M. (2007). Human prx1 gene is a target of Nrf2 and is up-regulated by hypoxia/reoxygenation: implication to tumor biology. *Cancer research* **67**(2), 546-54, 10.1158/0008-5472.CAN-06-2401.

Kim, Y. S. and Han, S. (2000). Nitric oxide protects Cu,Zn-superoxide dismutase from hydrogen peroxide-induced inactivation. *FEBS letters* **479**(1-2), 25-8.

Kirkwood, S. C., Siemers, E., Viken, R., Hodes, M. E., Conneally, P. M., Christian, J. C. and Foroud, T. (2002). Longitudinal personality changes among presymptomatic Huntington disease gene carriers. *Neuropsychiatry, neuropsychology, and behavioral neurology* **15**(3), 192-7.

Kita, H., Carmichael, J., Swartz, J., Muro, S., Wyttenbach, A., Matsubara, K., Rubinsztein, D. C. and Kato, K. (2002). Modulation of polyglutamine-induced cell death by genes identified by expression profiling. *Human molecular genetics* **11**(19), 2279-87.

Klepac, N., Relja, M., Klepac, R., Hecimovic, S., Babic, T. and Trkulja, V. (2007). Oxidative stress parameters in plasma of Huntington's disease patients, asymptomatic Huntington's disease gene carriers and healthy subjects : a cross-sectional study. *Journal of neurology* **254**(12), 1676-83, 10.1007/s00415-007-0611-y.

Kobayashi, M. and Yamamoto, M. (2006). Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Advances in enzyme regulation* **46**, 113-40, 10.1016/j.advenzreg.2006.01.007.

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y. S., Ueno, I., Sakamoto, A., Tong, K. I., Kim, M., Nishito, Y., Iemura, S., Natsume, T., Ueno, T., Kominami, E., Motohashi, H., Tanaka, K. and Yamamoto, M. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature cell biology* **12**(3), 213-23, 10.1038/ncb2021.

Koppula, S., Kumar, H., Kim, I. S. and Choi, D. K. (2012). Reactive oxygen species and inhibitors of inflammatory enzymes, NADPH oxidase, and iNOS in experimental models of Parkinson's disease. *Mediators of inflammation* **2012**, 823902, 10.1155/2012/823902.

Koppula, S., Kumar, H., More, S. V., Lim, H. W., Hong, S. M. and Choi, D. K. (2012). Recent updates in redox regulation and free radical scavenging effects by herbal products in experimental models of Parkinson's disease. *Molecules* **17**(10), 11391-420, 10.3390/molecules171011391.

Koroshetz, W. J., Jenkins, B. G., Rosen, B. R. and Beal, M. F. (1997). Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. *Annals of neurology* **41**(2), 160-5, 10.1002/ana.410410206.

Krause, K. H. (2004). Tissue distribution and putative physiological function of NOX family NADPH oxidases. *Japanese journal of infectious diseases* **57**(5), S28-9.

Kuemmerle, J. F. (2003). IGF-I elicits growth of human intestinal smooth muscle cells by activation of PI3K, PDK-1, and p70S6 kinase. *American journal of physiology. Gastrointestinal and liver physiology* **284**(3), G411-22, 10.1152/ajpgi.00310.2002.

Kumar, P., Kalonia, H. and Kumar, A. (2010). Huntington's disease: pathogenesis to animal models. *Pharmacological reports : PR* **62**(1), 1-14.

Kuwert, T., Lange, H. W., Langen, K. J., Herzog, H., Aulich, A. and Feinendegen, L. E. (1990). Cortical and subcortical glucose consumption measured by PET in patients with Huntington's disease. *Brain : a journal of neurology* **113 (Pt 5)**, 1405-23.

La Spada, A. (1999) [Updated 2011]. Spinal and Bulbar Muscular Atrophy. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1333/

Laffita-Mesa, J. M., Velázquez-Pérez, L. C, Santos Falcón, N., Cruz-Mariño, T., González Zaldívar, Y., Vázquez Mojena, Y., Almaguer-Gotay, D., Almaguer Mederos, L. E., Rodríguez Labrada, R.

(2012). Unexpanded and intermediate CAG polymorphisms at the SCA2 locus (ATXN2) in the Cuban population: evidence about the origin of expanded SCA2 alleles. *European Journal of Human Genetics* **20**(1):41-9. 10.1038/ejhg.2011.154. Epub 2011 Sep 21.

Lagoa, R., Lopez-Sanchez, C., Samhan-Arias, A. K., Ganan, C. M., Garcia-Martinez, V. and Gutierrez-Merino, C. (2009). Kaempferol protects against rat striatal degeneration induced by 3-nitropropionic acid. *Journal of neurochemistry* **111**(2), 473-87, 10.1111/j.1471-4159.2009.06331.x.

Landegent, J. E., Jansen in de Wal, N., Fisser-Groen, Y. M., Bakker, E., van der Ploeg, M. and Pearson, P. L. (1986). Fine mapping of the Huntington disease linked D4S10 locus by non-radioactive in situ hybridization. *Human genetics* **73**(4), 354-7.

Landwehrmeyer, G. B., McNeil, S. M., Dure, L. S. t., Ge, P., Aizawa, H., Huang, Q., Ambrose, C. M., Duyao, M. P., Bird, E. D., Bonilla, E. and et al. (1995). Huntington's disease gene: regional and cellular expression in brain of normal and affected individuals. *Annals of neurology* **37**(2), 218-30, 10.1002/ana.410370213.

Langbehn, D. R., Brinkman, R. R., Falush, D., Paulsen, J. S., Hayden, M. R. and International Huntington's Disease Collaborative, G. (2004). A new model for prediction of the age of onset and penetrance for Huntington's disease based on CAG length. *Clinical genetics* **65**(4), 267-77, 10.1111/j.1399-0004.2004.00241.x.

Langston, W., Circu, M. L. and Aw, T. Y. (2008). Insulin stimulation of gamma-glutamylcysteine ligase catalytic subunit expression increases endothelial GSH during oxidative stress: influence of low glucose. *Free radical biology & medicine* **45**(11), 1591-9, 10.1016/j.freeradbiomed.2008.09.013.

Lanska, D. J. (2000). George Huntington (1850-1916) and hereditary chorea. *Journal of the history of the neurosciences* **9**(1), 76-89, 10.1076/0964-704X(200004)9:1;1-2;FT076.

Laprairie, R. B., Warford, J. R., Hutchings, S., Robertson, G. S., Kelly, M. E. and Denovan-Wright, E. M. (2014). The cytokine and endocannabinoid systems are co-regulated by NF-kappaB p65/RelA in cell culture and transgenic mouse models of Huntington's disease and in striatal tissue from Huntington's disease patients. *Journal of neuroimmunology* **267**(1-2), 61-72, 10.1016/j.jneuroim.2013.12.008.

Lasker, A. G., Zee, D. S., Hain, T. C., Folstein, S. E. and Singer, H. S. (1987). Saccades in Huntington's disease: initiation defects and distractibility. *Neurology* **37**(3), 364-70.

Lastres-Becker, I., Rub, U. and Auburger, G. (2008). Spinocerebellar ataxia 2 (SCA2). *Cerebellum* **7**(2), 115-24, 10.1007/s12311-008-0019-y.

Lau, A., Tian, W., Whitman, S. A. and Zhang, D. D. (2013). The predicted molecular weight of Nrf2: it is what it is not. *Antioxidants & redox signaling* **18**(1), 91-3, 10.1089/ars.2012.4754.

Lawler, J. M., Barnes, W. S., Wu, G., Song, W. and Demaree, S. (2002). Direct antioxidant properties of creatine. *Biochemical and biophysical research communications* **290**(1), 47-52, 10.1006/bbrc.2001.6164.

Lazarev, V. F., Sverchinskyi, D. V., Ippolitova, M. V., Stepanova, A. V., Guzhova, I. V. and Margulis, B. A. (2013). Factors Affecting Aggregate Formation in Cell Models of Huntington's Disease and Amyotrophic Lateral Sclerosis. *Acta naturae* **5**(2), 81-9.

Leavitt, B. R., van Raamsdonk, J. M., Shehadeh, J., Fernandes, H., Murphy, Z., Graham, R. K., Wellington, C. L., Raymond, L. A. and Hayden, M. R. (2006). Wild-type huntingtin protects neurons from excitotoxicity. *Journal of neurochemistry* **96**(4), 1121-9, 10.1111/j.1471-4159.2005.03605.x.

LeBel, C. P., Ischiropoulos, H. and Bondy, S. C. (1992). Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chemical research in toxicology* **5**(2), 227-31.

Lee, H., Noh, J. Y., Oh, Y., Kim, Y., Chang, J. W., Chung, C. W., Lee, S. T., Kim, M., Ryu, H. and Jung, Y. K. (2012). IRE1 plays an essential role in ER stress-mediated aggregation of mutant huntingtin via the inhibition of autophagy flux. *Human molecular genetics* **21**(1), 101-14, 10.1093/hmg/ddr445.

Lee, J., Kosaras, B., Del Signore, S. J., Cormier, K., McKee, A., Ratan, R. R., Kowall, N. W. and Ryu, H. (2011). Modulation of lipid peroxidation and mitochondrial function improves neuropathology in Huntington's disease mice. *Acta neuropathologica* **121**(4), 487-98, 10.1007/s00401-010-0788-5.

Lee, J. M., Hanson, J. M., Chu, W. A. and Johnson, J. A. (2001). Phosphatidylinositol 3-kinase, not extracellular signal-regulated kinase, regulates activation of the antioxidant-responsive element in IMR-32 human neuroblastoma cells. *The Journal of biological chemistry* **276**(23), 20011-6, 10.1074/jbc.M100734200.

Legleiter, J., Mitchell, E., Lotz, G. P., Sapp, E., Ng, C., DiFiglia, M., Thompson, L. M. and Muchowski, P. J. (2010). Mutant huntingtin fragments form oligomers in a polyglutamine length-dependent

manner in vitro and in vivo. *The Journal of biological chemistry* **285**(19), 14777-90, 10.1074/jbc.M109.093708.
Lesort, M., Chun, W., Johnson, G. V. and Ferrante, R. J. (1999). Tissue transglutaminase is increased in Huntington's disease brain. *Journal of neurochemistry* **73**(5), 2018-27.
Lesort, M., Lee, M., Tucholski, J. and Johnson, G. V. (2003). Cystamine inhibits caspase activity. Implications for the treatment of polyglutamine disorders. *The Journal of biological chemistry* **278**(6), 3825-30, 10.1074/jbc.M205812200.
Li, C., Li, Y., He, L., Agarwal, A. R., Zeng, N., Cadenas, E. and Stiles, B. L. (2013). PI3K/AKT signaling regulates bioenergetics in immortalized hepatocytes. *Free radical biology & medicine* **60**, 29-40, 10.1016/j.freeradbiomed.2013.01.013.
Li, J. Y., Popovic, N. and Brundin, P. (2005). The use of the R6 transgenic mouse models of Huntington's disease in attempts to develop novel therapeutic strategies. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* **2**(3), 447-64, 10.1602/neurorx.2.3.447.
Li, S. H., Cheng, A. L., Zhou, H., Lam, S., Rao, M., Li, H. and Li, X. J. (2002). Interaction of Huntington disease protein with transcriptional activator Sp1. *Molecular and cellular biology* **22**(5), 1277-87.
Li, S. H. and Li, X. J. (2004). Huntingtin and its role in neuronal degeneration. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **10**(5), 467-75, 10.1177/1073858404266777.
Li, S. H. and Li, X. J. (2004). Huntingtin-protein interactions and the pathogenesis of Huntington's disease. *Trends in genetics : TIG* **20**(3), 146-54, 10.1016/j.tig.2004.01.008.
Li, S. H., Schilling, G., Young, W. S., 3rd, Li, X. J., Margolis, R. L., Stine, O. C., Wagster, M. V., Abbott, M. H., Franz, M. L., Ranen, N. G. and et al. (1993). Huntington's disease gene (IT15) is widely expressed in human and rat tissues. *Neuron* **11**(5), 985-93.
Li, W., Liu, H., Zhou, J. S., Cao, J. F., Zhou, X. B., Choi, A. M., Chen, Z. H. and Shen, H. H. (2012). Caveolin-1 inhibits expression of antioxidant enzymes through direct interaction with nuclear erythroid 2 p45-related factor-2 (Nrf2). *The Journal of biological chemistry* **287**(25), 20922-30, 10.1074/jbc.M112.352336.
Li, W., Yu, S. W. and Kong, A. N. (2006). Nrf2 possesses a redox-sensitive nuclear exporting signal in the Neh5 transactivation domain. *The Journal of biological chemistry* **281**(37), 27251-63, 10.1074/jbc.M602746200.
Li, Y., Zhu, H., Kuppusamy, P., Roubaud, V., Zweier, J. L. and Trush, M. A. (1998). Validation of lucigenin (bis-N-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *The Journal of biological chemistry* **273**(4), 2015-23.
Lievens, J. C., Iche, M., Laval, M., Faivre-Sarrailh, C. and Birman, S. (2008). AKT-sensitive or insensitive pathways of toxicity in glial cells and neurons in Drosophila models of Huntington's disease. *Human molecular genetics* **17**(6), 882-94, 10.1093/hmg/ddm360.
Lim, D., Fedrizzi, L., Tartari, M., Zuccato, C., Cattaneo, E., Brini, M. and Carafoli, E. (2008). Calcium homeostasis and mitochondrial dysfunction in striatal neurons of Huntington disease. *The Journal of biological chemistry* **283**(9), 5780-9, 10.1074/jbc.M704704200.
Lin, C. H., Tallaksen-Greene, S., Chien, W. M., Cearley, J. A., Jackson, W. S., Crouse, A. B., Ren, S., Li, X. J., Albin, R. L. and Detloff, P. J. (2001). Neurological abnormalities in a knock-in mouse model of Huntington's disease. *Human molecular genetics* **10**(2), 137-44.
Lin, J., Handschin, C. and Spiegelman, B. M. (2005). Metabolic control through the PGC-1 family of transcription coactivators. *Cell metabolism* **1**(6), 361-70, 10.1016/j.cmet.2005.05.004.
Lin, J., Wu, P. H., Tarr, P. T., Lindenberg, K. S., St-Pierre, J., Zhang, C. Y., Mootha, V. K., Jager, S., Vianna, C. R., Reznick, R. M., Cui, L., Manieri, M., Donovan, M. X., Wu, Z., Cooper, M. P., Fan, M. C., Rohas, L. M., Zavacki, A. M., Cinti, S., Shulman, G. I., Lowell, B. B., Krainc, D. and Spiegelman, B. M. (2004). Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell* **119**(1), 121-35, 10.1016/j.cell.2004.09.013.
Lin, M. T. and Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443**(7113), 787-95, 10.1038/nature05292.
Lin, Y. S., Cheng, T. H., Chang, C. P., Chen, H. M. and Chern, Y. (2013). Enhancement of brain-type creatine kinase activity ameliorates neuronal deficits in Huntington's disease. *Biochimica et biophysica acta* **1832**(6), 742-53, 10.1016/j.bbadis.2013.02.006.
Liot, G., Bossy, B., Lubitz, S., Kushnareva, Y., Sejbuk, N. and Bossy-Wetzel, E. (2009). Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an NMDA- and ROS-dependent pathway. *Cell death and differentiation* **16**(6), 899-909, 10.1038/cdd.2009.22.

Liu, C. S., Cheng, W. L., Kuo, S. J., Li, J. Y., Soong, B. W. and Wei, Y. H. (2008). Depletion of mitochondrial DNA in leukocytes of patients with poly-Q diseases. *Journal of the neurological sciences* **264**(1-2), 18-21, 10.1016/j.jns.2007.07.016.

Liu, Y., Hettinger, C. L., Zhang, D., Rezvani, K., Wang, X. and Wang, H. (2014). Sulforaphane enhances proteasomal and autophagic activities in mice and is a potential therapeutic reagent for Huntington's disease. *Journal of neurochemistry*, 10.1111/jnc.12647.

Lopes, C., Ribeiro, M., Duarte, A. I., Humbert, S., Saudou, F., Pereira de Almeida, L., Hayden, M. and Rego, A. C. (2013). IGF-1 Intranasal Administration Rescues Huntington's Disease Phenotypes in YAC128 Mice. *Molecular neurobiology*, 10.1007/s12035-013-8585-5.

Louis, E. D., Anderson, K. E., Moskowitz, C., Thorne, D. Z. and Marder, K. (2000). Dystonia-predominant adult-onset Huntington disease: association between motor phenotype and age of onset in adults. *Archives of neurology* **57**(9), 1326-30.

Lugo-Huitron, R., Ugalde Muniz, P., Pineda, B., Pedraza-Chaverri, J., Rios, C. and Perez-de la Cruz, V. (2013). Quinolinic acid: an endogenous neurotoxin with multiple targets. *Oxidative medicine and cellular longevity* **2013**, 104024, 10.1155/2013/104024.

Lunkes, A., Lindenberg, K. S., Ben-Haiem, L., Weber, C., Devys, D., Landwehrmeyer, G. B., Mandel, J. L. and Trottier, Y. (2002). Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. *Molecular cell* **10**(2), 259-69.

Madhavan, L., Ourednik, V. and Ourednik, J. (2008). Neural stem/progenitor cells initiate the formation of cellular networks that provide neuroprotection by growth factor-modulated antioxidant expression. *Stem cells* **26**(1), 254-65, 10.1634/stemcells.2007-0221.

Maglione, V., Cannella, M., Gradini, R., Cislaghi, G. and Squitieri, F. (2006). Huntingtin fragmentation and increased caspase 3, 8 and 9 activities in lymphoblasts with heterozygous and homozygous Huntington's disease mutation. *Mechanisms of ageing and development* **127**(2), 213-6, 10.1016/j.mad.2005.09.011.

Maglione, V., Marchi, P., Di Pardo, A., Lingrell, S., Horkey, M., Tidmarsh, E. and Sipione, S. (2010). Impaired ganglioside metabolism in Huntington's disease and neuroprotective role of GM1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**(11), 4072-80, 10.1523/JNEUROSCI.6348-09.2010.

Maher, J. M., Dieter, M. Z., Aleksunes, L. M., Slitt, A. L., Guo, G., Tanaka, Y., Scheffer, G. L., Chan, J. Y., Manautou, J. E., Chen, Y., Dalton, T. P., Yamamoto, M. and Klaassen, C. D. (2007). Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. *Hepatology* **46**(5), 1597-610, 10.1002/hep.21831.

Maldonado, P. D., Molina-Jijon, E., Villeda-Hernandez, J., Galvan-Arzate, S., Santamaria, A. and Pedraza-Chaverri, J. (2010). NAD(P)H oxidase contributes to neurotoxicity in an excitotoxic/prooxidant model of Huntington's disease in rats: protective role of apocynin. *Journal of neuroscience research* **88**(3), 620-9, 10.1002/jnr.22240.

Maldonado, P. D., Perez-De La Cruz, V., Torres-Ramos, M., Silva-Islas, C., Lecona-Vargas, R., Lugo-Huitron, R., Blanco-Ayala, T., Ugalde-Muniz, P., Vazquez-Cervantes, G. I., Fortoul, T. I., Ali, S. F. and Santamaria, A. (2012). Selenium-induced antioxidant protection recruits modulation of thioredoxin reductase during excitotoxic/pro-oxidant events in the rat striatum. *Neurochemistry international* **61**(2), 195-206, 10.1016/j.neuint.2012.05.004.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S. W. and Bates, G. P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* **87**(3), 493-506.

Mantamadiotis, T., Lemberger, T., Bleckmann, S. C., Kern, H., Kretz, O., Martin Villalba, A., Tronche, F., Kellendonk, C., Gau, D., Kapfhammer, J., Otto, C., Schmid, W. and Schutz, G. (2002). Disruption of CREB function in brain leads to neurodegeneration. *Nature genetics* **31**(1), 47-54, 10.1038/ng882.

Mao, Z., Choo, Y. S. and Lesort, M. (2006). Cystamine and cysteamine prevent 3-NP-induced mitochondrial depolarization of Huntington's disease knock-in striatal cells. *The European journal of neuroscience* **23**(7), 1701-10, 10.1111/j.1460-9568.2006.04686.x.

Mariani, E., Polidori, M. C., Cherubini, A. and Mecocci, P. (2005). Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **827**(1), 65-75, 10.1016/j.jchromb.2005.04.023.

Marin-Garcia, J., Akhmedov, A. T. and Moe, G. W. (2013). Mitochondria in heart failure: the emerging role of mitochondrial dynamics. *Heart failure reviews* **18**(4), 439-56, 10.1007/s10741-012-9330-2.

Maruyama, W., Hashizume, Y., Matsubara, K. and Naoi, M. (1996). Identification of 3-nitro-L-tyrosine, a product of nitric oxide and superoxide, as an indicator of oxidative stress in the human brain. *Journal of chromatography. B, Biomedical applications* **676**(1), 153-8.

Mastroberardino, P. G., Iannicola, C., Nardacci, R., Bernassola, F., De Laurenzi, V., Melino, G., Moreno, S., Pavone, F., Oliverio, S., Fesus, L. and Piacentini, M. (2002). 'Tissue' transglutaminase ablation reduces neuronal death and prolongs survival in a mouse model of Huntington's disease. *Cell death and differentiation* **9**(9), 873-80, 10.1038/sj.cdd.4401093.

Matilla-Duenas, A., Goold, R. and Giunti, P. (2008). Clinical, genetic, molecular, and pathophysiological insights into spinocerebellar ataxia type 1. *Cerebellum* **7**(2), 106-14, 10.1007/s12311-008-0009-0.

Matthews, R. T., Yang, L., Jenkins, B. G., Ferrante, R. J., Rosen, B. R., Kaddurah-Daouk, R. and Beal, M. F. (1998). Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**(1), 156-63.

Maxwell, S. R., Thomason, H., Sandler, D., Leguen, C., Baxter, M. A., Thorpe, G. H., Jones, A. F. and Barnett, A. H. (1997). Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. *European journal of clinical investigation* **27**(6), 484-90.

Mayr, B. and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature reviews. Molecular cell biology* **2**(8), 599-609, 10.1038/35085068.

Mazzola, J. L. and Sirover, M. A. (2001). Reduction of glyceraldehyde-3-phosphate dehydrogenase activity in Alzheimer's disease and in Huntington's disease fibroblasts. *Journal of neurochemistry* **76**(2), 442-9.

Mazzola, J. L. and Sirover, M. A. (2002). Alteration of nuclear glyceraldehyde-3-phosphate dehydrogenase structure in Huntington's disease fibroblasts. *Brain research. Molecular brain research* **100**(1-2), 95-101.

McConoughey, S. J., Basso, M., Niatsetskaya, Z. V., Sleiman, S. F., Smirnova, N. A., Langley, B. C., Mahishi, L., Cooper, A. J., Antonyak, M. A., Cerione, R. A., Li, B., Starkov, A., Chaturvedi, R. K., Beal, M. F., Coppola, G., Geschwind, D. H., Ryu, H., Xia, L., Iismaa, S. E., Pallos, J., Pasternack, R., Hils, M., Fan, J., Raymond, L. A., Marsh, J. L., Thompson, L. M. and Ratan, R. R. (2010). Inhibition of transglutaminase 2 mitigates transcriptional dysregulation in models of Huntington disease. *EMBO molecular medicine* **2**(9), 349-70, 10.1002/emmm.201000084.

McGill, J. K. and Beal, M. F. (2006). PGC-1alpha, a new therapeutic target in Huntington's disease? *Cell* **127**(3), 465-8, 10.1016/j.cell.2006.10.023.

Menalled, L., El-Khodor, B. F., Patry, M., Suarez-Farinas, M., Orenstein, S. J., Zahasky, B., Leahy, C., Wheeler, V., Yang, X. W., MacDonald, M., Morton, A. J., Bates, G., Leeds, J., Park, L., Howland, D., Signer, E., Tobin, A. and Brunner, D. (2009). Systematic behavioral evaluation of Huntington's disease transgenic and knock-in mouse models. *Neurobiology of disease* **35**(3), 319-36, 10.1016/j.nbd.2009.05.007.

Menalled, L. B., Sison, J. D., Dragatsis, I., Zeitlin, S. and Chesselet, M. F. (2003). Time course of early motor and neuropathological anomalies in a knock-in mouse model of Huntington's disease with 140 CAG repeats. *The Journal of comparative neurology* **465**(1), 11-26, 10.1002/cne.10776.

Mende-Mueller, L. M., Toneff, T., Hwang, S. R., Chesselet, M. F. and Hook, V. Y. (2001). Tissue-specific proteolysis of Huntingtin (htt) in human brain: evidence of enhanced levels of N- and C-terminal htt fragments in Huntington's disease striatum. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **21**(6), 1830-7.

Menzies, F. M., Huebener, J., Renna, M., Bonin, M., Riess, O. and Rubinsztein, D. C. (2010). Autophagy induction reduces mutant ataxin-3 levels and toxicity in a mouse model of spinocerebellar ataxia type 3. *Brain : a journal of neurology* **133**(Pt 1), 93-104, 10.1093/brain/awp292.

Michel, T. M., Camara, S., Tatschner, T., Frangou, S., Sheldrick, A. J., Riederer, P. and Grunblatt, E. (2010). Increased xanthine oxidase in the thalamus and putamen in depression. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry* **11**(2 Pt 2), 314-20, 10.3109/15622970802123695.

Michel, T. M., Sheldrick, A. J., Camara, S., Grunblatt, E., Schneider, F. and Riederer, P. (2011). Alteration of the pro-oxidant xanthine oxidase (XO) in the thalamus and occipital cortex of patients with schizophrenia. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry* **12**(8), 588-97, 10.3109/15622975.2010.526146.

Milakovic, T. and Johnson, G. V. (2005). Mitochondrial respiration and ATP production are significantly impaired in striatal cells expressing mutant huntingtin. *The Journal of biological chemistry* **280**(35), 30773-82, 10.1074/jbc.M504749200.

Mishra, S. and Murphy, L. J. (2006). The p53 oncoprotein is a substrate for tissue transglutaminase kinase activity. *Biochemical and biophysical research communications* **339**(2), 726-30, 10.1016/j.bbrc.2005.11.071.

Mitomi, Y., Nomura, T., Kurosawa, M., Nukina, N. and Furukawa, Y. (2012). Post-aggregation oxidation of mutant huntingtin controls the interactions between aggregates. *The Journal of biological chemistry* **287**(41), 34764-75, 10.1074/jbc.M112.387035.

Mochel, F., Charles, P., Seguin, F., Barritault, J., Coussieu, C., Perin, L., Le Bouc, Y., Gervais, C., Carcelain, G., Vassault, A., Feingold, J., Rabier, D. and Durr, A. (2007). Early energy deficit in Huntington disease: identification of a plasma biomarker traceable during disease progression. *PloS one* **2**(7), e647, 10.1371/journal.pone.0000647.

Mochel, F., Durant, B., Meng, X., O'Callaghan, J., Yu, H., Brouillet, E., Wheeler, V. C., Humbert, S., Schiffmann, R. and Durr, A. (2012). Early alterations of brain cellular energy homeostasis in Huntington disease models. *The Journal of biological chemistry* **287**(2), 1361-70, 10.1074/jbc.M111.309849.

Mochel, F., Duteil, S., Marelli, C., Jauffret, C., Barles, A., Holm, J., Sweetman, L., Benoist, J. F., Rabier, D., Carlier, P. G. and Durr, A. (2010). Dietary anaplerotic therapy improves peripheral tissue energy metabolism in patients with Huntington's disease. *European journal of human genetics : EJHG* **18**(9), 1057-60, 10.1038/ejhg.2010.72.

Moi, P., Chan, K., Asunis, I., Cao, A. and Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences of the United States of America* **91**(21), 9926-30.

Moinova, H. R. and Mulcahy, R. T. (1999). Up-regulation of the human gamma-glutamylcysteine synthetase regulatory subunit gene involves binding of Nrf-2 to an electrophile responsive element. *Biochemical and biophysical research communications* **261**(3), 661-8, 10.1006/bbrc.1999.1109.

Montine, T. J., Beal, M. F., Robertson, D., Cudkowicz, M. E., Biaggioni, I., O'Donnell, H., Zackert, W. E., Roberts, L. J. and Morrow, J. D. (1999). Cerebrospinal fluid F2-isoprostanes are elevated in Huntington's disease. *Neurology* **52**(5), 1104-5.

Montoya, A., Price, B. H., Menear, M. and Lepage, M. (2006). Brain imaging and cognitive dysfunctions in Huntington's disease. *Journal of psychiatry & neuroscience : JPN* **31**(1), 21-9.

Mookherjee, P., Quintanilla, R., Roh, M. S., Zmijewska, A. A., Jope, R. S. and Johnson, G. V. (2007). Mitochondrial-targeted active Akt protects SH-SY5Y neuroblastoma cells from staurosporine-induced apoptotic cell death. *Journal of cellular biochemistry* **102**(1), 196-210, 10.1002/jcb.21287.

Mora, A., Lipina, C., Tronche, F., Sutherland, C. and Alessi, D. R. (2005). Deficiency of PDK1 in liver results in glucose intolerance, impairment of insulin-regulated gene expression and liver failure. *The Biochemical journal* **385**(Pt 3), 639-48, 10.1042/BJ20041782.

Morrow, J. D., Chen, Y., Brame, C. J., Yang, J., Sanchez, S. C., Xu, J., Zackert, W. E., Awad, J. A. and Roberts, L. J. (1999). The isoprostanes: unique prostaglandin-like products of free-radical-initiated lipid peroxidation. *Drug metabolism reviews* **31**(1), 117-39, 10.1081/DMR-100101910.

Morrow, J. D., Harris, T. M. and Roberts, L. J., 2nd (1990). Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. *Analytical biochemistry* **184**(1), 1-10.

Morrow, J. D. and Roberts, L. J., 2nd (1996). The isoprostanes. Current knowledge and directions for future research. *Biochemical pharmacology* **51**(1), 1-9.

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *The Biochemical journal* **417**(1), 1-13, 10.1042/BJ20081386.

Nagaoka, U., Kim, K., Jana, N. R., Doi, H., Maruyama, M., Mitsui, K., Oyama, F. and Nukina, N. (2004). Increased expression of p62 in expanded polyglutamine-expressing cells and its association with polyglutamine inclusions. *Journal of neurochemistry* **91**(1), 57-68, 10.1111/j.1471-4159.2004.02692.x.

Nagata, E., Saiardi, A., Tsukamoto, H., Okada, Y., Itoh, Y., Satoh, T., Itoh, J., Margolis, R. L., Takizawa, S., Sawa, A. and Takagi, S. (2011). Inositol hexakisphosphate kinases induce cell death in Huntington disease. *The Journal of biological chemistry* **286**(30), 26680-6, 10.1074/jbc.M111.220749.

Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J. and Graham, R. M. (1994). Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* **264**(5165), 1593-6.

Nakaso, K., Yano, H., Fukuhara, Y., Takeshima, T., Wada-Isoe, K. and Nakashima, K. (2003). PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. *FEBS letters* **546**(2-3), 181-4.

Napoli, E., Wong, S., Hung, C., Ross-Inta, C., Bomdica, P. and Giulivi, C. (2013). Defective mitochondrial disulfide relay system, altered mitochondrial morphology and function in Huntington's disease. *Human molecular genetics* **22**(5), 989-1004, 10.1093/hmg/dds503.

Nasir, J., Floresco, S. B., O'Kusky, J. R., Diewert, V. M., Richman, J. M., Zeisler, J., Borowski, A., Marth, J. D., Phillips, A. G. and Hayden, M. R. (1995). Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell* **81**(5), 811-23.

Nelson, D. L. and Cox, M. M. (2004). Principles of Biochemistry Fourth Edition (Freeman Publishers), New York.

Neri, C. (2011). Value of Invertebrate Genetics and Biology to Develop Neuroprotective and Preventive Medicine in Huntington's Disease. In *Neurobiology of Huntington's Disease: Applications to Drug Discovery* (D. C. Lo and R. E. Hughes, Eds.), Boca Raton (FL).

Neuwald, A. F. and Hirano, T. (2000). HEAT repeats associated with condensins, cohesins, and other complexes involved in chromosome-related functions. *Genome research* **10**(10), 1445-52.

Nicholls, D. G. (2012). Fluorescence measurement of mitochondrial membrane potential changes in cultured cells. *Methods in molecular biology* **810**, 119-33, 10.1007/978-1-61779-382-0_8.

Niture, S. K., Jain, A. K., Shelton, P. M. and Jaiswal, A. K. (2011). Src subfamily kinases regulate nuclear export and degradation of transcription factor Nrf2 to switch off Nrf2-mediated antioxidant activation of cytoprotective gene expression. *The Journal of biological chemistry* **286**(33), 28821-32, 10.1074/jbc.M111.255042.

Niture, S. K. and Jaiswal, A. K. (2009). Prothymosin-alpha mediates nuclear import of the INrf2/Cul3 Rbx1 complex to degrade nuclear Nrf2. *The Journal of biological chemistry* **284**(20), 13856-68, 10.1074/jbc.M808084200.

Niture, S. K., Kaspar, J. W., Shen, J. and Jaiswal, A. K. (2010). Nrf2 signaling and cell survival. *Toxicology and applied pharmacology* **244**(1), 37-42, 10.1016/j.taap.2009.06.009.

Niture, S. K., Khatri, R. and Jaiswal, A. K. (2014). Regulation of Nrf2-an update. *Free radical biology & medicine* **66**, 36-44, 10.1016/j.freeradbiomed.2013.02.008.

Nucifora, F. C., Jr., Sasaki, M., Peters, M. F., Huang, H., Cooper, J. K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V. L., Dawson, T. M. and Ross, C. A. (2001). Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. *Science* **291**(5512), 2423-8, 10.1126/science.1056784.

Numakawa, T., Adachi, N., Richards, M., Chiba, S. and Kunugi, H. (2013). Brain-derived neurotrophic factor and glucocorticoids: reciprocal influence on the central nervous system. *Neuroscience* **239**, 157-72, 10.1016/j.neuroscience.2012.09.073.

Numazawa, S., Ishikawa, M., Yoshida, A., Tanaka, S. and Yoshida, T. (2003). Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. *American journal of physiology. Cell physiology* **285**(2), C334-42, 10.1152/ajpcell.00043.2003.

O'Brien, C. F., Miller, C., Goldblatt, D., Welle, S., Forbes, G., Lipinski, B., Panzik, J., Peck, R., Plumb, S., Oakes, D., Kurlan, R., Shoulson, I. (1990). Extraneural metabolism in early Huntington's disease. *Annals of neurology* **28**, 300-1.

O'Brien, J., Kla, K. M., Hopkins, I. B., Malecki, E. A. and McKenna, M. C. (2007). Kinetic parameters and lactate dehydrogenase isozyme activities support possible lactate utilization by neurons. *Neurochemical research* **32**(4-5), 597-607, 10.1007/s11064-006-9132-9.

O'Brien, J., Wilson, I., Orton, T. and Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European journal of biochemistry / FEBS* **267**(17), 5421-6.

Obrietan, K. and Hoyt, K. R. (2004). CRE-mediated transcription is increased in Huntington's disease transgenic mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**(4), 791-6, 10.1523/JNEUROSCI.3493-03.2004.

Okuda, S., Nishiyama, N., Saito, H. and Katsuki, H. (1996). Hydrogen peroxide-mediated neuronal cell death induced by an endogenous neurotoxin, 3-hydroxykynurenine. *Proceedings of the National Academy of Sciences of the United States of America* **93**(22), 12553-8.

Olah, J., Klivenyi, P., Gardian, G., Vecsei, L., Orosz, F., Kovacs, G. G., Westerhoff, H. V. and Ovadi, J. (2008). Increased glucose metabolism and ATP level in brain tissue of Huntington's disease transgenic mice. *The FEBS journal* **275**(19), 4740-55, 10.1111/j.1742-4658.2008.06612.x.

Oliveira, J. M., Chen, S., Almeida, S., Riley, R., Goncalves, J., Oliveira, C. R., Hayden, M. R., Nicholls, D. G., Ellerby, L. M. and Rego, A. C. (2006). Mitochondrial-dependent Ca2+ handling in Huntington's disease striatal cells: effect of histone deacetylase inhibitors. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26**(43), 11174-86, 10.1523/JNEUROSCI.3004-06.2006.

Ona, V. O., Li, M., Vonsattel, J. P., Andrews, L. J., Khan, S. Q., Chung, W. M., Frey, A. S., Menon, A. S., Li, X. J., Stieg, P. E., Yuan, J., Penney, J. B., Young, A. B., Cha, J. H. and Friedlander, R. M. (1999). Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature* **399**(6733), 263-7, 10.1038/20446.

Orr, A. L., Li, S., Wang, C. E., Li, H., Wang, J., Rong, J., Xu, X., Mastroberardino, P. G., Greenamyre, J. T. and Li, X. J. (2008). N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**(11), 2783-92, 10.1523/JNEUROSCI.0106-08.2008.

Orr, H. T. (2012). Cell biology of spinocerebellar ataxia. *The Journal of cell biology* **197**(2), 167-77, 10.1083/jcb.201105092.

Paglia, D. E. and Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of laboratory and clinical medicine* **70**(1), 158-69.

Pan, S., World, C. J., Kovacs, C. J. and Berk, B. C. (2009). Glucose 6-phosphate dehydrogenase is regulated through c-Src-mediated tyrosine phosphorylation in endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **29**(6), 895-901, 10.1161/ATVBAHA.109.184812.

Panov, A. V., Gutekunst, C. A., Leavitt, B. R., Hayden, M. R., Burke, J. R., Strittmatter, W. J. and Greenamyre, J. T. (2002). Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nature neuroscience* **5**(8), 731-6, 10.1038/nn884.

Papa, S., Martino, P. L., Capitanio, G., Gaballo, A., De Rasmo, D., Signorile, A. and Petruzzella, V. (2012). The oxidative phosphorylation system in mammalian mitochondria. *Advances in experimental medicine and biology* **942**, 3-37, 10.1007/978-94-007-2869-1_1.

Park, D., Choi, S. S. and Ha, K. S. (2010). Transglutaminase 2: a multi-functional protein in multiple subcellular compartments. *Amino acids* **39**(3), 619-31, 10.1007/s00726-010-0500-z.

Park, E. Y. and Rho, H. M. (2002). The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-p-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. *Molecular and cellular biochemistry* **240**(1-2), 47-55.

Parker, W. D., Jr., Boyson, S. J., Luder, A. S. and Parks, J. K. (1990). Evidence for a defect in NADH: ubiquinone oxidoreductase (complex I) in Huntington's disease. *Neurology* **40**(8), 1231-4.

Pasternak, J. J. (2005). Molecular Genetics of Neurological Disorders, in An Introduction to Human Molecular Genetics: Mechanisms of Inherited Diseases, Second Edition, John Wiley & Sons, Inc., Hoboken, NJ, USA. 10.1002/0471719188.ch14

Paulsen, J. S., Butters, N., Sadek, J. R., Johnson, S. A., Salmon, D. P., Swerdlow, N. R. and Swenson, M. R. (1995). Distinct cognitive profiles of cortical and subcortical dementia in advanced illness. *Neurology* **45**(5), 951-6.

Paulsen, J. S., Ready, R. E., Hamilton, J. M., Mega, M. S. and Cummings, J. L. (2001). Neuropsychiatric aspects of Huntington's disease. *Journal of neurology, neurosurgery, and psychiatry* **71**(3), 310-4.

Paulson, H. (1998) [Updated 2011]. Spinocerebellar Ataxia Type 3. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1196/

Penney, J. B., Jr., Young, A. B., Shoulson, I., Starosta-Rubenstein, S., Snodgrass, S. R., Sanchez-Ramos, J., Ramos-Arroyo, M., Gomez, F., Penchaszadeh, G., Alvir, J. and et al. (1990). Huntington's disease in Venezuela: 7 years of follow-up on symptomatic and asymptomatic individuals. *Movement disorders : official journal of the Movement Disorder Society* **5**(2), 93-9, 10.1002/mds.870050202.

Perez-De La Cruz, V., Carrillo-Mora, P. and Santamaria, A. (2012). Quinolinic Acid, an endogenous molecule combining excitotoxicity, oxidative stress and other toxic mechanisms. *International journal of tryptophan research : IJTR* **5**, 1-8, 10.4137/IJTR.S8158.

Perez-Severiano, F., Santamaria, A., Pedraza-Chaverri, J., Medina-Campos, O. N., Rios, C. and Segovia, J. (2004). Increased formation of reactive oxygen species, but no changes in glutathione peroxidase activity, in striata of mice transgenic for the Huntington's disease mutation. *Neurochemical research* **29**(4), 729-33.

Perluigi, M., Poon, H. F., Maragos, W., Pierce, W. M., Klein, J. B., Calabrese, V., Cini, C., De Marco, C. and Butterfield, D. A. (2005). Proteomic analysis of protein expression and oxidative modification in r6/2 transgenic mice: a model of Huntington disease. *Molecular & cellular proteomics : MCP* **4**(12), 1849-61, 10.1074/mcp.M500090-MCP200.

Perrin, V., Dufour, N., Raoul, C., Hassig, R., Brouillet, E., Aebischer, P., Luthi-Carter, R. and Deglon, N. (2009). Implication of the JNK pathway in a rat model of Huntington's disease. *Experimental neurology* **215**(1), 191-200, 10.1016/j.expneurol.2008.10.008.

Perroud, B., Jafar-Nejad, P., Wikoff, W. R., Gatchel, J. R., Wang, L., Barupal, D. K., Crespo-Barreto, J., Fiehn, O., Zoghbi, H. Y. and Kaddurah-Daouk, R. (2013). Pharmacometabolomic signature of

ataxia SCA1 mouse model and lithium effects. *PloS one* **8**(8), e70610, 10.1371/journal.pone.0070610.

Petersen, A., Hansson, O., Puschban, Z., Sapp, E., Romero, N., Castilho, R. F., Sulzer, D., Rice, M., DiFiglia, M., Przedborski, S. and Brundin, P. (2001). Mice transgenic for exon 1 of the Huntington's disease gene display reduced striatal sensitivity to neurotoxicity induced by dopamine and 6-hydroxydopamine. *The European journal of neuroscience* **14**(9), 1425-35.

Petrasch-Parwez, E., Nguyen, H. P., Lobbecke-Schumacher, M., Habbes, H. W., Wieczorek, S., Riess, O., Andres, K. H., Dermietzel, R. and Von Horsten, S. (2007). Cellular and subcellular localization of Huntingtin [corrected] aggregates in the brain of a rat transgenic for Huntington disease. *The Journal of comparative neurology* **501**(5), 716-30, 10.1002/cne.21272.

Pi, J., Bai, Y., Reece, J. M., Williams, J., Liu, D., Freeman, M. L., Fahl, W. E., Shugar, D., Liu, J., Qu, W., Collins, S. and Waalkes, M. P. (2007). Molecular mechanism of human Nrf2 activation and degradation: role of sequential phosphorylation by protein kinase CK2. *Free radical biology & medicine* **42**(12), 1797-806, 10.1016/j.freeradbiomed.2007.03.001.

Pickrell, A. M., Fukui, H., Wang, X., Pinto, M. and Moraes, C. T. (2011). The striatum is highly susceptible to mitochondrial oxidative phosphorylation dysfunctions. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**(27), 9895-904, 10.1523/JNEUROSCI.6223-10.2011.

Pinto, J. T., Khomenko, T., Szabo, S., McLaren, G. D., Denton, T. T., Krasnikov, B. F., Jeitner, T. M. and Cooper, A. J. (2009). Measurement of sulfur-containing compounds involved in the metabolism and transport of cysteamine and cystamine. Regional differences in cerebral metabolism. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **877**(28), 3434-41, 10.1016/j.jchromb.2009.05.041.

Polidori, M. C., Mecocci, P., Browne, S. E., Senin, U. and Beal, M. F. (1999). Oxidative damage to mitochondrial DNA in Huntington's disease parietal cortex. *Neuroscience letters* **272**(1), 53-6.

Polivka, J., Jr. and Janku, F. (2013). Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. *Pharmacology & therapeutics*, 10.1016/j.pharmthera.2013.12.004.

Pouladi, M. A., Xie, Y., Skotte, N. H., Ehrnhoefer, D. E., Graham, R. K., Kim, J. E., Bissada, N., Yang, X. W., Paganetti, P., Friedlander, R. M., Leavitt, B. R. and Hayden, M. R. (2010). Full-length huntingtin levels modulate body weight by influencing insulin-like growth factor 1 expression. *Human molecular genetics* **19**(8), 1528-38, 10.1093/hmg/ddq026.

Poyton, R. O., Ball, K. A. and Castello, P. R. (2009). Mitochondrial generation of free radicals and hypoxic signaling. *Trends in endocrinology and metabolism: TEM* **20**(7), 332-40, 10.1016/j.tem.2009.04.001.

Pringsheim, T., Wiltshire, K., Day, L., Dykeman, J., Steeves, T. and Jette, N. (2012). The incidence and prevalence of Huntington's disease: a systematic review and meta-analysis. *Movement disorders : official journal of the Movement Disorder Society* **27**(9), 1083-91, 10.1002/mds.25075.

Puigserver, P. and Spiegelman, B. M. (2003). Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocrine reviews* **24**(1), 78-90, 10.1210/er.2002-0012.

Pulst, S. M. (1998) [Updated 2013]. Spinocerebellar Ataxia Type 2. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1275/

Qiu, X., Brown, K., Hirschey, M. D., Verdin, E. and Chen, D. (2010). Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell metabolism* **12**(6), 662-7, 10.1016/j.cmet.2010.11.015.

Quintanilla, R. A., Jin, Y. N., Fuenzalida, K., Bronfman, M. and Johnson, G. V. (2008). Rosiglitazone treatment prevents mitochondrial dysfunction in mutant huntingtin-expressing cells: possible role of peroxisome proliferator-activated receptor-gamma (PPARgamma) in the pathogenesis of Huntington disease. *The Journal of biological chemistry* **283**(37), 25628-37, 10.1074/jbc.M804291200.

Rada, P., Rojo, A. I., Chowdhry, S., McMahon, M., Hayes, J. D. and Cuadrado, A. (2011). SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. *Molecular and cellular biology* **31**(6), 1121-33, 10.1128/MCB.01204-10.

Rada, P., Rojo, A. I., Evrard-Todeschi, N., Innamorato, N. G., Cotte, A., Jaworski, T., Tobon-Velasco, J. C., Devijver, H., Garcia-Mayoral, M. F., Van Leuven, F., Hayes, J. D., Bertho, G. and Cuadrado, A. (2012). Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis. *Molecular and cellular biology* **32**(17), 3486-99, 10.1128/MCB.00180-12.

Raha, S. and Robinson, B. H. (2001). Mitochondria, oxygen free radicals, and apoptosis. *American journal of medical genetics* **106**(1), 62-70, 10.1002/ajmg.1398.

Rajput, P. S., Kharmate, G., Norman, M., Liu, S. H., Sastry, B. R., Brunicardi, C. F. and Kumar, U. (2011). Somatostatin receptor 1 and 5 double knockout mice mimic neurochemical changes of Huntington's disease transgenic mice. *PloS one* **6**(9), e24467, 10.1371/journal.pone.0024467.

Rangone, H., Pardo, R., Colin, E., Girault, J. A., Saudou, F. and Humbert, S. (2005). Phosphorylation of arfaptin 2 at Ser260 by Akt Inhibits PolyQ-huntingtin-induced toxicity by rescuing proteasome impairment. *The Journal of biological chemistry* **280**(23), 22021-8, 10.1074/jbc.M407528200.

Ravikumar, B., Stewart, A., Kita, H., Kato, K., Duden, R. and Rubinsztein, D. C. (2003). Raised intracellular glucose concentrations reduce aggregation and cell death caused by mutant huntingtin exon 1 by decreasing mTOR phosphorylation and inducing autophagy. *Human molecular genetics* **12**(9), 985-94.

Raymond, C. R., Ireland, D. R. and Abraham, W. C. (2003). NMDA receptor regulation by amyloid-beta does not account for its inhibition of LTP in rat hippocampus. *Brain research* **968**(2), 263-72.

Rebec, G. V., Barton, S. J. and Ennis, M. D. (2002). Dysregulation of ascorbate release in the striatum of behaving mice expressing the Huntington's disease gene. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**(2), RC202.

Rebec, G. V., Barton, S. J., Marseilles, A. M. and Collins, K. (2003). Ascorbate treatment attenuates the Huntington behavioral phenotype in mice. *Neuroreport* **14**(9), 1263-5, 10.1097/01.wnr.0000081868.45938.12.

Rebec, G. V., Conroy, S. K. and Barton, S. J. (2006). Hyperactive striatal neurons in symptomatic Huntington R6/2 mice: variations with behavioral state and repeated ascorbate treatment. *Neuroscience* **137**(1), 327-36, 10.1016/j.neuroscience.2005.08.062.

Rechler, M. M., Schilling, E. E., King, G. S., Fraioli, F., Rosenberg, A. M., Higa, O. Z., Podskalny, J. M., Grunfeld, C., Nissley, P. and Kahn, C. R. (1980). Receptors for insulin and insulin-like growth factors in disease. *Advances in biochemical psychopharmacology* **21**, 489-97.

Rechler, M. M., Zapf, J., Nissley, S. P., Froesch, E. R., Moses, A. C., Podskalny, J. M., Schilling, E. E. and Humbel, R. E. (1980). Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. *Endocrinology* **107**(5), 1451-9.

Rego, A. C., Monteiro, N. M., Silva, A. P., Gil, J., Malva, J. O. and Oliveira, C. R. (2003). Mitochondrial apoptotic cell death and moderate superoxide generation upon selective activation of non-desensitizing AMPA receptors in hippocampal cultures. *Journal of neurochemistry* **86**(4), 792-804.

Reiner, A., Albin, R. L., Anderson, K. D., D'Amato, C. J., Penney, J. B. and Young, A. B. (1988). Differential loss of striatal projection neurons in Huntington disease. *Proceedings of the National Academy of Sciences of the United States of America* **85**(15), 5733-7.

Reynolds, D. S., Carter, R. J. and Morton, A. J. (1998). Dopamine modulates the susceptibility of striatal neurons to 3-nitropropionic acid in the rat model of Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **18**(23), 10116-27.

Reynolds, N. C., Jr., Prost, R. W. and Mark, L. P. (2005). Heterogeneity in 1H-MRS profiles of presymptomatic and early manifest Huntington's disease. *Brain research* **1031**(1), 82-9, 10.1016/j.brainres.2004.10.030.

Reynolds, N. C., Prost, R. W., Mark, L. P. and Joseph, S. A. (2008). MR-spectroscopic findings in juvenile-onset Huntington's disease. *Movement disorders : official journal of the Movement Disorder Society* **23**(13), 1931-5, 10.1002/mds.22245.

Rhodes, L. E., Freeman, B. K., Auh, S., Kokkinis, A. D., La Pean, A., Chen, C., Lehky, T. J., Shrader, J. A., Levy, E. W., Harris-Love, M., Di Prospero, N. A. and Fischbeck, K. H. (2009). Clinical features of spinal and bulbar muscular atrophy. *Brain : a journal of neurology* **132**(Pt 12), 3242-51, 10.1093/brain/awp258.

Ribeiro, M., Rosenstock, T. R., Cunha-Oliveira, T., Ferreira, I. L., Oliveira, C. R. and Rego, A. C. (2012). Glutathione redox cycle dysregulation in Huntington's disease knock-in striatal cells. *Free radical biology & medicine* **53**(10), 1857-67, 10.1016/j.freeradbiomed.2012.09.004.

Ribeiro, M., Silva, A. C., Rodrigues, J., Naia, L. and Rego, A. C. (2013). Oxidizing effects of exogenous stressors in Huntington's disease knock-in striatal cells--protective effect of cystamine and creatine. *Toxicological sciences : an official journal of the Society of Toxicology* **136**(2), 487-99, 10.1093/toxsci/kft199.

Richfield, E. K., Maguire-Zeiss, K. A., Cox, C., Gilmore, J. and Voorn, P. (1995). Reduced expression of preproenkephalin in striatal neurons from Huntington's disease patients. *Annals of neurology* **37**(3), 335-43, 10.1002/ana.410370309.

Richfield, E. K., Maguire-Zeiss, K. A., Vonkeman, H. E. and Voorn, P. (1995). Preferential loss of preproenkephalin versus preprotachykinin neurons from the striatum of Huntington's disease patients. *Annals of neurology* **38**(6), 852-61, 10.1002/ana.410380605.

Richfield, E. K., O'Brien, C. F., Eskin, T. and Shoulson, I. (1991). Heterogeneous dopamine receptor changes in early and late Huntington's disease. *Neuroscience letters* **132**(1), 121-6.

Richter, C., Park, J. W. and Ames, B. N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proceedings of the National Academy of Sciences of the United States of America* **85**(17), 6465-7.

Rigamonti, D., Bauer, J. H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., Clementi, E., Hackam, A., Hayden, M. R., Li, Y., Cooper, J. K., Ross, C. A., Govoni, S., Vincenz, C. and Cattaneo, E. (2000). Wild-type huntingtin protects from apoptosis upstream of caspase-3. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**(10), 3705-13.

Rigamonti, D., Sipione, S., Goffredo, D., Zuccato, C., Fossale, E. and Cattaneo, E. (2001). Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *The Journal of biological chemistry* **276**(18), 14545-8, 10.1074/jbc.C100044200.

Robinson, K. M., Janes, M. S. and Beckman, J. S. (2008). The selective detection of mitochondrial superoxide by live cell imaging. *Nature protocols* **3**(6), 941-7, 10.1038/nprot.2008.56.

Rocchi, A. and Pennuto, M. (2013). New routes to therapy for spinal and bulbar muscular atrophy. *Journal of molecular neuroscience : MN* **50**(3), 514-23, 10.1007/s12031-013-9978-7.

Rohrer, D., Salmon, D. P., Wixted, J. T. and Paulsen, J. S. (1999). The disparate effects of Alzheimer's disease and Huntington's disease on semantic memory. *Neuropsychology* **13**(3), 381-8.

Rosenstock, T. R., de Brito, O. M., Lombardi, V., Louros, S., Ribeiro, M., Almeida, S., Ferreira, I. L., Oliveira, C. R. and Rego, A. C. (2011). FK506 ameliorates cell death features in Huntington's disease striatal cell models. *Neurochemistry international* **59**(5), 600-9, 10.1016/j.neuint.2011.04.009.

Rosenstock, T. R., Duarte, A. I. and Rego, A. C. (2010). Mitochondrial-associated metabolic changes and neurodegeneration in Huntington's disease - from clinical features to the bench. *Current drug targets* **11**(10), 1218-36.

Rotwein, P., Burgess, S. K., Milbrandt, J. D. and Krause, J. E. (1988). Differential expression of insulin-like growth factor genes in rat central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* **85**(1), 265-9.

Ruan, Q., Lesort, M., MacDonald, M. E. and Johnson, G. V. (2004). Striatal cells from mutant huntingtin knock-in mice are selectively vulnerable to mitochondrial complex II inhibitor-induced cell death through a non-apoptotic pathway. *Human molecular genetics* **13**(7), 669-81, 10.1093/hmg/ddh082.

Rue, L., Lopez-Soop, G., Gelpi, E., Martinez-Vicente, M., Alberch, J. and Perez-Navarro, E. (2013). Brain region- and age-dependent dysregulation of p62 and NBR1 in a mouse model of Huntington's disease. *Neurobiology of disease* **52**, 219-28, 10.1016/j.nbd.2012.12.008.

Ruiz, C., Casarejos, M. J., Gomez, A., Solano, R., de Yebenes, J. G. and Mena, M. A. (2012). Protection by glia-conditioned medium in a cell model of Huntington disease. *PLoS currents* **4**, e4fbca54a2028b, 10.1371/4fbca54a2028b.

Ryan, A. B., Zeitlin, S. O. and Scrable, H. (2006). Genetic interaction between expanded murine Hdh alleles and p53 reveal deleterious effects of p53 on Huntington's disease pathogenesis. *Neurobiology of disease* **24**(2), 419-27, 10.1016/j.nbd.2006.08.002.

Ryu, J. K., Kim, S. U. and McLarnon, J. G. (2004). Blockade of quinolinic acid-induced neurotoxicity by pyruvate is associated with inhibition of glial activation in a model of Huntington's disease. *Experimental neurology* **187**(1), 150-9, 10.1016/j.expneurol.2004.01.006.

Sadagurski, M., Cheng, Z., Rozzo, A., Palazzolo, I., Kelley, G. R., Dong, X., Krainc, D. and White, M. F. (2011). IRS2 increases mitochondrial dysfunction and oxidative stress in a mouse model of Huntington disease. *The Journal of clinical investigation* **121**(10), 4070-81, 10.1172/JCI46305.

Saleh, N., Moutereau, S., Azulay, J. P., Verny, C., Simonin, C., Tranchant, C., El Hawajri, N., Bachoud-Levi, A. C., Maison, P. and Huntington French Speaking, G. (2010). High insulinlike growth factor I is associated with cognitive decline in Huntington disease. *Neurology* **75**(1), 57-63, 10.1212/WNL.0b013e3181e62076.

Sanchez, I., Xu, C. J., Juo, P., Kakizaka, A., Blenis, J. and Yuan, J. (1999). Caspase-8 is required for cell death induced by expanded polyglutamine repeats. *Neuron* **22**(3), 623-33.

Sanchez-Pernaute, R., Garcia-Segura, J. M., del Barrio Alba, A., Viano, J. and de Yebenes, J. G. (1999). Clinical correlation of striatal 1H MRS changes in Huntington's disease. *Neurology* **53**(4), 806-12.

Santamaria, A., Perez-Severiano, F., Rodriguez-Martinez, E., Maldonado, P. D., Pedraza-Chaverri, J., Rios, C. and Segovia, J. (2001). Comparative analysis of superoxide dismutase activity between acute

pharmacological models and a transgenic mouse model of Huntington's disease. *Neurochemical research* **26**(4), 419-24.

Sarantos, M. R., Papanikolaou, T., Ellerby, L. M. and Hughes, R. E. (2012). Pizotifen Activates ERK and Provides Neuroprotection in vitro and in vivo in Models of Huntington's Disease. *Journal of Huntington's disease* **1**(2), 195-210, 10.3233/JHD-120033.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**(5712), 1098-101, 10.1126/science.1106148.

Sathasivam, K., Hobbs, C., Turmaine, M., Mangiarini, L., Mahal, A., Bertaux, F., Wanker, E. E., Doherty, P., Davies, S. W. and Bates, G. P. (1999). Formation of polyglutamine inclusions in non-CNS tissue. *Human molecular genetics* **8**(5), 813-22.

Saudou, F., Finkbeiner, S., Devys, D. and Greenberg, M. E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* **95**(1), 55-66.

Scaduto, R. C., Jr. and Grotyohann, L. W. (1999). Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophysical journal* **76**(1 Pt 1), 469-77, 10.1016/S0006-3495(99)77214-0.

Scarpulla, R. C. (2008). Nuclear control of respiratory chain expression by nuclear respiratory factors and PGC-1-related coactivator. *Annals of the New York Academy of Sciences* **1147**, 321-34, 10.1196/annals.1427.006.

Scarpulla, R. C. (2008). Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiological reviews* **88**(2), 611-38, 10.1152/physrev.00025.2007.

Schaffar, G., Breuer, P., Boteva, R., Behrends, C., Tzvetkov, N., Strippel, N., Sakahira, H., Siegers, K., Hayer-Hartl, M. and Hartl, F. U. (2004). Cellular toxicity of polyglutamine expansion proteins: mechanism of transcription factor deactivation. *Molecular cell* **15**(1), 95-105, 10.1016/j.molcel.2004.06.029.

Schapiro, M., Cecil, K. M., Doescher, J., Kiefer, A. M. and Jones, B. V. (2004). MR imaging and spectroscopy in juvenile Huntington disease. *Pediatric radiology* **34**(8), 640-3, 10.1007/s00247-004-1159-y.

Schapiro, M., Cecil, K. M., Doescher, J., Kiefer, A. M. and Jones, B. V. (2004). MR imaging and spectroscopy in juvenile Huntington disease. *Pediatric radiology* **34**(8), 640-3, 10.1007/s00247-004-1159-y.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H. and Wanker, E. E. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell* **90**(3), 549-58.

Schilling, G., Becher, M. W., Sharp, A. H., Jinnah, H. A., Duan, K., Kotzuk, J. A., Slunt, H. H., Ratovitski, T., Cooper, J. K., Jenkins, N. A., Copeland, N. G., Price, D. L., Ross, C. A. and Borchelt, D. R. (1999). Intranuclear inclusions and neuritic aggregates in transgenic mice expressing a mutant N-terminal fragment of huntingtin. *Human molecular genetics* **8**(3), 397-407.

Schwarcz, R. and Kohler, C. (1983). Differential vulnerability of central neurons of the rat to quinolinic acid. *Neuroscience letters* **38**(1), 85-90.

Senatorov, V. V., Charles, V., Reddy, P. H., Tagle, D. A. and Chuang, D. M. (2003). Overexpression and nuclear accumulation of glyceraldehyde-3-phosphate dehydrogenase in a transgenic mouse model of Huntington's disease. *Molecular and cellular neurosciences* **22**(3), 285-97.

Serrano, F., Kolluri, N. S., Wientjes, F. B., Card, J. P. and Klann, E. (2003). NADPH oxidase immunoreactivity in the mouse brain. *Brain research* **988**(1-2), 193-8.

Sharon, I., Sharon, R., Wilkens, J. P., Ersan, T. (2010). Huntington Disease Dementia. emedicine, WebMD. Medscape. Retrieved 2010-05-16.

Sharp, A. H., Loev, S. J., Schilling, G., Li, S. H., Li, X. J., Bao, J., Wagster, M. V., Kotzuk, J. A., Steiner, J. P., Lo, A. and et al. (1995). Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* **14**(5), 1065-74.

Shelbourne, P. F., Killeen, N., Hevner, R. F., Johnston, H. M., Tecott, L., Lewandoski, M., Ennis, M., Ramirez, L., Li, Z., Iannicola, C., Littman, D. R. and Myers, R. M. (1999). A Huntington's disease CAG expansion at the murine Hdh locus is unstable and associated with behavioural abnormalities in mice. *Human molecular genetics* **8**(5), 763-74.

Shelton, J. G., Steelman, L. S., White, E. R. and McCubrey, J. A. (2004). Synergy between PI3K/Akt and Raf/MEK/ERK pathways in IGF-1R mediated cell cycle progression and prevention of apoptosis in hematopoietic cells. *Cell cycle* **3**(3), 372-9.

Shih, A. Y., Imbeault, S., Barakauskas, V., Erb, H., Jiang, L., Li, P. and Murphy, T. H. (2005). Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *The Journal of biological chemistry* **280**(24), 22925-36, 10.1074/jbc.M414635200.

Shih, A. Y., Johnson, D. A., Wong, G., Kraft, A. D., Jiang, L., Erb, H., Johnson, J. A. and Murphy, T. H. (2003). Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**(8), 3394-406.

Shimohata, T., Nakajima, T., Yamada, M., Uchida, C., Onodera, O., Naruse, S., Kimura, T., Koide, R., Nozaki, K., Sano, Y., Ishiguro, H., Sakoe, K., Ooshima, T., Sato, A., Ikeuchi, T., Oyake, M., Sato, T., Aoyagi, Y., Hozumi, I., Nagatsu, T., Takiyama, Y., Nishizawa, M., Goto, J., Kanazawa, I., Davidson, I., Tanese, N., Takahashi, H. and Tsuji, S. (2000). Expanded polyglutamine stretches interact with TAFII130, interfering with CREB-dependent transcription. *Nature genetics* **26**(1), 29-36, 10.1038/79139.

Shirendeb, U., Reddy, A. P., Manczak, M., Calkins, M. J., Mao, P., Tagle, D. A. and Reddy, P. H. (2011). Abnormal mitochondrial dynamics, mitochondrial loss and mutant huntingtin oligomers in Huntington's disease: implications for selective neuronal damage. *Human molecular genetics* **20**(7), 1438-55, 10.1093/hmg/ddr024.

Shirendeb, U. P., Calkins, M. J., Manczak, M., Anekonda, V., Dufour, B., McBride, J. L., Mao, P. and Reddy, P. H. (2012). Mutant huntingtin's interaction with mitochondrial protein Drp1 impairs mitochondrial biogenesis and causes defective axonal transport and synaptic degeneration in Huntington's disease. *Human molecular genetics* **21**(2), 406-20, 10.1093/hmg/ddr475.

Sian, J., Dexter, D. T., Lees, A. J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P. and Marsden, C. D. (1994). Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. *Annals of neurology* **36**(3), 348-55, 10.1002/ana.410360305.

Siddiqui, A., Rivera-Sanchez, S., Castro Mdel, R., Acevedo-Torres, K., Rane, A., Torres-Ramos, C. A., Nicholls, D. G., Andersen, J. K. and Ayala-Torres, S. (2012). Mitochondrial DNA damage is associated with reduced mitochondrial bioenergetics in Huntington's disease. *Free radical biology & medicine* **53**(7), 1478-88, 10.1016/j.freeradbiomed.2012.06.008.

Siegel, G. J., Agranoff, B. W., Albers, W., Fisher, S. K., Uhler, M. D., eds (1999). Basic Neurochemistry - Molecular, Cellular and Medical Aspects. Philadelphia: Lippincott-Raven; ISBN 10: 0-397-51820-X.

Silva, A. C., Almeida, S., Laco, M., Duarte, A. I., Domingues, J., Oliveira, C. R., Januario, C. and Rego, A. C. (2013). Mitochondrial respiratory chain complex activity and bioenergetic alterations in human platelets derived from pre-symptomatic and symptomatic Huntington's disease carriers. *Mitochondrion* **13**(6), 801-9, 10.1016/j.mito.2013.05.006.

Slow, E. J., van Raamsdonk, J., Rogers, D., Coleman, S. H., Graham, R. K., Deng, Y., Oh, R., Bissada, N., Hossain, S. M., Yang, Y. Z., Li, X. J., Simpson, E. M., Gutekunst, C. A., Leavitt, B. R. and Hayden, M. R. (2003). Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Human molecular genetics* **12**(13), 1555-67.

Smith, K. M., Matson, S., Matson, W. R., Cormier, K., Del Signore, S. J., Hagerty, S. W., Stack, E. C., Ryu, H. and Ferrante, R. J. (2006). Dose ranging and efficacy study of high-dose coenzyme Q10 formulations in Huntington's disease mice. *Biochimica et biophysica acta* **1762**(6), 616-26, 10.1016/j.bbadis.2006.03.004.

Song, W., Chen, J., Petrilli, A., Liot, G., Klinglmayr, E., Zhou, Y., Poquiz, P., Tjong, J., Pouladi, M. A., Hayden, M. R., Masliah, E., Ellisman, M., Rouiller, I., Schwarzenbacher, R., Bossy, B., Perkins, G. and Bossy-Wetzel, E. (2011). Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. *Nature medicine* **17**(3), 377-82, 10.1038/nm.2313.

Sorbi, S., Bird, E. D. and Blass, J. P. (1983). Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain. *Annals of neurology* **13**(1), 72-8, 10.1002/ana.410130116.

Sorensen, S. A. and Fenger, K. (1992). Causes of death in patients with Huntington's disease and in unaffected first degree relatives. *Journal of medical genetics* **29**(12), 911-4.

Soriano, F. X., Leveille, F., Papadia, S., Higgins, L. G., Varley, J., Baxter, P., Hayes, J. D. and Hardingham, G. E. (2008). Induction of sulfiredoxin expression and reduction of peroxiredoxin hyperoxidation by the neuroprotective Nrf2 activator 3H-1,2-dithiole-3-thione. *Journal of neurochemistry* **107**(2), 533-43, 10.1111/j.1471-4159.2008.05648.x.

Sorolla, M. A., Reverter-Branchat, G., Tamarit, J., Ferrer, I., Ros, J. and Cabiscol, E. (2008). Proteomic and oxidative stress analysis in human brain samples of Huntington disease. *Free radical biology & medicine* **45**(5), 667-78, 10.1016/j.freeradbiomed.2008.05.014.

Sorolla, M. A., Rodriguez-Colman, M. J., Tamarit, J., Ortega, Z., Lucas, J. J., Ferrer, I., Ros, J. and Cabiscol, E. (2010). Protein oxidation in Huntington disease affects energy production and vitamin B6 metabolism. *Free radical biology & medicine* **49**(4), 612-21, 10.1016/j.freeradbiomed.2010.05.016.

Sosa, V., Moline, T., Somoza, R., Paciucci, R., Kondoh, H. and ME, L. L. (2013). Oxidative stress and cancer: an overview. *Ageing research reviews* **12**(1), 376-90, 10.1016/j.arr.2012.10.004.

Squitieri, F., Falleni, A., Cannella, M., Orobello, S., Fulceri, F., Lenzi, P. and Fornai, F. (2010). Abnormal morphology of peripheral cell tissues from patients with Huntington disease. *Journal of neural transmission* **117**(1), 77-83, 10.1007/s00702-009-0328-4.

Stack, C., Ho, D., Wille, E., Calingasan, N. Y., Williams, C., Liby, K., Sporn, M., Dumont, M. and Beal, M. F. (2010). Triterpenoids CDDO-ethyl amide and CDDO-trifluoroethyl amide improve the behavioral phenotype and brain pathology in a transgenic mouse model of Huntington's disease. *Free radical biology & medicine* **49**(2), 147-58, 10.1016/j.freeradbiomed.2010.03.017.

Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S. and Beal, M. F. (2004). Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**(36), 7779-88, 10.1523/JNEUROSCI.1899-04.2004.

Steffan, J. S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L. C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y. Z., Cattaneo, E., Pandolfi, P. P., Thompson, L. M. and Marsh, J. L. (2004). SUMO modification of Huntingtin and Huntington's disease pathology. *Science* **304**(5667), 100-4, 10.1126/science.1092194.

Steffan, J. S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B. L., Kazantsev, A., Schmidt, E., Zhu, Y. Z., Greenwald, M., Kurokawa, R., Housman, D. E., Jackson, G. R., Marsh, J. L. and Thompson, L. M. (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. *Nature* **413**(6857), 739-43, 10.1038/35099568.

Steffan, J. S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y. Z., Gohler, H., Wanker, E. E., Bates, G. P., Housman, D. E. and Thompson, L. M. (2000). The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proceedings of the National Academy of Sciences of the United States of America* **97**(12), 6763-8, 10.1073/pnas.100110097.

Stoy, N., Mackay, G. M., Forrest, C. M., Christofides, J., Egerton, M., Stone, T. W. and Darlington, L. G. (2005). Tryptophan metabolism and oxidative stress in patients with Huntington's disease. *Journal of neurochemistry* **93**(3), 611-23, 10.1111/j.1471-4159.2005.03070.x.

St-Pierre, J., Buckingham, J. A., Roebuck, S. J. and Brand, M. D. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *The Journal of biological chemistry* **277**(47), 44784-90, 10.1074/jbc.M207217200.

Strand, A. D., Aragaki, A. K., Shaw, D., Bird, T., Holton, J., Turner, C., Tapscott, S. J., Tabrizi, S. J., Schapira, A. H., Kooperberg, C. and Olson, J. M. (2005). Gene expression in Huntington's disease skeletal muscle: a potential biomarker. *Human molecular genetics* **14**(13), 1863-76, 10.1093/hmg/ddi192.

Strong, T. V., Tagle, D. A., Valdes, J. M., Elmer, L. W., Boehm, K., Swaroop, M., Kaatz, K. W., Collins, F. S. and Albin, R. L. (1993). Widespread expression of the human and rat Huntington's disease gene in brain and nonneural tissues. *Nature genetics* **5**(3), 259-65, 10.1038/ng1193-259.

Subramony, S. H., Ashizawa, T. (1998) [Updated 2011]. Spinocerebellar Ataxia Type 1. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1184/

Sun, Z., Chin, Y. E. and Zhang, D. D. (2009). Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Molecular and cellular biology* **29**(10), 2658-72, 10.1128/MCB.01639-08.

Sun, Z., Huang, Z. and Zhang, D. D. (2009). Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response. *PloS one* **4**(8), e6588, 10.1371/journal.pone.0006588.

Sun, Z., Wu, T., Zhao, F., Lau, A., Birch, C. M. and Zhang, D. D. (2011). KPNA6 (Importin {alpha}7)-mediated nuclear import of Keap1 represses the Nrf2-dependent antioxidant response. *Molecular and cellular biology* **31**(9), 1800-11, 10.1128/MCB.05036-11.

Sun, Z., Zhang, S., Chan, J. Y. and Zhang, D. D. (2007). Keap1 controls postinduction repression of the Nrf2-mediated antioxidant response by escorting nuclear export of Nrf2. *Molecular and cellular biology* **27**(18), 6334-49, 10.1128/MCB.00630-07.

Suvorova, E. S., Lucas, O., Weisend, C. M., Rollins, M. F., Merrill, G. F., Capecchi, M. R. and Schmidt, E. E. (2009). Cytoprotective Nrf2 pathway is induced in chronically txnrd 1-deficient hepatocytes. *PloS one* **4**(7), e6158, 10.1371/journal.pone.0006158.

Szabadkai, G., Simoni, A. M., Chami, M., Wieckowski, M. R., Youle, R. J. and Rizzuto, R. (2004). Drp-1-dependent division of the mitochondrial network blocks intraorganellar Ca2+ waves and protects against Ca2+-mediated apoptosis. *Molecular cell* **16**(1), 59-68, 10.1016/j.molcel.2004.09.026.

Tabrizi, S. J., Cleeter, M. W., Xuereb, J., Taanman, J. W., Cooper, J. M. and Schapira, A. H. (1999). Biochemical abnormalities and excitotoxicity in Huntington's disease brain. *Annals of neurology* **45**(1), 25-32.

Tabrizi, S. J., Workman, J., Hart, P. E., Mangiarini, L., Mahal, A., Bates, G., Cooper, J. M. and Schapira, A. H. (2000). Mitochondrial dysfunction and free radical damage in the Huntington R6/2 transgenic mouse. *Annals of neurology* **47**(1), 80-6.

Taherzadeh-Fard, E., Saft, C., Akkad, D. A., Wieczorek, S., Haghikia, A., Chan, A., Epplen, J. T. and Arning, L. (2011). PGC-1alpha downstream transcription factors NRF-1 and TFAM are genetic modifiers of Huntington disease. *Molecular neurodegeneration* **6**(1), 32, 10.1186/1750-1326-6-32.

Taherzadeh-Fard, E., Saft, C., Andrich, J., Wieczorek, S. and Arning, L. (2009). PGC-1alpha as modifier of onset age in Huntington disease. *Molecular neurodegeneration* **4**, 10, 10.1186/1750-1326-4-10.

Tallaksen-Greene, S. J., Crouse, A. B., Hunter, J. M., Detloff, P. J. and Albin, R. L. (2005). Neuronal intranuclear inclusions and neuropil aggregates in HdhCAG(150) knockin mice. *Neuroscience* **131**(4), 843-52, 10.1016/j.neuroscience.2004.10.037.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. *Biochemical and biophysical research communications* **135**(2), 397-402.

Tanigawa, S., Lee, C. H., Lin, C. S., Ku, C. C., Hasegawa, H., Qin, S., Kawahara, A., Korenori, Y., Miyamori, K., Noguchi, M., Lee, L. H., Lin, Y. C., Steve Lin, C. L., Nakamura, Y., Jin, C., Yamaguchi, N., Eckner, R., Hou, D. X. and Yokoyama, K. K. (2013). Jun dimerization protein 2 is a critical component of the Nrf2/MafK complex regulating the response to ROS homeostasis. *Cell death & disease* **4**, e921, 10.1038/cddis.2013.448.

Tanito, M., Agbaga, M. P. and Anderson, R. E. (2007). Upregulation of thioredoxin system via Nrf2-antioxidant responsive element pathway in adaptive-retinal neuroprotection in vivo and in vitro. *Free radical biology & medicine* **42**(12), 1838-50, 10.1016/j.freeradbiomed.2007.03.018.

Tasset, I., Perez-De La Cruz, V., Elinos-Calderon, D., Carrillo-Mora, P., Gonzalez-Herrera, I. G., Luna-Lopez, A., Konigsberg, M., Pedraza-Chaverri, J., Maldonado, P. D., Ali, S. F., Tunez, I. and Santamaria, A. (2010). Protective effect of tert-butylhydroquinone on the quinolinic-acid-induced toxicity in rat striatal slices: role of the Nrf2-antioxidant response element pathway. *Neuro-Signals* **18**(1), 24-31, 10.1159/000243650.

Telenius, H., Kremer, H. P., Theilmann, J., Andrew, S. E., Almqvist, E., Anvret, M., Greenberg, C., Greenberg, J., Lucotte, G., Squitieri, F. and et al. (1993). Molecular analysis of juvenile Huntington disease: the major influence on (CAG)n repeat length is the sex of the affected parent. *Human molecular genetics* **2**(10), 1535-40.

The Huntington's Disease Collaborative Research Group. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72**(6), 971-83.

Theodore, M., Kawai, Y., Yang, J., Kleshchenko, Y., Reddy, S. P., Villalta, F. and Arinze, I. J. (2008). Multiple nuclear localization signals function in the nuclear import of the transcription factor Nrf2. *The Journal of biological chemistry* **283**(14), 8984-94, 10.1074/jbc.M709040200.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M. and Biswal, S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer research* **62**(18), 5196-203.

Tkac, I., Dubinsky, J. M., Keene, C. D., Gruetter, R. and Low, W. C. (2007). Neurochemical changes in Huntington R6/2 mouse striatum detected by in vivo 1H NMR spectroscopy. *Journal of neurochemistry* **100**(5), 1397-406, 10.1111/j.1471-4159.2006.04323.x.

Torres Aleman, I. (2005). Role of insulin-like growth factors in neuronal plasticity and neuroprotection. *Advances in experimental medicine and biology* **567**, 243-58.

Toyoshima, Y., Onodera, O., Yamada, M., et al. (2005) [Updated 2012]. Spinocerebellar Ataxia Type 17. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1438/

Trettel, F., Rigamonti, D., Hilditch-Maguire, P., Wheeler, V. C., Sharp, A. H., Persichetti, F., Cattaneo, E. and MacDonald, M. E. (2000). Dominant phenotypes produced by the HD mutation in STHdh(Q111) striatal cells. *Human molecular genetics* **9**(19), 2799-809.

Tretter, L., Takacs, K., Hegedus, V. and Adam-Vizi, V. (2007). Characteristics of alpha-glycerophosphate-evoked H2O2 generation in brain mitochondria. *Journal of neurochemistry* **100**(3), 650-63, 10.1111/j.1471-4159.2006.04223.x.

Trottier, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E. C. and Mandel, J. L. (1995). Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form. *Nature genetics* **10**(1), 104-10, 10.1038/ng0595-104.

Trushina, E., Dyer, R. B., Badger, J. D., 2nd, Ure, D., Eide, L., Tran, D. D., Vrieze, B. T., Legendre-Guillemin, V., McPherson, P. S., Mandavilli, B. S., Van Houten, B., Zeitlin, S., McNiven, M., Aebersold, R., Hayden, M., Parisi, J. E., Seeberg, E., Dragatsis, I., Doyle, K., Bender, A., Chacko, C. and McMurray, C. T. (2004). Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro. *Molecular and cellular biology* **24**(18), 8195-209, 10.1128/MCB.24.18.8195-8209.2004.

Trushina, E., Singh, R. D., Dyer, R. B., Cao, S., Shah, V. H., Parton, R. G., Pagano, R. E. and McMurray, C. T. (2006). Mutant huntingtin inhibits clathrin-independent endocytosis and causes accumulation of cholesterol in vitro and in vivo. *Human molecular genetics* **15**(24), 3578-91, 10.1093/hmg/ddl434.

Tsang, T. M., Woodman, B., McLoughlin, G. A., Griffin, J. L., Tabrizi, S. J., Bates, G. P. and Holmes, E. (2006). Metabolic characterization of the R6/2 transgenic mouse model of Huntington's disease by high-resolution MAS 1H NMR spectroscopy. *Journal of proteome research* **5**(3), 483-92, 10.1021/pr050244o.

Tseng, Y. H., Ueki, K., Kriauciunas, K. M. and Kahn, C. R. (2002). Differential roles of insulin receptor substrates in the anti-apoptotic function of insulin-like growth factor-1 and insulin. *The Journal of biological chemistry* **277**(35), 31601-11, 10.1074/jbc.M202932200.

Tsuji, S. (1999) [Updated 2010]. DRPLA. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1491/

Tsuji, S. (2012). Dentatorubral-pallidoluysian atrophy. *Handbook of clinical neurology* **103**, 587-94, 10.1016/B978-0-444-51892-7.00041-3.

Tsunemi, T., Ashe, T. D., Morrison, B. E., Soriano, K. R., Au, J., Roque, R. A., Lazarowski, E. R., Damian, V. A., Masliah, E. and La Spada, A. R. (2012). PGC-1alpha rescues Huntington's disease proteotoxicity by preventing oxidative stress and promoting TFEB function. *Science translational medicine* **4**(142), 142ra97, 10.1126/scitranslmed.3003799.

Tunez, I., Drucker-Colin, R., Jimena, I., Medina, F. J., Munoz Mdel, C., Pena, J. and Montilla, P. (2006). Transcranial magnetic stimulation attenuates cell loss and oxidative damage in the striatum induced in the 3-nitropropionic model of Huntington's disease. *Journal of neurochemistry* **97**(3), 619-30, 10.1111/j.1471-4159.2006.03724.x.

Tunez, I., Montilla, P., del Carmen Munoz, M., Medina, F. J. and Drucker-Colin, R. (2006). Effect of transcranial magnetic stimulation on oxidative stress induced by 3-nitropropionic acid in cortical synaptosomes. *Neuroscience research* **56**(1), 91-5, 10.1016/j.neures.2006.05.012.

Tunez, I., Sanchez-Lopez, F., Aguera, E., Fernandez-Bolanos, R., Sanchez, F. M. and Tasset-Cuevas, I. (2011). Important role of oxidative stress biomarkers in Huntington's disease. *Journal of medicinal chemistry* **54**(15), 5602-6, 10.1021/jm200605a.

Turner, D. A. and Adamson, D. C. (2011). Neuronal-astrocyte metabolic interactions: understanding the transition into abnormal astrocytoma metabolism. *Journal of neuropathology and experimental neurology* **70**(3), 167-76, 10.1097/NEN.0b013e31820e1152.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of physiology* **552**(Pt 2), 335-44, 10.1113/jphysiol.2003.049478.

Twig, G., Elorza, A., Molina, A. J., Mohamed, H., Wikstrom, J. D., Walzer, G., Stiles, L., Haigh, S. E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B. F., Yuan, J., Deeney, J. T., Corkey, B. E. and Shirihai, O. S. (2008). Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal* **27**(2), 433-46, 10.1038/sj.emboj.7601963.

Twig, G., Hyde, B. and Shirihai, O. S. (2008). Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. *Biochimica et biophysica acta* **1777**(9), 1092-7, 10.1016/j.bbabio.2008.05.001.

Uchida, K. (2003). 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress in lipid research* **42**(4), 318-43.

Uchida, K., Kanematsu, M., Morimitsu, Y., Osawa, T., Noguchi, N. and Niki, E. (1998). Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. *The Journal of biological chemistry* **273**(26), 16058-66.

Unno, T., Wakamori, M., Koike, M., Uchiyama, Y., Ishikawa, K., Kubota, H., Yoshida, T., Sasakawa, H., Peters, C., Mizusawa, H. and Watase, K. (2012). Development of Purkinje cell degeneration in a knockin mouse model reveals lysosomal involvement in the pathogenesis of SCA6. *Proceedings of the National Academy of Sciences of the United States of America* **109**(43), 17693-8, 10.1073/pnas.1212786109.

Valencia, A., Reeves, P. B., Sapp, E., Li, X., Alexander, J., Kegel, K. B., Chase, K., Aronin, N. and DiFiglia, M. (2010). Mutant huntingtin and glycogen synthase kinase 3-beta accumulate in neuronal lipid rafts of a presymptomatic knock-in mouse model of Huntington's disease. *Journal of neuroscience research* **88**(1), 179-90, 10.1002/jnr.22184.

Valencia, A., Sapp, E., Kimm, J. S., McClory, H., Reeves, P. B., Alexander, J., Ansong, K. A., Masso, N., Frosch, M. P., Kegel, K. B., Li, X. and DiFiglia, M. (2013). Elevated NADPH oxidase activity contributes to oxidative stress and cell death in Huntington's disease. *Human molecular genetics* **22**(6), 1112-31, 10.1093/hmg/dds516.

van den Bogaard, S. J., Dumas, E. M., Teeuwisse, W. M., Kan, H. E., Webb, A., Roos, R. A. and van der Grond, J. (2011). Exploratory 7-Tesla magnetic resonance spectroscopy in Huntington's disease provides in vivo evidence for impaired energy metabolism. *Journal of neurology* **258**(12), 2230-9, 10.1007/s00415-011-6099-5.

van der Burg, J. M., Bjorkqvist, M. and Brundin, P. (2009). Beyond the brain: widespread pathology in Huntington's disease. *Lancet neurology* **8**(8), 765-74, 10.1016/S1474-4422(09)70178-4.

van Ommen, B., Koster, A., Verhagen, H. and van Bladeren, P. J. (1992). The glutathione conjugates of tert-butyl hydroquinone as potent redox cycling agents and possible reactive agents underlying the toxicity of butylated hydroxyanisole. *Biochemical and biophysical research communications* **189**(1), 309-14.

Van Raamsdonk, J. M., Metzler, M., Slow, E., Pearson, J., Schwab, C., Carroll, J., Graham, R. K., Leavitt, B. R. and Hayden, M. R. (2007). Phenotypic abnormalities in the YAC128 mouse model of Huntington disease are penetrant on multiple genetic backgrounds and modulated by strain. *Neurobiology of disease* **26**(1), 189-200, 10.1016/j.nbd.2006.12.010.

Van Raamsdonk, J. M., Murphy, Z., Slow, E. J., Leavitt, B. R. and Hayden, M. R. (2005). Selective degeneration and nuclear localization of mutant huntingtin in the YAC128 mouse model of Huntington disease. *Human molecular genetics* **14**(24), 3823-35, 10.1093/hmg/ddi407.

Van Raamsdonk, J. M., Pearson, J., Bailey, C. D., Rogers, D. A., Johnson, G. V., Hayden, M. R. and Leavitt, B. R. (2005). Cystamine treatment is neuroprotective in the YAC128 mouse model of Huntington disease. *Journal of neurochemistry* **95**(1), 210-20, 10.1111/j.1471-4159.2005.03357.x.

Van Raamsdonk, J. M., Pearson, J., Slow, E. J., Hossain, S. M., Leavitt, B. R. and Hayden, M. R. (2005). Cognitive dysfunction precedes neuropathology and motor abnormalities in the YAC128 mouse model of Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**(16), 4169-80, 10.1523/JNEUROSCI.0590-05.2005.

Van Raamsdonk, J. M., Warby, S. C. and Hayden, M. R. (2007). Selective degeneration in YAC mouse models of Huntington disease. *Brain research bulletin* **72**(2-3), 124-31, 10.1016/j.brainresbull.2006.10.018.

van Roon-Mom, W. M., Pepers, B. A., t Hoen, P. A., Verwijmeren, C. A., den Dunnen, J. T., Dorsman, J. C. and van Ommen, G. B. (2008). Mutant huntingtin activates Nrf2-responsive genes and impairs dopamine synthesis in a PC12 model of Huntington's disease. *BMC molecular biology* **9**, 84, 10.1186/1471-2199-9-84.

Veskoukis, A. S., Tsatsakis, A. M. and Kouretas, D. (2012). Dietary oxidative stress and antioxidant defense with an emphasis on plant extract administration. *Cell stress & chaperones* **17**(1), 11-21, 10.1007/s12192-011-0293-3.

Vida, C., Corpas, I., De la Fuente, M. and Gonzalez, E. M. (2011). Age-related changes in xanthine oxidase activity and lipid peroxidation, as well as in the correlation between both parameters, in plasma and several organs from female mice. *Journal of physiology and biochemistry* **67**(4), 551-8, 10.1007/s13105-011-0100-8.

Villeneuve, N. F., Tian, W., Wu, T., Sun, Z., Lau, A., Chapman, E., Fang, D. and Zhang, D. D. (2013). USP15 negatively regulates Nrf2 through deubiquitination of Keap1. *Molecular cell* **51**(1), 68-79, 10.1016/j.molcel.2013.04.022.

Vincent, E. E., Elder, D. J., Thomas, E. C., Phillips, L., Morgan, C., Pawade, J., Sohail, M., May, M. T., Hetzel, M. R. and Tavare, J. M. (2011). Akt phosphorylation on Thr308 but not on Ser473 correlates with Akt protein kinase activity in human non-small cell lung cancer. *British journal of cancer* **104**(11), 1755-61, 10.1038/bjc.2011.132.

Vis, J. C., Verbeek, M. M., de Waal, R. M., ten Donkelaar, H. J. and Kremer, B. (2001). The mitochondrial toxin 3-nitropropionic acid induces differential expression patterns of apoptosis-related markers in rat striatum. *Neuropathology and applied neurobiology* **27**(1), 68-76.

Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *The Journal of biological chemistry* **269**(7), 5241-8.

Volonte, D., Liu, Z., Musille, P. M., Stoppani, E., Wakabayashi, N., Di, Y. P., Lisanti, M. P., Kensler, T. W. and Galbiati, F. (2013). Inhibition of nuclear factor-erythroid 2-related factor (Nrf2) by caveolin-1 promotes stress-induced premature senescence. *Molecular biology of the cell* **24**(12), 1852-62, 10.1091/mbc.E12-09-0666.

Vonsattel, J. P. (2008). Huntington disease models and human neuropathology: similarities and differences. *Acta neuropathologica* **115**(1), 55-69, 10.1007/s00401-007-0306-6.

Vonsattel, J. P. and DiFiglia, M. (1998). Huntington disease. *Journal of neuropathology and experimental neurology* **57**(5), 369-84.

Vonsattel, J. P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D. and Richardson, E. P., Jr. (1985). Neuropathological classification of Huntington's disease. *Journal of neuropathology and experimental neurology* **44**(6), 559-77.

Walker, F. O. (2007). Huntington's disease. *Lancet* **369**(9557), 218-28, 10.1016/S0140-6736(07)60111-1.

Wallimann, T. (2007). Introduction--creatine: cheap ergogenic supplement with great potential for health and disease. *Sub-cellular biochemistry* **46**, 1-16.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *The Biochemical journal* **281 (Pt 1)**, 21-40.

Wang, H., Lim, P. J., Karbowski, M. and Monteiro, M. J. (2009). Effects of overexpression of huntingtin proteins on mitochondrial integrity. *Human molecular genetics* **18**(4), 737-52, 10.1093/hmg/ddn404.

Wang, J. Q., Chen, Q., Wang, X., Wang, Q. C., Wang, Y., Cheng, H. P., Guo, C., Sun, Q., Chen, Q. and Tang, T. S. (2013). Dysregulation of mitochondrial calcium signaling and superoxide flashes cause mitochondrial genomic DNA damage in Huntington disease. *The Journal of biological chemistry* **288**(5), 3070-84, 10.1074/jbc.M112.407726.

Wang, L., Chen, Y., Sternberg, P. and Cai, J. (2008). Essential roles of the PI3 kinase/Akt pathway in regulating Nrf2-dependent antioxidant functions in the RPE. *Investigative ophthalmology & visual science* **49**(4), 1671-8, 10.1167/iovs.07-1099.

Wang, R., Luo, Y., Ly, P. T., Cai, F., Zhou, W., Zou, H. and Song, W. (2012). Sp1 regulates human huntingtin gene expression. *Journal of molecular neuroscience : MN* **47**(2), 311-21, 10.1007/s12031-012-9739-z.

Warby, S. C., Chan, E. Y., Metzler, M., Gan, L., Singaraja, R. R., Crocker, S. F., Robertson, H. A. and Hayden, M. R. (2005). Huntingtin phosphorylation on serine 421 is significantly reduced in the striatum and by polyglutamine expansion in vivo. *Human molecular genetics* **14**(11), 1569-77, 10.1093/hmg/ddi165.

Warby, S. C., Graham, R. K., Hayden, M. R. (1998) [Updated 2010]. Huntington Disease. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2014. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1305/

Warby, S. C., Montpetit, A., Hayden, A. R., Carroll, J. B., Butland, S. L., Visscher, H., Collins, J. A., Semaka, A., Hudson, T. J. and Hayden, M. R. (2009). CAG expansion in the Huntington disease gene is associated with a specific and targetable predisposing haplogroup. *American journal of human genetics* **84**(3), 351-66, 10.1016/j.ajhg.2009.02.003.

Warby, S. C., Visscher, H., Collins, J. A., Doty, C. N., Carter, C., Butland, S. L., Hayden, A. R., Kanazawa, I., Ross, C. J. and Hayden, M. R. (2011). HTT haplotypes contribute to differences in Huntington disease prevalence between Europe and East Asia. *European journal of human genetics : EJHG* **19**(5), 561-6, 10.1038/ejhg.2010.229.

Weiss, A., Klein, C., Woodman, B., Sathasivam, K., Bibel, M., Regulier, E., Bates, G. P. and Paganetti, P. (2008). Sensitive biochemical aggregate detection reveals aggregation onset before symptom development in cellular and murine models of Huntington's disease. *Journal of neurochemistry* **104**(3), 846-58, 10.1111/j.1471-4159.2007.05032.x.

Wellington, C. L., Ellerby, L. M., Gutekunst, C. A., Rogers, D., Warby, S., Graham, R. K., Loubser, O., van Raamsdonk, J., Singaraja, R., Yang, Y. Z., Gafni, J., Bredesen, D., Hersch, S. M., Leavitt, B. R., Roy, S., Nicholson, D. W. and Hayden, M. R. (2002). Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**(18), 7862-72.

Wellington, C. L., Ellerby, L. M., Hackam, A. S., Margolis, R. L., Trifiro, M. A., Singaraja, R., McCutcheon, K., Salvesen, G. S., Propp, S. S., Bromm, M., Rowland, K. J., Zhang, T., Rasper, D., Roy, S., Thornberry, N., Pinsky, L., Kakizuka, A., Ross, C. A., Nicholson, D. W., Bredesen, D. E. and Hayden, M. R. (1998). Caspase cleavage of gene products associated with triplet expansion

disorders generates truncated fragments containing the polyglutamine tract. *The Journal of biological chemistry* **273**(15), 9158-67.

Wellington, C. L., Singaraja, R., Ellerby, L., Savill, J., Roy, S., Leavitt, B., Cattaneo, E., Hackam, A., Sharp, A., Thornberry, N., Nicholson, D. W., Bredesen, D. E. and Hayden, M. R. (2000). Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. *The Journal of biological chemistry* **275**(26), 19831-8, 10.1074/jbc.M001475200.

Wexler, N. S., Lorimer, J., Porter, J., Gomez, F., Moskowitz, C., Shackell, E., Marder, K., Penchaszadeh, G., Roberts, S. A., Gayan, J., Brocklebank, D., Cherny, S. S., Cardon, L. R., Gray, J., Dlouhy, S. R., Wiktorski, S., Hodes, M. E., Conneally, P. M., Penney, J. B., Gusella, J., Cha, J. H., Irizarry, M., Rosas, D., Hersch, S., Hollingsworth, Z., MacDonald, M., Young, A. B., Andresen, J. M., Housman, D. E., De Young, M. M., Bonilla, E., Stillings, T., Negrette, A., Snodgrass, S. R., Martinez-Jaurrieta, M. D., Ramos-Arroyo, M. A., Bickham, J., Ramos, J. S., Marshall, F., Shoulson, I., Rey, G. J., Feigin, A., Arnheim, N., Acevedo-Cruz, A., Acosta, L., Alvir, J., Fischbeck, K., Thompson, L. M., Young, A., Dure, L., O'Brien, C. J., Paulsen, J., Brickman, A., Krch, D., Peery, S., Hogarth, P., Higgins, D. S., Jr., Landwehrmeyer, B. and Project, U. S.-V. C. R. (2004). Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. *Proceedings of the National Academy of Sciences of the United States of America* **101**(10), 3498-503, 10.1073/pnas.0308679101.

Weydt, P., Pineda, V. V., Torrence, A. E., Libby, R. T., Satterfield, T. F., Lazarowski, E. R., Gilbert, M. L., Morton, G. J., Bammler, T. K., Strand, A. D., Cui, L., Beyer, R. P., Easley, C. N., Smith, A. C., Krainc, D., Luquet, S., Sweet, I. R., Schwartz, M. W. and La Spada, A. R. (2006). Thermoregulatory and metabolic defects in Huntington's disease transgenic mice implicate PGC-1alpha in Huntington's disease neurodegeneration. *Cell metabolism* **4**(5), 349-62, 10.1016/j.cmet.2006.10.004.

Wheeler, V. C., Auerbach, W., White, J. K., Srinidhi, J., Auerbach, A., Ryan, A., Duyao, M. P., Vrbanac, V., Weaver, M., Gusella, J. F., Joyner, A. L. and MacDonald, M. E. (1999). Length-dependent gametic CAG repeat instability in the Huntington's disease knock-in mouse. *Human molecular genetics* **8**(1), 115-22.

Wheeler, V. C., Gutekunst, C. A., Vrbanac, V., Lebel, L. A., Schilling, G., Hersch, S., Friedlander, R. M., Gusella, J. F., Vonsattel, J. P., Borchelt, D. R. and MacDonald, M. E. (2002). Early phenotypes that presage late-onset neurodegenerative disease allow testing of modifiers in Hdh CAG knock-in mice. *Human molecular genetics* **11**(6), 633-40.

Wheeler, V. C., White, J. K., Gutekunst, C. A., Vrbanac, V., Weaver, M., Li, X. J., Li, S. H., Yi, H., Vonsattel, J. P., Gusella, J. F., Hersch, S., Auerbach, W., Joyner, A. L. and MacDonald, M. E. (2000). Long glutamine tracts cause nuclear localization of a novel form of huntingtin in medium spiny striatal neurons in HdhQ92 and HdhQ111 knock-in mice. *Human molecular genetics* **9**(4), 503-13.

White, J. K., Auerbach, W., Duyao, M. P., Vonsattel, J. P., Gusella, J. F., Joyner, A. L. and MacDonald, M. E. (1997). Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. *Nature genetics* **17**(4), 404-10, 10.1038/ng1297-404.

Wilcox, G. (2005). Insulin and insulin resistance. *The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists* **26**(2), 19-39.

Wilcox, K. C., Zhou, L., Jordon, J. K., Huang, Y., Yu, Y., Redler, R. L., Chen, X., Caplow, M. and Dokholyan, N. V. (2009). Modifications of superoxide dismutase (SOD1) in human erythrocytes: a possible role in amyotrophic lateral sclerosis. *The Journal of biological chemistry* **284**(20), 13940-7, 10.1074/jbc.M809687200.

Wild, A. C., Moinova, H. R. and Mulcahy, R. T. (1999). Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *The Journal of biological chemistry* **274**(47), 33627-36.

Woodman, B., Butler, R., Landles, C., Lupton, M. K., Tse, J., Hockly, E., Moffitt, H., Sathasivam, K. and Bates, G. P. (2007). The Hdh(Q150/Q150) knock-in mouse model of HD and the R6/2 exon 1 model develop comparable and widespread molecular phenotypes. *Brain research bulletin* **72**(2-3), 83-97, 10.1016/j.brainresbull.2006.11.004.

Woznicki, D. T. and Walker, J. B. (1979). Formation of a supplemental long time-constant reservoir of high energy phosphate by brain in vivo and in vitro and its reversible depletion by potassium depolarization. *Journal of neurochemistry* **33**(1), 75-80.

Wu, J., Lin, F. and Qin, Z. (2007). Sequestration of glyceraldehyde-3-phosphate dehydrogenase to aggregates formed by mutant huntingtin. *Acta biochimica et biophysica Sinica* **39**(11), 885-90.

Wu, Y. T., Ouyang, W., Lazorchak, A. S., Liu, D., Shen, H. M. and Su, B. (2011). mTOR complex 2 targets Akt for proteasomal degradation via phosphorylation at the hydrophobic motif. *The Journal of biological chemistry* **286**(16), 14190-8, 10.1074/jbc.M111.219923.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C. and Spiegelman, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**(1), 115-24, 10.1016/S0092-8674(00)80611-X.

Wyss, M. and Kaddurah-Daouk, R. (2000). Creatine and creatinine metabolism. *Physiological reviews* **80**(3), 1107-213.

Wyttenbach, A., Sauvageot, O., Carmichael, J., Diaz-Latoud, C., Arrigo, A. P. and Rubinsztein, D. C. (2002). Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Human molecular genetics* **11**(9), 1137-51.

Xu, C., Yuan, X., Pan, Z., Shen, G., Kim, J. H., Yu, S., Khor, T. O., Li, W., Ma, J. and Kong, A. N. (2006). Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. *Molecular cancer therapeutics* **5**(8), 1918-26, 10.1158/1535-7163.MCT-05-0497.

Xun, Z., Rivera-Sanchez, S., Ayala-Pena, S., Lim, J., Budworth, H., Skoda, E. M., Robbins, P. D., Niedernhofer, L. J., Wipf, P. and McMurray, C. T. (2012). Targeting of XJB-5-131 to mitochondria suppresses oxidative DNA damage and motor decline in a mouse model of Huntington's disease. *Cell reports* **2**(5), 1137-42, 10.1016/j.celrep.2012.10.001.

Yamamoto, A., Cremona, M. L. and Rothman, J. E. (2006). Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway. *The Journal of cell biology* **172**(5), 719-31, 10.1083/jcb.200510065.

Yang, L., Calingasan, N. Y., Thomas, B., Chaturvedi, R. K., Kiaei, M., Wille, E. J., Liby, K. T., Williams, C., Royce, D., Risingsong, R., Musiek, E. S., Morrow, J. D., Sporn, M. and Beal, M. F. (2009). Neuroprotective effects of the triterpenoid, CDDO methyl amide, a potent inducer of Nrf2-mediated transcription. *PloS one* **4**(6), e5757, 10.1371/journal.pone.0005757.

Yang, L., Calingasan, N. Y., Wille, E. J., Cormier, K., Smith, K., Ferrante, R. J. and Beal, M. F. (2009). Combination therapy with coenzyme Q10 and creatine produces additive neuroprotective effects in models of Parkinson's and Huntington's diseases. *Journal of neurochemistry* **109**(5), 1427-39, 10.1111/j.1471-4159.2009.06074.x.

Yart, A., Laffargue, M., Mayeux, P., Chretien, S., Peres, C., Tonks, N., Roche, S., Payrastre, B., Chap, H. and Raynal, P. (2001). A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor. *The Journal of biological chemistry* **276**(12), 8856-64, 10.1074/jbc.M006966200.

Yoshioka, H., Niizuma, K., Katsu, M., Okami, N., Sakata, H., Kim, G. S., Narasimhan, P. and Chan, P. H. (2011). NADPH oxidase mediates striatal neuronal injury after transient global cerebral ischemia. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **31**(3), 868-80, 10.1038/jcbfm.2010.166.

Yu, M., Li, H., Liu, Q., Liu, F., Tang, L., Li, C., Yuan, Y., Zhan, Y., Xu, W., Li, W., Chen, H., Ge, C., Wang, J. and Yang, X. (2011). Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cellular signalling* **23**(5), 883-92, 10.1016/j.cellsig.2011.01.014.

Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M. and Dawson, V. L. (2002). Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**(5579), 259-63, 10.1126/science.1072221.

Yu, Z. X., Li, S. H., Evans, J., Pillarisetti, A., Li, H. and Li, X. J. (2003). Mutant huntingtin causes context-dependent neurodegeneration in mice with Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**(6), 2193-202.

Zainelli, G. M., Ross, C. A., Troncoso, J. C., Fitzgerald, J. K. and Muma, N. A. (2004). Calmodulin regulates transglutaminase 2 cross-linking of huntingtin. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**(8), 1954-61, 10.1523/JNEUROSCI.4424-03.2004.

Zainelli, G. M., Ross, C. A., Troncoso, J. C. and Muma, N. A. (2003). Transglutaminase cross-links in intranuclear inclusions in Huntington disease. *Journal of neuropathology and experimental neurology* **62**(1), 14-24.

Zala, D., Colin, E., Rangone, H., Liot, G., Humbert, S. and Saudou, F. (2008). Phosphorylation of mutant huntingtin at S421 restores anterograde and retrograde transport in neurons. *Human molecular genetics* **17**(24), 3837-46, 10.1093/hmg/ddn281.

Zala, D., Hinckelmann, M. V., Yu, H., Lyra da Cunha, M. M., Liot, G., Cordelieres, F. P., Marco, S. and Saudou, F. (2013). Vesicular glycolysis provides on-board energy for fast axonal transport. *Cell* **152**(3), 479-91, 10.1016/j.cell.2012.12.029.

Zanella, A., Izzo, C., Meola, G., Mariani, M., Colotti, M. T., Silani, V., Pellegata, G. and Scarlato, G. (1980). Metabolic impairment and membrane abnormality in red cells from Huntington's disease. *Journal of the neurological sciences* **47**(1), 93-103.

Zeitlin, S., Liu, J. P., Chapman, D. L., Papaioannou, V. E. and Efstratiadis, A. (1995). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nature genetics* **11**(2), 155-63, 10.1038/ng1095-155.

Zemskov, E. A., Jana, N. R., Kurosawa, M., Miyazaki, H., Sakamoto, N., Nekooki, M. and Nukina, N. (2003). Pro-apoptotic protein kinase C delta is associated with intranuclear inclusions in a transgenic model of Huntington's disease. *Journal of neurochemistry* **87**(2), 395-406.

Zemskov, E. A. and Nukina, N. (2003). Impaired degradation of PKCalpha by proteasome in a cellular model of Huntington's disease. *Neuroreport* **14**(11), 1435-8, 10.1097/01.wnr.0000082020.91120.35.

Zhai, W., Jeong, H., Cui, L., Krainc, D. and Tjian, R. (2005). In vitro analysis of huntingtin-mediated transcriptional repression reveals multiple transcription factor targets. *Cell* **123**(7), 1241-53, 10.1016/j.cell.2005.10.030.

Zhang, D. D. and Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Molecular and cellular biology* **23**(22), 8137-51.

Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J. and Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Molecular and cellular biology* **24**(24), 10941-53, 10.1128/MCB.24.24.10941-10953.2004.

Zhang, H., Liu, H., Dickinson, D. A., Liu, R. M., Postlethwait, E. M., Laperche, Y. and Forman, H. J. (2006). gamma-Glutamyl transpeptidase is induced by 4-hydroxynonenal via EpRE/Nrf2 signaling in rat epithelial type II cells. *Free radical biology & medicine* **40**(8), 1281-92, 10.1016/j.freeradbiomed.2005.11.005.

Zhang, M., An, C., Gao, Y., Leak, R. K., Chen, J. and Zhang, F. (2013). Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Progress in neurobiology* **100**, 30-47, 10.1016/j.pneurobio.2012.09.003.

Zhang, S. F., Hennessey, T., Yang, L., Starkova, N. N., Beal, M. F. and Starkov, A. A. (2011). Impaired brain creatine kinase activity in Huntington's disease. *Neuro-degenerative diseases* **8**(4), 194-201, 10.1159/000321681.

Zhang, Y., Leavitt, B. R., van Raamsdonk, J. M., Dragatsis, I., Goldowitz, D., MacDonald, M. E., Hayden, M. R. and Friedlander, R. M. (2006). Huntingtin inhibits caspase-3 activation. *The EMBO journal* **25**(24), 5896-906, 10.1038/sj.emboj.7601445.

Zhang, Z., Blake, D. R., Stevens, C. R., Kanczler, J. M., Winyard, P. G., Symons, M. C., Benboubetra, M. and Harrison, R. (1998). A reappraisal of xanthine dehydrogenase and oxidase in hypoxic reperfusion injury: the role of NADH as an electron donor. *Free radical research* **28**(2), 151-64.

Zhu, H., Itoh, K., Yamamoto, M., Zweier, J. L. and Li, Y. (2005). Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS letters* **579**(14), 3029-36, 10.1016/j.febslet.2005.04.058.

Zourlidou, A., Gidalevitz, T., Kristiansen, M., Landles, C., Woodman, B., Wells, D. J., Latchman, D. S., de Belleroche, J., Tabrizi, S. J., Morimoto, R. I. and Bates, G. P. (2007). Hsp27 overexpression in the R6/2 mouse model of Huntington's disease: chronic neurodegeneration does not induce Hsp27 activation. *Human molecular genetics* **16**(9), 1078-90, 10.1093/hmg/ddm057.

Zuccato, C., Belyaev, N., Conforti, P., Ooi, L., Tartari, M., Papadimou, E., MacDonald, M., Fossale, E., Zeitlin, S., Buckley, N. and Cattaneo, E. (2007). Widespread disruption of repressor element-1 silencing transcription factor/neuron-restrictive silencer factor occupancy at its target genes in Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**(26), 6972-83, 10.1523/JNEUROSCI.4278-06.2007.

Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B. R., Goffredo, D., Conti, L., MacDonald, M. E., Friedlander, R. M., Silani, V., Hayden, M. R., Timmusk, T., Sipione, S. and Cattaneo, E. (2001). Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science* **293**(5529), 493-8, 10.1126/science.1059581.

Zuccato, C., Tartari, M., Crotti, A., Goffredo, D., Valenza, M., Conti, L., Cataudella, T., Leavitt, B. R., Hayden, M. R., Timmusk, T., Rigamonti, D. and Cattaneo, E. (2003). Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nature genetics* **35**(1), 76-83, 10.1038/ng1219.

Accessed websites:

Creatine Safety, Tolerability, & Efficacy in Huntington's Disease (CREST-E). Phase 3 HD clinical trial. ClinicalTrials.gov Identifier:
NCT00712426. Accessed 7/5/2013
http://clinicaltrials.gov/show/NCT00712426

Raptor Pharmaceutical Corp. (Nasdaq:RPTP) Phase 2/3 clinical trial of RP103 (delayed-release cysteamine) for the potential treatment of Huntington's disease (HD) in collaboration with the Centre Hospitalier Universitaire d'Angers (CHU d'Angers). Accessed 7/5/2013 and 21/02/2014
http://www.raptorpharma.com/
http://ir.raptorpharma.com/releasedetail.cfm?ReleaseID=826962

"It is good to have an end to journey toward; but
it is the journey that matters, in the end"
Ernest Hemingway

Universidade de Coimbra

OXIDATIVE STRESS IN HUNTINGTON ’S DISEASE KNOCK-IN STRIATAL CELLS

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