Departament de Biologia Cel·lular, Immunologia i Neurociències
Facultat de Medicina

Characterization of the mechanisms underlying alterations in macroautophagy and survival signalling in Huntington's disease

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Above all, don't fear difficult moments.
The best comes from them.

Rita Levi-Montalcini

A tots aquells que m'heu donat el vostre suport dia a dia. Al Joan, a la Pilar, a l'Emma, al Pau, al Martí, al Dídac, als meus amics i a l'Adrià.

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RESUM

La malaltia de Huntington és un trastorn neurodegeneratiu progressiu causat per una expansió de repeticions del triplet CAG (més de 37) en l'exó 1 del gen de la huntingtina que genera una proteïna aberrant. Aquest canvi en la proteïna provoca una pèrdua selectiva de neurones GABAèrgiques de projecció en el nucli estriat, tot i que també s'han detectat alteracions i degeneració en altres àrees de l'encèfal, generant una simptomatologia complexa que engloba alteracions motores, cognitives i emocionals. Un marcador de la patologia és la formació d'agregats que tenen una ultraestructura fibril·lar. Aquestes estructures estan principalment formades per fragments N-terminals de la huntingtina mutada, que són generats per l'acció de proteases.

Un dels processos que condueix a la mort selectiva de les neurones és l'activació de l'apoptosi. L'equilibri entre vies pro-apoptòtiques i vies de supervivència és el que regula el destí de la cèl·lula. D'aquesta manera, la via de supervivència més important en la cèl·lula, la via de PI3K/AKT, es troba sobre-activada en el nucli estriat de diferents models cel·lulars i animals de la malaltia de Huntington. A més, els nivells de PHLPP1, una fosfatasa que defosforila AKT, es troben disminuïts en la malaltia de Huntington, contribuint en la sobre-activació de la via d'AKT al llarg de la patologia.

Una proteïna que es troba en la via de l'AKT i que juga un paper central en la supervivència cel·lular, la transcripció, la traducció proteica i l'autofàgia, és mTOR. mTOR és a la vegada un substrat indirecte d'AKT i quinasa que fosforila directament AKT, segons si forma part del complex mTORC1 o mTORC2 i amb quines proteïnes s'associï. El fet que la via d'AKT es trobi alterada en la malaltia de Huntington ens suggereix que l'activitat de mTOR també podria estar afectada. Per tant, ens va interessar estudiar per una banda la via de mTOR, i per l'altra, un dels processos que aquesta via regula, la macroautofàgia (a partir d'ara serà anomenat simplement com a autofàgia). A més a més, l'autofàgia participa en l'eliminació d'agregats proteics i, la seva activació, s'ha proposat com a mecanisme terapèutic per a la malaltia de Huntington.

La fosfatasa PHLPP1 s'ha descrit que també regula les PKCs. En el moment en què PKC es defosforila, es degrada. Aquests experiments juntament amb evidències de que una isoforma de les PKC presenta una expressió gènica reduïda, suggereixen que hi podrien haver alteracions en la via de les PKC en la malaltia de Huntington. En mamífers s'han descrit 12 isoformes diferents de les PKC, que estan distribuïdes en categories segons la seva activació. Alhora, les PKCs regulen tant processos de proliferació cel·lular i diferenciació, com d'apoptosis.

Per tant, en aquesta tesi doctoral hem estudiat possibles alteracions en l'autofàgia selectiva i les vies de senyalització de mTOR i de PKC, per tal d'analitzar la seva participació en la degeneració que es produeix en la malaltia de Huntington.

Totes aquestes vies s'han estudiat, principalment, en un model animal de la malaltia de Huntington, el ratolí R6/1, que sobre-expressa un fragment de la huntingtina mutada humana, l'exó-1, amb 145 repeticions CAG. S'ha descrit que en aquests animals els símptomes motors apareixen entre les 15 i les 21 setmanes d'edat, tot i que ja s'observen agregats de huntingtina mutada a partir de les 8 setmanes d'edat. Els símptomes cognitius tenen una aparició anterior que els símptomes motors, cap a les 12 setmanes d'edat.

Per tal d'estudiar el procés d'autofàgia selectiva al llarg de la malaltia de Huntington, es van analitzar els nivells i la localització intracel·lular de dues proteïnes, p62/SQSTM1 (p62) i NBR1, que reconeixen específicament components cel·lulars que s'han de degradar per autofàgia. Aquestes dues proteïnes tenen un domini d'interacció amb els autofagosomes i es degraden en el moment en què els autofagosomes es fusionen amb els lisosomes. Per això, alteracions en els nivells de p62 i NBR1 s'han associat a canvis en l'activitat autofàgica. Els nivells de p62 i NBR1 van ser estudiats en el model de ratolí R6/1 en diferents àrees cerebrals (nucli estriat, hipocamp i escorça cerebral) al llarg de la progressió de la malaltia. En comparació amb els animals control, els nivells proteics de p62 i NBR1 es van trobar alterats en els animals R6/1. Els nivells de p62 i NBR1 disminuïen a un estadi inicial de la malaltia, les 12 setmanes d'edat dels ratolins R6/1, en totes les zones cerebrals estudiades, suggerint una major degradació d'aquestes proteïnes degut a una major activitat autofàgica. A les 30 setmanes d'edat, un estadi avançat de la patologia, els nivells de p62 i NBR1 incrementaven tant en el nucli estriat com en l'hipocamp. Els nivells de les dues proteïnes en l'escorça cerebral es mantenien reduïts des de les 12 setmanes d'edat fins a etapes avançades de la patologia. Per tal d'entendre la causa d'aquesta desregulació es va analitzar la seva expressió gènica en el nucli estriat i en l'escorça cerebral als mateixos estadis de la patologia. En les dues zones cerebrals es van observar canvis en l'expressió de p62, amb un increment a partir de les 8 setmanes d'edat, però no es van trobar canvis en l'expressió de NBR1. Com que el patró d'expressió no era suficient per entendre les alteracions dels nivells de proteics, vam pensar que altres mecanismes hi podien estar implicats, com per exemple la interacció d'aquestes proteïnes amb els agregats de huntingtina mutada. Es van realitzar immunoprecipitacions d'extractes proteics del nucli estriat i de l'escorça cerebral

d'animals de 30 setmanes. Es va observar que tant p62 com NBR1 co-immunoprecipitaven amb la huntingtina mutada. Mitjançant immunohistoquímica es va detectar que p62 no només interaccionava amb els agregats de huntingtina mutada, sinó que ho feia principalment amb els agregats nuclears. Aquest resultat suggeria un canvi en la distribució nucli-citoplasma de p62, que es va confirmar mitjançant *western blot* de fraccions citoplasmàtiques i nuclears. En el nucli estriat dels animals R6/1 a les 12 setmanes d'edat, quan observàvem disminució dels nivells totals de les dues proteïnes, p62 era principalment citoplasmàtica, mentre que a les 30 setmanes, s'acumulava en les fraccions nuclears d'animals R6/1 en comparació amb els control. p62 també s'acumulava en fraccions nuclears d'extractes d'hipocamp, i en menor grau en l'escorça cerebral a les 30 setmanes d'edat. Es van realitzar tincions immunohistoquímiques en mostres de malalts de Huntington i es va detectar que, mentre que p62 es trobava principalment en agregats nuclears, NBR1 tenia una localització principalment citoplasmàtica i molt lligada al marcatge de lipofucsina, confirmant els resultats que observàvem en el model animal. p62 viatja del nucli al citoplasma mitjançant l'exportina-1. S'ha descrit que els nivells proteics d'exportina-1 disminueixen amb l'edat en mostres d'animals R6/2, un altre model de la malaltia de Huntington. Per tal de saber si la reducció dels nivells d'exportina-1 amb l'edat podien tenir algun paper en l'acumulació de p62, es van analitzar els nivells d'exportina-1 en les diferents àrees cerebrals a les 12 i a les 30 setmanes d'edat en l'animal R6/1. Mentre que els nivells d'exportina-1 disminuïen des de l'edat presimptomàtica fins a un estadi avançat de la patologia en el nucli estriat i en l'hipocamp, es trobaven inalterats en l'escorça cerebral.

Per tal de determinar si aquesta disminució amb l'edat de l'exportina-1 podia tenir algun efecte sobre l'autofàgia, mitjançant l'acumulació nuclear de proteïnes importants per aquest procés, es va tractar una línia cel·lular estable d'origen estriatal ($STHdh^{7Q/7Q}$) amb Leptomicina B (LMB), inhibidor irreversible de l'exportina-1. Després del tractament es van analitzar els nivells d'autofagosomes totals utilitzant el marcador LC3II, que és una proteïna associada específicament a la membrana dels autofagosomes. Es va observar un increment significatiu dels nivells de LC3II en les cèl·lules tractades amb LMB respecte les cèl·lules control. Per tal de saber si aquest increment era resultat d'una major síntesi d'autofagosomes, es va bloquejar la degradació d'aquests co-tractant durant 6h amb Bafilomicina A1 (BafA1). No s'observava cap increment addicional quan es co-tractava amb els dos fàrmacs, fet que suggeria que els increments de LC3II no eren deguts a una major síntesi d'autofagosomes. Per tal de saber si la degradació dels autofagosomes, pas essencial per la finalització del procés d'autofàgia, podia estar alterada, es van transfectar les

cèl·lules amb un plàsmid que expressa mRFP-GFP-LC3 en tàndem. Els autofagosomes són positius pels dos fluoròfors. Quan els autofagosomes es fusionen amb un lisosoma, s'acidifiquen i el GFP queda atenuat i es degrada ràpidament per l'acció de les hidrolases lisosomals. Aleshores, aquestes estructures només són positives per mRFP. Es va calcular el percentatge d'autofagosomes no acidificats (percentatge de col·localització) respecte el total d'estructures autofàgiques (estructures positives per mRFP). En presència de LMB, el percentatge d'autofagosomes respecte el total d'estructures autofàgiques incrementava, indicant una degradació ineficient dels autofagosomes i per tant un defecte en el procés d'autofàgia. Finalment, per saber si aquest defecte en l'autofàgia podia alterar la degradació de proteïnes de vida mitja llarga, es van fer estudis de proteòlisi mitjançant un marcador radioactiu. L'experiment va ser realitzat en una condició control i també sota diferents condicions per portar el sistema a l'extrem, en deprivació de sèrum per incrementar l'autofàgia o amb un tractament de NH_4Cl i leupeptina per inhibir-la. En cap de les condicions el tractament amb LMB retardava la degradació de proteïnes de vida mitja llarga.

Els resultats obtinguts durant el desenvolupament d'aquest primer objectiu suggereixen que en fases inicials de la malaltia de Huntington l'autofàgia selectiva es troba incrementada, ja que els nivells proteics de p62 i NBR1 disminueixen de forma similar en totes les àrees cerebrals del model R6/1. En un estadi avançat de la malaltia, p62 es va acumulant al nucli degut a la interacció amb els agregats de huntingtina mutada i a la reducció amb l'edat dels nivells d'exportina-1. El fet que NBR1 no es localitzi als agregats nuclears de huntingtina mutada, fa pensar que podria ser una proteïna essencial en mantenir el procés d'autofàgia en la malaltia de Huntington.

Les alteracions detectades en el flux de l'autofàgia selectiva, podrien ser induïdes per canvis en l'activitat de la quinasa mTOR. És per això que ens vam plantejar estudiar la via de senyalització de mTOR al llarg de la progressió de la malaltia per entendre la seva contribució en la desregulació de l'autofàgia, i per la sobre-activació específicament estriatal de la quinasa de supervivència AKT. Per això, es va estudiar la via de mTOR en l'estriat del model R6/1 en unes edats semblants a les que s'havia analitzat la fosforilació d'AKT. S'han descrit sis fosforilacions diferents per mTOR, de les quals la fosforilació en la serina 2448 és la que està regulada per AKT, mentre que la fosforilació en la serina 2481 està més relacionada amb la seva activació. Es van detectar increments significatius d'aquests dos residus fosforilats a partir de les 12 setmanes d'edat en el nucli estriat dels ratolins mutants en comparació amb els

control, mentre que els nivells totals de mTOR es mantenien invariables. En altres models de la malaltia de Huntington s'ha trobat mTOR segrestada als agregats nuclears de huntingtina mutada. Ens vam preguntar si pmTOR hi podia quedar també segrestada i així acumular-s'hi. Es van analitzar les formes fosforilades de mTOR en fraccions enriquides en nucli i citoplasma del nucli estriat de ratolins R6/1 de 30 setmanes d'edat, i no es van detectar canvis en la seva localització respecte els animals control. Per immunohistoquímica, tampoc es va observar colocalització de pmTOR, ni mTOR, amb els agregats de huntingtina mutada. Aquests resultats ens van suggerir que la via de mTOR es trobava alterada i incrementada en la malaltia de Huntington. mTOR, però, no treballa sola sinó en associació amb altres proteïnes, formant els complexes mTORC1 i mTORC2. Raptor i Rictor s'associen a mTOR per definir, respectivament, aquests dos complexes, i són les dues proteïnes que determinen l'especificitat de substrat. D'aquesta manera, mTORC1 regula la traducció i l'autofàgia, mentre que mTORC2 regula la supervivència cel·lular i el citoesquelet. Per tal de conèixer quins dels dos complexes es troba més afectat en la patologia, ens vam plantejar d'estudiar els nivells per *western blot* de les proteïnes Rictor i Raptor. Els nivells proteics de Rictor, però no de Raptor es van trobar incrementats al nucli estriat dels ratolins R6/1 a totes les edats estudiades, i el mateix efecte es va observar en mostres de putamen d'humans afectats de la malaltia de Huntington. Aquest resultat ens va suggerir que el complex mTORC2, però no mTORC1 es trobava sobre-activat en la malaltia. Per demostrar aquesta hipòtesi, es van analitzar diferents substrats específics dels dos complexes. Es van analitzar ULK1, S6K i 4EBP1, que són regulades pel complex mTORC1, i per altra banda es van analitzar SGK i PKCα, que estan, conjuntament amb AKT, regulades pel complex mTORC2. No es van trobar alteracions en cap de les fosforilacions dels substrats de mTORC1 i de mTORC2, a part de 4EBP1 i AKT. Els nivells de la forma fosforilada de 4EBP1 incrementaven des de les 8 setmanes d'edat, molt abans de que pAKT i pmTOR incrementessin. Els resultats que s'han obtingut fins al moment suggereixen que la sobre-activació de la via d'AKT podria ser induïda per increments en l'activitat mTORC2, en part deguda a l'augment dels nivells proteics de Rictor i de pmTOR, i que podrien tenir un paper compensatori en la malaltia de Huntington davant de la toxicitat induïda per la huntingtina mutada. Tot i això, mTOR, no sembla tenir cap efecte sobre el procés d'autofàgia, ja que no es van trobar canvis en la fosforilació de ULK1, proteïna essencial per la formació dels autofagosomes en l'autofàgia. Per tant, els canvis en activitat autofàgica, detectats mitjançant l'anàlisi dels nivells de p62 i NBR1, semblen ser deguts a una regulació mitjançant un mecanisme independent de mTOR.

Finalment, vam analitzar al llarg de la patologia els nivells totals de dues isoformes de les PKC convencionals, la PKCα i la PKCβII, i una isoforma de les PKC novedoses, la PKCδ, en el nucli estriat, escorça cerebral i hipocamp del model R6/1. Vam detectar un lleuger descens dels nivells de les dues isoformes convencionals en totes les àrees cerebrals a partir de les 12 setmanes d'edat. En canvi, els nivells de PKCδ estaven molt reduïts a totes les àrees cerebrals examinades ja a partir de les 8 setmanes d'edat, i seguien disminuint fins a arribar a ser molt reduïts a etapes avançades de la patologia. Addicionalment es va detectar una disminució dels nivells de PKCδ en mostres humanes de putamen afectats de la malaltia de Huntington. A partir d'aquest punt vam decidir focalitzar l'estudi en la PKCδ, ja que aquesta era la isoforma que presentava unes alteracions majors i que, a més, és pro-apoptòtica. No vam trobar diferències en els nivells d'un fragment catalíticament actiu de PKCδ generat per la caspasa-3 que es transloca al nucli de les cèl·lules generant fragmentació del DNA i apoptosis. A més, vam observar que els nivells estaven reduïts en tota la cèl·lula sense haver-hi diferències de reducció dels seus nivells proteics entre el nucli i el citoplasma, essent PKCδ una proteïna principalment citoplasmàtica. Finalment, vam analitzar si aquesta disminució de PKCδ podia ser deguda a un increment en la seva degradació, per un mecanisme independent a PHLPP, ja que aquesta es troba reduïda en els mateixos models animals de la malaltia. S'ha descrit que un increment en la fosforilació de la treonina 505 incrementa la degradació de la proteïna. Els nivells d'aquesta fosforilació es van trobar incrementats a les 30, però no a les 8, setmanes d'edat en el model animal R6/1 en el nucli estriat, l'hipocamp i l'escorça cerebral. Aquests resultats ens suggerien que la disminució de la proteïna pro-apoptòtica PKCδ, mediada per processos de degradació, podrien ser un mecanisme compensatori activat per les cèl·lules en resposta a l'expressió de huntingtina mutada par tal d'intentar sobreviure. Per demostrar aquesta hipòtesi vam transfectar cèl·lules estriatals amb un plàsmid per sobre-expressar PKCδ, juntament amb un plàsmid per sobre-expressar l'exó 1 de la huntingtina normal i mutada. Fent un recompte de nuclis apoptòtics, vam observar que la sobre-expressió de PKCδ en cèl·lules que expressen l'exó 1 de la huntingtina mutada, però no la normal, induïa més mort cel·lular que la que s'induïa per l'única sobre-expressió de la huntingtina mutada. Aquest últim resultat indica que una reducció de PKCδ des de fases inicials de la patologia podria ser un mecanisme de supervivència generat per les cèl·lules per tal de contrarestar la toxicitat de la huntingtina mutada.

Tots aquests resultats indiquen que en la malaltia de Huntington hi ha un equilibri entre senyals apoptòtiques i mecanismes de supervivència. La huntingtina mutada

genera toxicitat que pot ser contrarestada per diferents mecanismes, com la degradació de la mateixa huntingtina induint l'autofàgia selectiva, o l'activació de senyals de supervivència com la inducció de la via de l'AKT-mTOR i la inhibició de la proteïna pro-apoptòtica PKCδ. Per tant, la regulació d'aquestes vies de supervivència seria una bona teràpia per evitar la degeneració neuronal en la malaltia de Huntington.

ABBREVIATIONS

4EBP1	4E-binding protein 1
AKAP	A-Kinase anchoring protein
AKT	Protein kinase B
AMBRA1	Autophagy/beclin-1 regulator-1
AMPK	5' AMP-activated protein kinase
aPKC	Atypical PKC
Atg	Autophagy-related genes
BafA1	Bafilomycin A1
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
CBP	CREB-binding protein
CDK5	Cyclin-dependent kinase 5
CFP	Cyan fluorescent protein
cPKC	Conventional PKC
DAG	Diacylglicerol
Deptor	DEP domain-containing mTOR-interacting protein
DNA	Deoxyribonucleic acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-ligating enzyme
FIP200	Focal adhesion kinase family interacting protein 200 kDa
FKBP12	12 kDa FK506-binding protein
GABA	Y-Aminobutyric acid
GFP	Green fluorescent protein
GPe	Globus pallidus pars externa
GPi	Globus pallidus pars interna
GSK3β	Glycogen synthase-3β
HA	Haemmagglutinin
HDAC6	Histone deacetylase
HEAT	Huntingtin, Elongation fator3, PR65/A subunit of protein phosphatase 2A and mTOR
HIP14	Huntingtin-interacting protein 14
HSC70	Heat shock 70 kDa protein
IKKβ	IkappaB kinase
JNK1	c-Jun N-terminal kinase-1
LAMP2A	Lysosome-associated membrane protein
LIR	LC3 interaction region

LMB	Leptomycin B
Lys	Lysine
MAPK	Mitogen-activated protein kinase
mGluR1	Metabotropic glutamate receptor 1
mLST8/GβL	Mammalian lethal with SEC13 protein 8
mRFP	Monomeric red fluorescent protein
mRNA	Messenger ribonucleic acid
mSin1	Mammalian stress-activated MAPK-interacting protein 1
mTOR	Mammalian target of rapamycin kinase
mTORC1	mTOR complex
NBR1	Neighbour of BRCA1 gene 1
NDP52	Nuclear dot protein 52
NES	Nuclear export signal
NLS	Nuclear localization signal
NMDA	N-Methyl-D-aspartic acid
nPKC	Novel PKC
p62	p62/Sequestosome1
PB1	Phox and Bem1
PDK1	3-Phosphoinositide dependent protein kinase-1
PE	Phosphatidylethanolamine
PH	Pleckstrin homology
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PolyQ	Polyglutamine
PP2A	Protein phosphatase 2A
PRAS40	40 KDa Pro-rich AKT substrate
Protor	Protein observed with Rictor
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RACK	Receptors for activated C kinases
Rag	Ras-related GTPase
Raptor	Regulatory-associated protein of mTOR

Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
S6K1	p70 ribosomal S6 protein kinase-1
Ser	Serine
SGK	Serum glucocorticoid-regulated kinase
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
SUMO	Small ubiquitin-like modifier
tf-LC3	Tandem fluorescent-tagged LC3
TFEB	Transcription factor EB
Thr	Threonine
TrkA	Tropomyosin receptor kinase A
TSC	Tuberous sclerosis protein
UBA	Ubiquitin-associated
ULK1	UNC-51–like kinase
UPS	Ubiquitin-protasome system
Vps	Vacuolar protein sorting
Xpo-1	Exportin-1
YAC	Yeast artificial chromosome
δCF	PKCδ catalytic fragment

SUMMARY

I. INTRODUCTION

Neurodegenerative diseases are characterized by neuronal loss and synaptic abnormalities, which are chronic and progressive. Intriguingly, these abnormalities most commonly appear later in adulthood and less likely in youth. Mature neurons have the ability to activate survival mechanisms in response to harmful stimuli that would act as brakes of neurodegeneration. Therefore, whether a neuron lives or dies in pathological conditions is the result of a complex balance between anti- and pro-apoptotic signals, respectively. This balance might strongly regulate the onset of the disease. Here, we have characterized some putative compensatory pro-survival processes that could be involved in delaying Huntington's disease pathology.

1. - HUNTINGTON'S DISEASE

Huntington's disease is an autosomal hereditary progressive neuropathological disorder characterized by a mutation on the chromosome 4p16.3 (Gilliam *et al.,* 1987; Wexler *et al.,* 1987) of the *It15* gene, which encodes for the protein huntingtin (HDCRG, 1993). The mutant gene contains an expanded number of CAG repeats in the N-terminal of the sequence, more precisely at the exon 1 (which is about 88 amino acids long) of the gene. This mutation leads to lengthening of the polyglutamine (polyQ) chain of huntingtin, and thus to alter its biological functions if the mutation is long enough (HDCRG, 1993). The disease occurs when the threshold of about 36 polyQ is exceeded (Kremer *et al.,* 1994). The mutation generates a mutant protein with altered folding and high propensity to generate intracellular aggregates (DiFiglia *et al.,* 1997). The onset of symptoms has been inversely correlated with the lengthening of the mutation. Juvenile or early-onset Huntington's disease, which is the most severe outcome, is associated with CAG repeat lengths greater than 50, whereas adult-onset of the disease is associated with CAG repeats from 40 to 50 (Rubinsztein *et al.,* 1996). Individuals with CAG repeats in the range of 35 to 40 show incomplete penetrance of the mutant allele. Finally, normal CAG repeat length is usually about 17-20. Intervals of 20-35 CAG repeats are considered rare, and not pathological, but are meiotically unstable and can expand into the disease range in some cases (Imarisio *et al.,* 2008).

The disease was first described in 1872 by George Huntington (1850-1916), who named the disease Chorea (from the Greek *choreia* that means dance) due to the marked and characteristic feature of involuntary movements in those who are affected (Huntington, 1872). The incidence is about 5-7 individuals within 100,000, although in some areas such as Tasmania and the area around Lake Maracaibo in Venezuela the

incidence increases significantly. In contrast, in most African and Asian populations the prevalence of the disease is very low (Walker, 2007).

Huntington's disease is manifested with a triad of motor, cognitive and also psychiatric symptomatologies. Movement disorder for most patients follows a biphasic pattern, while initially dyskinetic movements predominate, later on, they decrease to bradykinetic movements characterized by rigidity (Young *et al.,* 1986). Chorea, which is common at early Huntington's disease and wanes with disease progression, characterizes the dyskinetic movements and refers to the rapid, irregular, and jerky movement of the limbs, trunk, and face. Patients with an early-onset Huntington's disease might not develop chorea (Sturrock & Leavitt, 2010). Cognitive dysfunction in Huntington's disease, often spares long-term memory, but is characterized by a loss of executive functions, such as organising, planning, checking, or adapting alternatives, and delays the acquisition of new motor skills. Speech deteriorates faster than comprehension. Psychiatric symptoms, however, arise with some frequency but do not progress with disease severity. Depression, anxiety and irritability are typical, and suicide happens with more frequency than in general population. Manic and psychotic symptoms can also develop (Walker, 2007). Besides the triad, other symptoms do appear such as significant weight loss and, occasionally and with more frequency in juvenile Huntington's disease, epileptic seizures (Gonzalez-Alegre & Afifi, 2006; Morales *et al.,* 1989). Before all these symptoms start to emerge gradually, individuals are healthy and have no detectable clinical abnormalities. Death occurs normally 12-15 years from the time of symptomatic onset (Vonsattel, 2008). The cause of death is infection in about 45% of cases, being pneumonia responsible for around 25% of deaths in Huntington's disease (Sturrock & Leavitt, 2010).

1.1 - NEUROPATHOLOGY

Although huntingtin is expressed in all the tissues, some areas of the brain present enhanced susceptibility to the mutated protein compared to other areas and tissues in the body. Atrophy of the striatum (caudate nucleus and putamen) and thinning of the cortex are the most significant neuropathological abnormalities, although atrophy also occurs in other brain areas (Vonsattel *et al.,* 1985) (Fig. 1). It has been suggested that this brain atrophy may start before the onset of clinical symptoms (Aylward *et al.,* 2004; Kipps *et al.,* 2005). The neuropathologist Jean Paul Vonsattel developed in 1985 a scale to grade the striatal neuropathology in Huntington's disease, which consists of 5

grades from 0 (no gross pathological abnormality) to 4 (severe pathology) (Vonsattel *et al*., 1985) (Fig. 1). Grade 0 appears indistinguishable from normal brains after gross examination. However, 30–40% neuronal loss can be detected only in the head of the caudate nucleus upon histological examination. Grade 1 shows atrophy, neuronal loss, and astrogliosis in the tail and, in some cases, the body of the caudate nucleus. Grades 2 and 3 are characterized by progressive and severe striatal atrophy, and grade 4 includes the most severe Huntington's disease cases with atrophy of the striatum and up to 95% neuronal loss (Vonsattel *et al*., 1985).

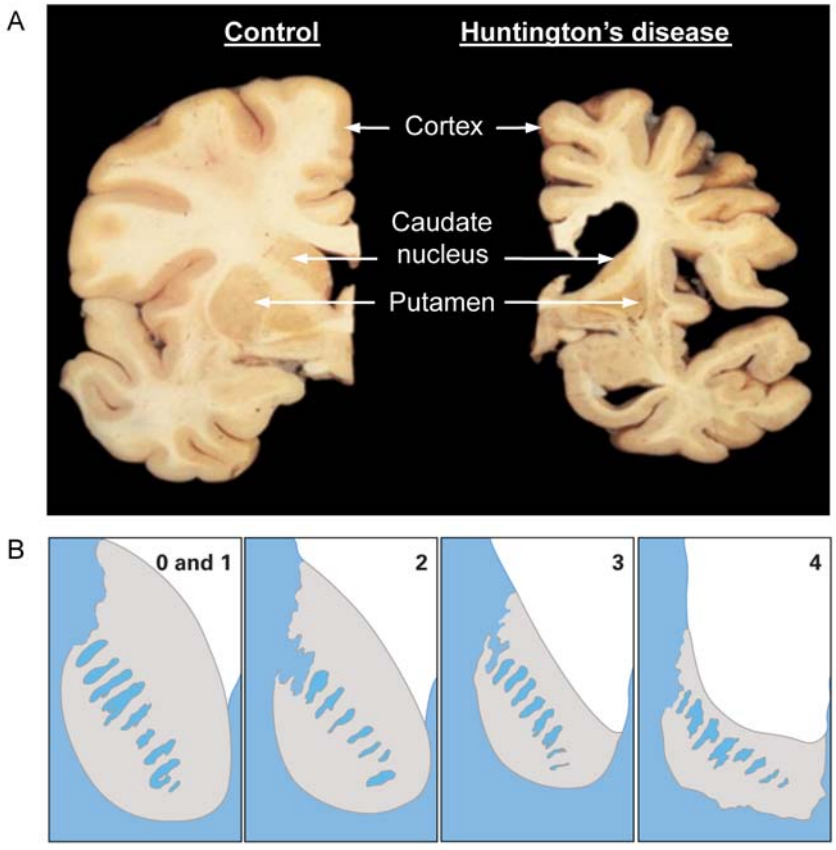

Figure 1.- Huntington's disease pathology. (A) Human brain coronal sections that show the degeneration that occurs in the caudate nucleus and putamen as well as the cortical atrophy in Huntington's disease. Image adapted from http://hdroster.iu.edu. (B) Scheme that represents the degrees of the striatal neuropathology in the Vonsattel grade scale. A brain in grade 0 appears macroscopically indistinguishable from normal brains, but there is already a 30–40% neuronal loss in the striatum. The neuropathology progresses from grade 1 until grade 4, with progressive atrophy, increasing astrogliosis and neuronal loss. At grade 4 there is up to 95% neuronal loss in the striatum. Figure adapted from Ellison & Love, 2004.

The striatum is a member of the interconnected subcortical nuclei group named basal ganglia, together with the globus pallidus pars externa (GPe) and interna (GPi), the subthalamic nucleus (STN) and the substantia nigra pars compacta (SNc), and

pars reticulata (SNr). The striatum is a convergence point for glutamatergic inputs from cortex and thalamus, as well as dopaminergic afferents from the midbrain. It is also the source of basal ganglia circuits that are critical for motor function and procedural learning (Bolam *et al.,* 2000). Two main types of neurons compose the striatum, the medium-sized spiny projection neurons and interneurons. Medium-sized spiny neurons represent the 90-95% of neuronal population within the striatum, and their excitability is modulated by interneurons (Kreitzer, 2009). Medium-sized spiny neurons are GABAergic striatal efferent neurons that connect with the GPe and GPi by two different pathways, the 'direct' and 'indirect' (Fig. 2). Neurons in the 'direct pathway' project directly from putamen to the GPi/SNr, and express GABA and substance P. Neurons in the 'indirect pathway', express GABA and enkephalin, and project to the GPe, which in turn project to the GPi/SNr (Gerfen & Surmeier, 2011; Obeso *et al.,* 2008) (Fig. 2).

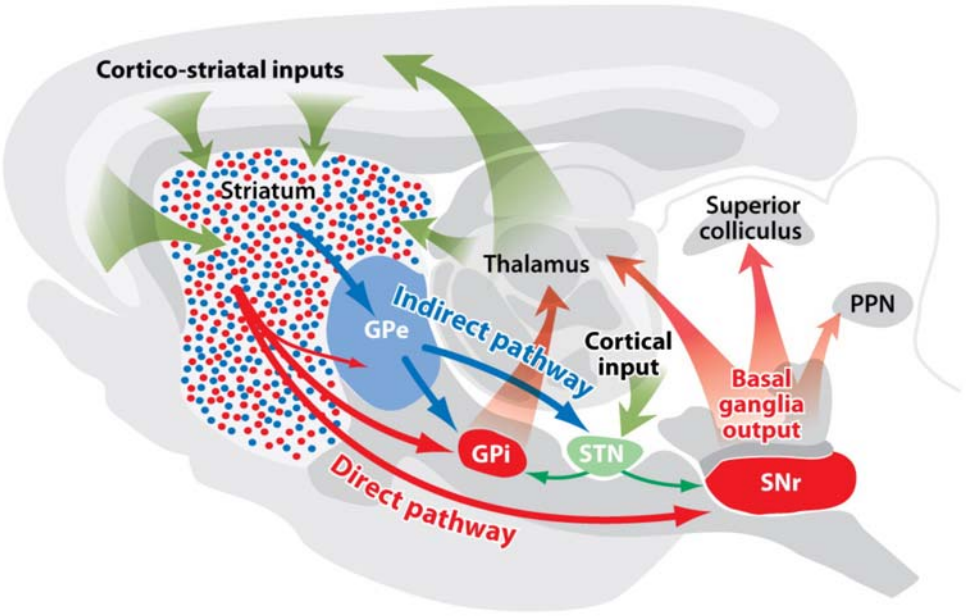

Figure 2.- Basal ganglia circuits. The striatum receives glutamatergic (green arrows) inputs from the cortex and the thalamus and projects to the GPe and GPi/SNr by two different GABAergic (blue and red arrows) pathways, the 'indirect' and 'direct', respectively. In Huntington's disease the 'indirect' pathway is affected earlier than the 'direct' pathway. Figure adapted from Gerfen & Surmeier, 2011.

Robert Ferrante and colleagues revealed that not the whole striatal neuronal population is prone to degenerate. While medium-sized spiny neurons were found to preferentially degenerate in Huntington's disease, interneurons were relatively spared (Ferrante *et al.,* 1985). Further immunohistochemical studies performed by Anne Young's group, revealed different degrees of degeneration of distinct medium-sized spiny neuron populations in Huntington's disease. In early and middle stages of Huntington's disease, the 'indirect pathway' was much more affected than the 'direct

pathway'. At a stage 4 of the disease, striatal efferent projections were almost all depleted, with the exception of some striatal projections to the GPi/SNc (Reiner *et al.,* 1988). Degeneration of these two neuronal populations severely impairs both basal ganglia circuits and gives rise to the motor abnormalities that occur in Huntington's disease (Graybiel, 2000).

Although the striatum is highly affected in Huntington's disease, the disease is far from being only characterized by motor symptoms. Nowadays, it is well established that the degeneration that occurs in Huntington's disease is a more widespread phenomenon within the brain, which could explain the clinical heterogeneity and complexity of Huntington's disease. By means of magnetic resonance imaging (MRI)-based morphometric analysis, almost all brain structures, including cerebral cortex, globus pallidus, amygdala, hippocampus, brainstem, and cerebellum, volumes were found reduced at mid-stages of Huntington's disease (Jeste *et al.,* 1984; Rosas *et al.,* 2003). In grades 3 and 4, the cerebral cortex, particularly the efferent layers III, V, and VI, are markedly affected (Hedreen *et al.,* 1991; Heinsen *et al.,* 1994; Rosas *et al.,* 2008).

1.2 - HUNTINGTIN PROTEIN

Huntingtin is encoded by a single gene, which is 200 Kb long and possesses 67 exons, being the first of them the one containing the mutation (HDCRG, 1993). The translated huntingtin is a 348 KDa protein with 3144 amino acids (aa) (HDCRG, 1993), which shows ubiquitous expression, being brain and testis the organs that express huntingtin at highest levels, and neocortex, cerebellar cortex, striatum and hippocampus the main expression sites within the brain (Borrell-Pages *et al.,* 2006). Although a small proportion is also found in the nucleus (Kegel *et al.,* 2002), huntingtin is a primarily cytoplasmic protein that is known to be associated with the plasma membrane, endocytic and autophagic vesicles, endosomal compartments, the endoplasmic reticulum, the Golgi apparatus, mitochondria and microtubules (Imarisio *et al.,* 2008).

Huntingtin is a multi-domain protein and thus has several interacting partners and functions (Fig. 3). A considerable fraction of huntingtin contains tandem arrays of Huntingtin, Elongation fator3, PR65/A subunit of protein phosphatase 2A and mTOR (HEAT) repeats that are important for protein interactions and protein complex

formation (Andrade & Bork, 1995). Moreover, it is predicted that huntingtin contains from 28 to 36 HEAT repeats that could stack together generating an elongated superhelical solenoid with diameter of 200Å (Li *et al.,* 2006; Takano & Gusella, 2002). Both a cluster of the first three HEAT repeats flanked by positively charged regions (amino acids residues 172–372), and the huntingtin N-terminal stretch of about 17 aa do play an important role in targeting huntingtin to the various intracellular membrane-bound organelles (Atwal *et al.,* 2007; Kegel *et al.,* 2005; Rockabrand *et al.,* 2007). It is unknown whether huntingtin contains nuclear localization signal (NLS), although a conserved nuclear export signal (NES) is found near its C-terminus (Xia *et al.,* 2003), which would account for the small proportion of huntingtin present in the nucleus. Its nuclear entrance could be triggered by other non-described means of transport. Another important region within huntingtin sequence is the poly-proline stretch, which is localized just after the polyQ chain. This domain also triggers an important structural role in the mutant huntingtin (Darnell *et al.,* 2007).

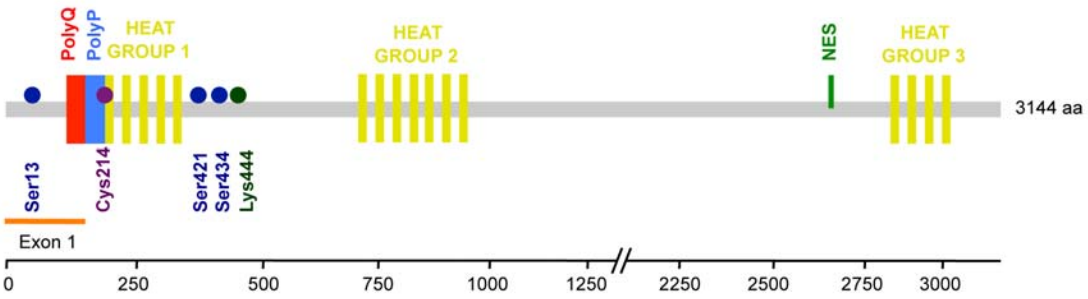

Figure 3.- Schematic diagram of the huntingtin protein. The diagram represents the huntingtin amino acid sequence with the polyglutamnie (PolyQ) and poly-proline (PolyP) tracts, the HEAT repeat domains and the nuclear exporting signal (NES). The following post-translational modifications are indicated: Phosphorylation at Ser13, Ser421 and Ser434 (blue), palmytoilation at Cys214 (purple), and acetylation at Lys444 (green). Figure adapted from Zuccato *et al.,* 2010.

Huntingtin and mutant huntingtin undergo posttranslational modifications with different preferences. The competition of both SUMOylation and ubiquitination events that normally occur at lysines localized at the first 17 aa from the N-terminal huntingtin just before the polyQ stretch, regulate huntingtin fate, localization and function. It has been proposed that mutant huntingtin would be less ubiquitinated since it would have higher preference for SUMOylation events than the wild-type protein. This would permit mutant huntingtin escape from degradation fate (Steffan *et al.,* 2004). Huntingtin-interacting protein 14 (HIP14) induces huntingtin palmitoylation at cystein 214 (Huang *et al.,* 2004; Yanai *et al.,* 2006). A mutant huntingtin palmitoylation-resistant form induces increased cell toxicity, and HIP14 interacts less with mutant huntingtin than

with the wild-type. Thus, mutant huntingtin would potentially be less palmitoylated and consequently play a role in the pathology (Yanai et al., 2006). Mutant, but not the wild-type, huntingtin is also acetylated at lysine 444, which targets the protein to the macroautophagy pathway (Jeong *et al.,* 2009). Both huntingtin and mutant huntingtin can undergo proteolytic cleavage, generating small N-terminal fragments that, only in the case of mutant huntingtin, have been described to be highly toxic and with high tendency to aggregate (Hodgson *et al.,* 1999; Wellington *et al.,* 2002). Interestingly, phosphorylation reduces caspase-mediated mutant huntingtin cleavage and attenuate toxicity (Luo *et al.,* 2005; Warby *et al.,* 2009). Several reports show that huntingtin and mutant huntingtin can be phosphorylated on serine (Ser) 421 by protein kinase B (also named AKT) (Humbert *et al.,* 2002; Warby *et al.,* 2005) and at Ser434 by the cyclin-dependent kinase 5 (CDK5) (Luo *et al.,* 2005). Also IkappaB kinase (IKKβ) complex phosphorylates huntingtin and mutant huntingtin at Ser13, which promote modification of the adjacent lysine residues and target huntingtin clearance by the proteasome and the lysosome. In contrast, mutant huntingtin is less efficiently phosphorylated, which would ultimately contribute to disease (Thompson *et al.,* 2009).

The large size of the protein that makes isolation and analysis difficult, the lack of obvious homology with other proteins, its ubiquitous localization within the cell and promiscuous interactions with more than 200 partners identified to date, make difficult to understand all the cellular roles of huntingtin (Imarisio et al., 2008) (Fig. 4). Huntingtin has been associated with embryonic development due to the fact that knockout of the gene encoding for huntingtin in the mouse results in lethality early in embryogenesis (Embryonic day 8.5), prior to organogenesis (Duyao *et al.,* 1995; Nasir *et al.,* 1995; Zeitlin *et al.,* 1995). Huntingtin is also involved in cell viability with an anti-apoptotic role (Dragatsis *et al.,* 2000; Zeitlin et al., 1995), in transcription regulation by interacting with an array of proteins involved in the regulation of mRNA productions such as REST/NRSF (Zuccato *et al.,* 2003), in facilitating transport along microtubules by directly interacting with the dynein/dynactin microtubule-based motor complex (Colin *et al.,* 2008), in negatively regulating the activity of glutamate receptors (Sun *et al.,* 2001), in metabotropic glutamate receptor 1 (mGluR1) signalling via its interaction with optineurin (Anborgh *et al.,* 2005), and in several other reported processes.

Although the polyQ stretch has received much attention for its pathogenic properties when expanded, it is possibly not essential for normal huntingtin function, since mice with deletion of the short CAG triplet repeat exhibit no gross phenotypic differences in comparison to control littermates (Clabough & Zeitlin, 2006). Thus,

mutation and lengthening of this stretch probably confers different posttranslational modifications and different structure that would all lead to interact distinctly with its partners or with new ones and, thus, acquisition of new toxic functions (Fig. 4).

1.3 - MOUSE MODELS OF HUNTINGTON'S DISEASE

Since Huntington's disease is caused by a single mutation, the introduction of the mutant gene into non-human primate, mouse, fly, fish, worm and even sheep has permitted the generation of disease models that have helped to advance in the mechanistic insights of neurodegeneration.

However, genetic knowledge of the disease is very recent, and the first transgenic mouse model of the disease was not developed until 1996 (Mangiarini *et al.,* 1996). Before that, models of the disease were induced by either excitotoxic lesioning or mitochondrial dysfunction. The first toxic model of Huntington's disease was developed in 1976 and consisted on intrastriatal administration of kainic acid (a kainate receptor agonist) (Coyle & Schwarcz, 1976). It was followed by other models such as intrastriatal injection of quinolinic acid (an NMDA receptor agonist) or systemic administration of 3-nitropropionic acid (inhibitor of the complex II of the mitochondrial respiratory chain) (Beal *et al.,* 1993; Beal *et al.,* 1986). These insults induced in rats a selective degeneration of medium-sized spiny neurons, while sparing interneurons. Although these approaches could lead to the selective degeneration observed in Huntington's disease, in these models mutant huntingtin was lacking. Thus, the generation of several transgenic models has permitted to better understand the progression of the pathogenesis that underlies Huntington's disease and to evaluate the potential of new therapeutic approaches.

The wide array of animals that have been generated since the first transgenic mouse, provide a broad phenotype variety. While some models do hardly express visible phenotype until getting very old, others display neuropathology early from birth. This broad phenotype variety does not only exist between species but also in between distinct models generated in the same species. The species that has been most extensively used for the study of Huntington's disease is the mouse, and many different mouse models have been generated (Table 1).

Mouse model	Promoter	CAG repeats	Aggregates	Motor symptoms	Cognitive deficits	Lifespan	References
R6/1	Human *Huntingtin*	116	9 W	18 W (rota-rod)	12 weeks	32-40 W	Canals *et al.,* 2004; Giralt *et al.,* 2011b; Mangiarini *et al.,* 1996; Naver *et al.,* 2003
R6/2	Human *Huntingtin*	144	3-4.5 W	6-8 W (rota-rod)	4-8 weeks	13-16 W	Carter *et al.,* 1999; Davies *et al.,* 1997; Lione *et al.,* 1999; Mangiarini *et al.*, 1996
N171-82Q	Mouse prion protein	82	6.5 M	12 W (rota-rod)	Not reported	16-22 W	Schilling *et al.,* 1999
HD94-tet off	CAMKIIa-tTA	94	12 W	4 W (clasping)	Not reported	Normal	Martin-Aparicio *et al.,* 2001; Yamamoto *et al.*, 2000
YAC72	Human *Huntingtin*	72	No	16 M (rota-rod)	Not reported	Normal	Hodgson *et al.*, 1999; Seo *et al.,* 2008
YAC128	Human *Huntingtin*	120	18 M	6 M (rota-rod)	8.5 M	Normal	Slow *et al.,* 2003; Van Raamsdonk *et al.,* 2005
Hdh 94Q	Mouse *Huntingtin*	94	18 M	4 M (decreased locomotion)	4 M	Normal	Menalled *et al.,* 2003; Trueman *et al.,* 2007
Hdh 111Q	Mouse *Huntingtin*	109	10 M	24 M (gait deficits)	8 M in $Hdh^{Q7/Q111}$	Normal	Giralt *et al.,* 2012; Wheeler *et al.,* 2000
Hdh 140Q	Mouse *Huntingtin*	140	4 M	4 M (decreased locomotion)	Not reported	Normal	Menalled *et al.,* 2003
Hdh 150Q	Mouse *Huntingtin*	150	10-14 M	15-40 W (gait and rota-rod)	No	Normal	Heng *et al.* 2007; Lin *et al.*, 2001

Table 1. Genetically modified mouse models of Huntington's disease. The table includes information about the promoter under which the mutation is expressed, the CAG repeat number, the onset of mutant huntingtin aggregation, motor and cognitive symptoms, and lifespan. Weeks (W); months (M).

All these mouse models differ from each other with regard to the type of mutation expressed, portion of the protein included in the transgene, promoter employed, expression levels of mutant protein and even background strain, making each of them unique (Alberch *et al.,* 2008). While some strains display early neuropathology and mortality, others progress so slowly that visible phenotype is not appreciated until mice

get very old. This permits the study of different progression degrees of the human pathology. Importantly, none of these models do recapitulate with total reliability the phenotypic aspects of human pathology.

Mice models can be classified into categories based on the genetic introduction of the mutation, (1) mice that express only a N-terminal fragment of the mutant huntingtin gene, and (2) mice that express the full-length mutant huntingtin gene, being the first group the one that usually expresses a more severe phenotype of the disease.

1.3.1 - N-terminal mutant huntingtin models

R6/1 and R6/2 mice were the first transgenic models to be developed by Gillian Bates' group and collaborators in 1996, and, from all the mouse models generated, they present the most aggressive phenotype. They carry a human genomic fragment containing huntingtin promoter, mutant huntingtin exon 1 with CAG repeats and the first 262 base pair (bp) of human huntingtin intron 1 sequence (Mangiarini *et al.,* 1996). The differences between both lines are the copies of the transgene that have been integrated in the genome and the expression level of the transgene. R6/1 mice integrated in their genome one single copy of the transgenic fragment while R6/2 integrated three copies of it. Moreover, R6/1 transgene expression is 31% while R6/2 is 75% relative to the endogenous huntingtin expression level (Mangiarini *et al.,* 1996). R6/1 mouse model exhibits weight loss at 22 weeks of age, and R6/2 mice, which develop a more aggressive phenotype than R6/1 mice, show weight loss at 8 weeks of age (Mangiarini *et al.,* 1996). Neuronal atrophy is a general event within the brain in both mice strains that curses together with ventricular enlargement but minimal neuronal loss (Canals *et al.,* 2004; Mangiarini *et al.,* 1996; Turmaine *et al.,* 2000). R6/2 mice also develop astrogliosis (Giralt *et al.,* 2011a).

N171-82Q mice carry a N-terminal fragment from the human mutant huntingtin gene, which is longer than the exon 1 in R6 models. In these mice, a slight number of cortical (4%) and striatal (1.8%) apoptotic neurons have been detected at 5 months of age, as well as reactive gliosis at 4 months of age (Yu *et al.,* 2003).

Finally, the generation of a conditional mouse model expressing in the forebrain an N-terminal fragment of the mutant huntingtin under an inducible promoter (HD94) has favoured the study of Huntington's disease phenotype reversibility. Silencing the

expression of mutant huntingtin, even relatively late in pathology, resulted in not only halting the disease progression but also reversal of aggregate formation and motor decline (Yamamoto *et al.,* 2000). Interestingly, these mice show a significant progressive striatal neuronal loss from 17 to 22 months of age, which can be partially reverted by silencing the expression of mutant huntingtin during these months (Diaz-Hernandez *et al.,* 2005).

1.3.2 - Full-length mutant huntingtin models

Mice that express the full-length mutant huntingtin gene can be grouped in those in which mutant huntingtin is delivered in a yeast artificial chromosome (YAC) and those in which a knock-in mutation has been performed to modify the endogenous murine huntingtin.

Several YAC models that express different repeat length have been generated. Although all the YAC lines suffer from brain atrophy, the one with 128 CAG repeats presents the most severe pathology (Slow *et al.,* 2003). It is the only model that, besides striatal and cortical atrophy, develops quantitive striatal neuronal loss at 12 months of age (15-18%) (Slow *et al.*, 2003).

Finally, knock-in models are more faithful genetic models of the human condition because they carry the mutation in its appropriate genomic context. These animals, however, present a late onset of the disease and a mild progression of the pathology. None of the knock-in lines generated, develop neuronal loss (Menalled, 2005; Wheeler *et al.,* 2002; Yu *et al.,* 2003), although reactive gliosis can be detected in 111 and 150 CAGs knock-in models (Lin *et al.,* 2001; Wheeler *et al.,* 2002).

Stable cell lines, as a tool to study *in vitro* mutant huntingtin effects, have been generated from the HdhQ9 and HdhQ111 mice. Striatal precursors at embryonic day 14 were immortalized with the simian vacuolating virus 40 Large T antigen. The generated cell lines are the control STHdh$^{7Q/7Q}$ and the Huntington's disease cellular models STHdh$^{7Q/111Q}$ and STHdh$^{111Q/111Q}$ (Trettel *et al.,* 2000).

1.4 - MOLECULAR MECHANISMS INVOLVED IN NEURONAL DYSFUNCTION IN HUNTINGTON'S DISEASE

Although mutant huntingtin is ubiquitously expressed, neurons, and more precisely striatal medium-sized spiny neurons cells, are primarily affected in Huntington's disease. All along the disease progression stressful events are continuously generated. However, the exact mechanisms that drive to the selective neuronal vulnerability and neuronal degeneration are still under debate. Much research has been performed to understand the process by which mutant huntingtin triggers neurodegeneration, and why some specific cells are primarily affected in Huntington's disease. Several studies have indicated that huntingtin may be a scaffold protein involved in orchestrating sets of proteins for intracellular transport and signalling processes. Huntingtin has been implicated in vesicle transport and cytoskeletal anchoring (Gutekunst *et al.,* 1998) as well as in clathrin-mediated endocytosis, neuronal transport processes and postsynaptic signalling (Harjes & Wanker, 2003; Landles & Bates, 2004; Li & Li, 2004).

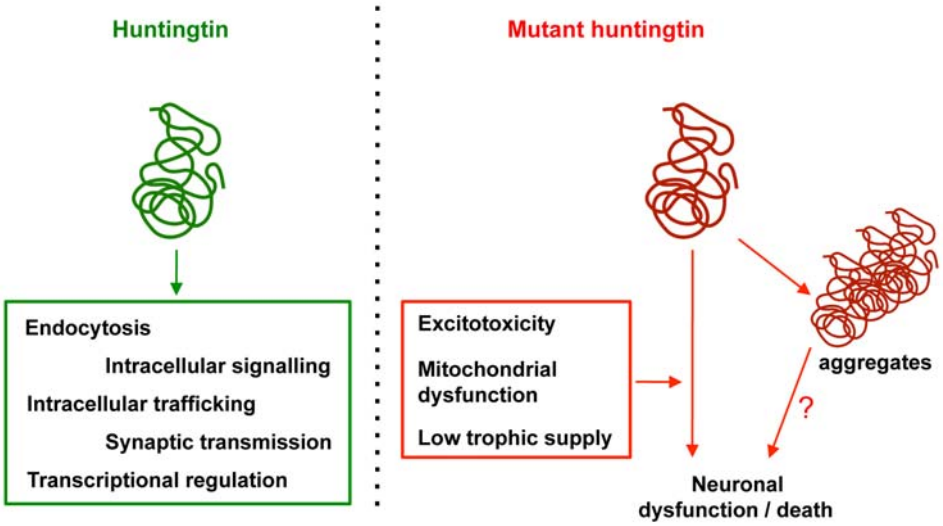

Figure 4.- Huntingtin and mutant huntingtin functions. The diagram shows huntingtin functions and possible toxic mechanisms triggering cell dysfunction/death together with mutant huntingtin. Figure adapted from Perez-Navarro *et al.,* 2006.

Moreover, huntingtin is also localized in the nucleus, where it interacts with proteins involved in gene transcription (Harjes & Wanker, 2003; Landles & Bates, 2004; Li & Li, 2004). Thus, it has been suggested that a loss of huntingtin function triggered by the mutation may contribute to the neuropathology of Huntington's disease (Cattaneo *et al.,* 2005) (Fig. 4). Together with this loss of function, several mechanisms, intrinsic of each neuronal population such as excitotoxicity, mitochondrial dysfunction and low

trophic supply, can also modulate neuronal degeneration in the presence of mutant huntingtin (Perez-Navarro *et al.,* 2006) (Fig. 4). Finally, mutant huntingtin is very unstable and unfolds improperly giving rise to protein aggregates. It is under debate whether these aggregates could enhance mutant huntingtin-induced toxicity or be protective for cells (Arrasate *et al.,* 2004; Scherzinger *et al.,* 1997) (Fig. 4).

1.4.1 - Aggregation in Huntington's disease

Huntington's disease is characterized by the presence of protein aggregates that accumulate within cells, resembling what has been observed in various forms of spinocerebellar ataxia, as well as in other neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Bano *et al.,* 2011).

DiFiglia and collaborators performed in 1997 an extended immunohistochemical analysis of human brain post-mortem tissue, using an antiserum raised against N-terminal huntingtin epitope. The most important finding was the presence of these mutant huntingtin clusters within nucleus of neurons that degenerate in Huntington's disease. They found large nuclear inclusion bodies that were positioned variably throughout the nucleus, adjacent to or distant from the nucleolus, in neurons from all the cortical layers and in medium-sized spiny neurons, but they were not present in cerebellar neurons. Nuclear inclusion bodies were also absent in the brains of control individuals. Interestingly, they found a correlation between the onset of the disease and the frequency of the nuclear inclusions, being those more frequent in juvenile than in adult Huntington's disease patients. The fact that with the antiserum against an epitope at the internal site of huntingtin they failed to detect nuclear inclusions, suggested that these clusters contained a cleaved fragment of N-terminal mutant huntingtin (DiFiglia *et al.,* 1997). Similar findings were obtained in transgenic mice, with some differences though. In the transgenic mice nuclear inclusions were found also in neurons from the cerebellum, and dense accumulation of mutant huntingtin in the cytoplasm of neurons was also observed (Davies *et al.,* 1997). Immunohistochemisrty of human brain tissue using another N-terminal antibody revealed also cytoplasmic aggregates, which were named neuropil aggregates (Gutekunst *et al.,* 1999). These aggregates, seem to be present long before symptoms or neuropathology is detectable, suggesting that inclusion formation precedes the onset of symptoms in both transgenic mice and in human brain (Davies *et al.,* 1997; Gutekunst *et al.,* 1999). Finally, in the conditional animal model of Huntington's disease, not only symptomatic amelioration of the

behavioural phenotype was observed, but also disappearance of inclusions when abolishing the transgene expression (Yamamoto *et al.,* 2000)

These findings, in addition to results showing that the same polyQ length threshold causes both aggregates and pathogenicity, have implicated aggregates in triggering neurodegeneration (Scherzinger *et al.,* 1997). However, the function of aggregate formation along the progression of the disease has not yet been elucidated, and the toxic effect still remains controversial. Nevertheless, it has been suggested that aggregate formation promoters might protect neurons by decreasing the levels of toxic diffuse forms of mutant huntingtin (Arrasate *et al.,* 2004; Bodner *et al.,* 2006). Although the mechanism by which mutant huntingtin could trigger toxicity is unknown, it is well known that mutant huntingtin is not only prone to aggregate with itself but also has the tendency to aggregate with other selected proteins (Busch *et al.,* 2003; Steffan *et al.,* 2000). This suggests, that besides the toxic function that mutant huntingtin has *per se*, it can induce additional cell toxicity by sequestering and impairing the function of other proteins.

Aggregates are formed not only by one single specific mutant huntingtin fragment, but also by a broad range of different N-terminal mutant huntingtin fragments (Hoffner *et al.,* 2005). Mutant huntingtin is target of a number of specific proteases such as caspases, calpains and matrix metalloproteinases, which generate these N-terminal fragments. It was suggested that huntingtin cleavage by caspases is a normal physiological event, since caspase-cleaved huntingtin was detected both in wild-type and Huntington's disease mouse models as well as in control human and Huntington's disease brains (Wellington *et al.,* 2002), although this caspase-dependent cleavage is more efficient as polyQ length increases (Goldberg *et al.,* 1996). In contrast to the controversy raised around the toxicity of mutant huntingtin aggregates, unanimity does exist when considering the toxicity of soluble or oligomerized N-terminal mutant huntingtin fragments (Graham *et al.,* 2006; Wellington *et al.,* 2002). In addition, small N-terminal fragments have been observed to redistribute to the nucleus and be required for neurodegeneration (Hodgson *et al.,* 1999). It has been recently suggested that the polyQ sequence possesses NES activity itself (Chan *et al.,* 2011), and thus mutant huntingtin but not the wild-type, would interact with exportin-1 (Xpo-1) to translocate out from the nucleus (Chan *et al.,* 2011; Hilditch-Maguire *et al.,* 2000). A reduction in the levels of Xpo-1 with age, which occurs in a Huntington's disease mouse model, could account for the permanence of mutant huntingtin within the nucleus and contribute to cellular toxicity and aggregation (Chan *et al.,* 2011).

It has been hypothesized that the fact that unfolded proteins do not accumulate in aggregates in unstressed cells, despite their continued production, is due in part to the existence of a cellular 'quality control' machinery, which ensures the fidelity of transcription and translation, by chaperoning nascent or unfolded proteins, and by selectively degrading these improperly folded proteins (Kopito, 2000; Ross & Poirier, 2004). Thus, a proper efficiency of degradation mechanisms within the cells is highly important to avoid neurodegeneration in Huntington's disease.

2 - MECHANISMS THAT COULD TRIGGER CELL SURVIVAL IN HUNTINGTON'S DISEASE

2.1 - MECHANISMS TO DEGRADE ABERRANT PROTEINS OR ORGANELLES

Every eukaryotic cell has two main systems to degrade intracellular components, the ubiquitin-proteasome system (UPS) and autophagy. While the proteasome is responsible for rapid protein turnover, autophagy degrades long-lived proteins and other intracellular components such as organelles, lipid deposits and even pathogens. Upon failure of both systems in degrading misfolded proteins, protein aggregates start to accumulate.

2.1.1 - UPS

The UPS plays an essential role in the degradation and clearance of soluble short-lived, misfolded and mutant proteins in eukaryotic cells, in both cytoplasm and nucleus (Rockel *et al.,* 2005). Therefore, it is a main regulator of several crucial processes, such as cell division, transcription, antigen processing and signal transduction (Ding & Keller, 2001; Goldberg *et al.,* 1995; Low, 2011).

The UPS consists of both substrate recruiting and substrate-degradation machinery. On one hand, the substrate-degradation machinery, called the 26S proteasome, is a large elongated cylinder with multiple peptidase activities, composed by a 20S core particle capped by a 19S regulatory particle at one or both ends. In the inner part of the 20S complex, there are three types of catalytic subunits that execute the corresponding catalytic activities of the proteasome: trypsin-like, chymotrypsin-like,

and peptidylglutamyl-peptide hydrolyzing activity (Li & Li, 2011). The 19S functions in recognizing polyubiquitinated proteins, leading the substrate to enter in the proteolytic chamber and unfolding the substrate to be able to fit inside the narrow chamber (Ciechanover, 2005). On the other hand, an ubiquitin chain tag is required for the substrate to be recognized by the 19S. Ubiquitin is a small and heat-stable protein, which is highly conserved among species and plays a role, among others, in recruiting substrate for degradation (Ciechanover, 2005). Ubiquitins are progressively added to the substrate through iso-peptide bonds that link the terminal residue of one ubiquitin and the internal lysine 48 of the previously conjugated one (Thrower *et al.,* 2000). This reaction requires the sequential actions of ubiquitin-activating (E1), -conjugating (E2) and -ligating (E3) enzymes (Tan *et al.,* 2008). Once the 19S complexes have recognized polyubiquitinated substrates, deubiquitnating enzymes disassemble, then, polyubiquitin chains into ubiquitin monomers that can be reused (Kawakami *et al.,* 1999).

Important mediators of the UPS, that help ubiquitinated proteins entering the proteasome, are chaperones (Slavotinek & Biesecker, 2001). These proteins not only prevent improper folding and aggregation but also facilitate formation of a correct conformation of misfolded proteins, often through cycles of ATP-regulated binding and release. Molecular chaperones typically recognize and bind to the exposed hydrophobic residues of misfolded proteins, by non-covalent interaction (Hartl, 1996; Slavotinek & Biesecker, 2001).

Although the UPS is the primary machinery of polyubiquitinated protein degradation, it has been reported that it fails in the digestion of stable protein complexes or aggregates (Venkatraman *et al.,* 2004). In the same study they showed that the UPS is not capable of degrading long polyQ stretches and cuts only at the flanking residues of the stretch. This leads to the generation of fragments that are more hydrophobic and aggregate more readily.

Thus, lysosomal degradation has emerged as a crucial mechanism for maintaining cellular homeostasis in diseases characterized by protein aggregation.

2.1.2 - Autophagy

Autophagy, which from the Greek means, ‘self eating’, is a process within the cell relevant in mediating the degradation of cell components inside the lysosomes. It is a

catabolic process to degrade and recycle cell components, therefore it is important in maintaining both protein and organelle turnover. The macromolecules that result from the degradation can be reused again.

From a reductionist point of view, autophagy is an adaptation to starvation. Autophagy is activated as soon as a cell lacks nutrient supply, since the cell is forced to catabolise its own reserves to keep alive until the situation improves (Mizushima *et al.,* 2008). This adaptation process has been observed from single-cell organisms to more complex organisms such as mammals. Autophagy-deficient yeasts rapidly die under poor nutrition conditions (Tsukada & Ohsumi, 1993). Moreover, during embryogenesis autophagic level is low, but after birth autophagy is immediately up-regulated until the levels restore to basal, 1 or 2 days later. This phenomenon has been proposed to be an adaptation to the nutrient interruption that the neonate faces just after birth until nutrient supply is restored through milk. In agreement, homozygous knockout neonate mice deficient for Atg5 or Atg7, two essential proteins in autophagy, die within 1 day, although minimal abnormalities are detected at birth (Komatsu *et al.,* 2005; Kuma *et al.,* 2004).

However, besides induced autophagy, constitutive autophagy at a low level is needed, since down-regulation of the process has been proposed to contribute to pathological conditions such as neurodegeneration, hepatic dysfunction and cardiovascular disorders (Hara *et al.,* 2006; Komatsu *et al.,* 2005, 2006; Takemura *et al.,* 2009).

Although autophagy initially seems to be protective, in the past few years it has been also linked to a type of programmed cell death. There is a complex interplay between autophagy and apoptosis, since both processes can work in synergy but also one can counteract the other (Eisenberg-Lerner *et al.,* 2009). Some proteins from the autophagy machinery have been linked to activate apoptosis, for example Atg5 can undergo calpain-mediated cleavage to generate a pro-apoptotic fragment that functions in the intrinsic mitochondrial death pathway, or p62/SQSTM1 (p62) induces caspase-8 aggregation leading to full activation of the protease (Jin *et al.,* 2009; Yousefi *et al.,* 2006).

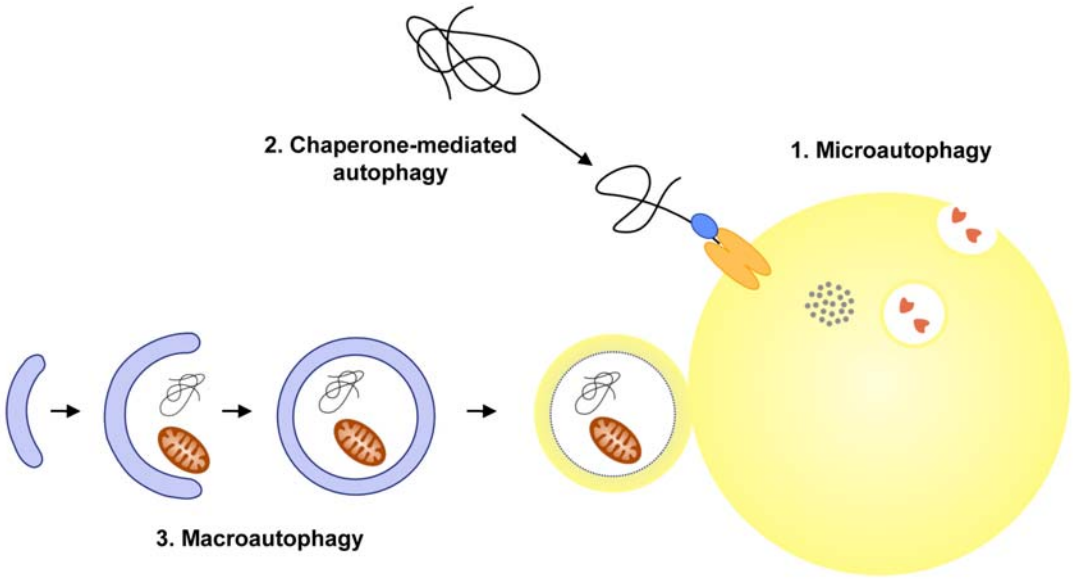

Figure 5.- Types of autophagy. The diagram shows the three ways by which the cell delivers the autophagic cargo into the lysosomes: microautophagy, chaperone-mediated autophagy and macroautophagy. Figure adapted from Martinez-Vicente & Cuervo, 2007.

Lysosomes are central in the autophagic process since they contain all the acid hydrolases needed for degradation. The way in which the autophagic cargo is delivered within the lysosomes distinguishes types of autophagy (Cuervo, 2010). There are three ways of delivering the cargo into the lysosomes: microautophagy, chaperone-mediated autophagy and macroautophagy (Fig. 5).

2.1.2.1 - Microautophagy

In the case of microautophagy the cargo is sequestered by the lysosome itself. Cytosolic components are internalized through invaginations at the lysosome membrane and, once in the lumen, the whole internalized vesicle is degraded (Shpilka & Elazar, 2011). Organelles such as peroxisomes or mitochondria can be engulfed and self-digested through microautophagy processes (Farre *et al.,* 2009). By electron microscope abnormal lysosomal structure morphology can be detected, such as elongated forms with arm-like membrane extensions or large lysosomes with multiple submembrane compartments. These kinds of structures are interpreted as microautophagic events. However, since there is a lack of reliable markers to track and

specific techniques to analyze microautophagy, aside from electron microscope, the knowledge of this process in mammalian cells is limited (Mijaljica *et al.,* 2011).

Multivesicular bodies, which are defined as organelles with vesicular structures trapped in their lumen, degrade mainly transmembranal proteins by internalization and sorting to the lysosomes. To get degraded, these multivesicular bodies fuse to an autophagosome from the macroautophagy pathway forming a hybrid organelle called amphisome. In a final step this intermediate structure will get degraded when fusing to a lysosome (Fader & Colombo, 2009). It has been suggested that alterations in the multivesicular bodies biogenesis block the degradation of some proteins through autophagy (Rusten & Simonsen, 2008). Since the formation of multivesicular bodies in late endosomes resembles that of microautophagy, some groups have hypothesized that microautophagy can occur also in late endosomes (Cuervo, 2010; Fader & Colombo, 2009; Sahu *et al.,* 2011).

2.1.2.2 - Chaperone-mediated autophagy

Chaperone-mediated autophagy differs from the other two autophagic pathways in many ways. It does not involve vesicle formation, it is saturable, it is a process for degradation of cytosolic soluble proteins, and it is highly selective. In this case, cargo, which is recognized by cytosolic chaperones, directly translocates into the lysosome across the lysosomal membrane with the help of a set of lysosomal proteins (Kon & Cuervo, 2010).

Proteins destined for lysosomal degradation have the pentapeptide motif KFERQ in its sequence. This tag is only recognized by the cytosolic chaperone Heat shock 70 kDa protein (HSC70), which delivers the cargo to the lysosomes. Mutations in the tag sequence impair both recognition and degradation of proteins. More than one KFERQ motif in the sequence of a protein does not imply more degradation, since translocation of proteins into the lysosomal lumen is a saturable process. Delivery of the cargo into the lysosomes does not involve vesicle formation nor membrane budding, but the presence of the single-span lysosome-associated membrane protein (LAMP2A) on the lysosomal surface, which acts as a receptor for chaperone-mediated autophagy through the C-terminal (Cuervo, 2010). The substrate binding to LAMP2A monomers drives its multimerization to a 700 KDa complex that has been elucidated to be essential for protein translocation. HSC70, together with a subset of co-chaperones, help unfolding the substrate at the lysosomal surface. Finally, to complete the translocation of substrate proteins into lysosomes, a form of HSC70 resident in the

lysosomal lumen is needed. Although the mechanism by which lysosomal-HSC70 mediates substrate translocation is still unclear, blocking the chaperone strongly impairs chaperone-mediated autophagy (Bejarano & Cuervo, 2010).

2.1.2.3 - Macroautophagy

In the case of macroautophagy, the cargo delivery to the lysosomes is mediated by another 400-900 nm vacuolar structure called autophagosome. When autophagosomes are forming, they engulf a portion of cytoplasm and retain it in its lumen. Since autophagosomes do not have the enzyme machinery to degrade its content they fuse to lysosomes in a later step. From now on, this process will be simply referred as autophagy.

The sequestration of the cargo is triggered by a double-bilayered membrane, called phagophore, which expands until enclosure around a portion of cytoplasm. The resulting double-layered organelle is the aforementioned autophagosome. Autophagosome formation, thus, requires three steps: phagophore nucleation, phagophore expansion and autophagosome maturation (Geng & Klionsky, 2008). The molecular basis of this process was not understood until 1993, when Ohsumi and co-workers isolated in yeast the first autophagy-related genes (Atg), which elucidated the core autophagic machinery (Tsukada & Ohsumi, 1993). To date, 34 different Atgs and their orthologs in mammals have been identified, implying a conserved autophagic mechanism from yeast to higher eukaryotes (Weidberg *et al.,* 2011) (Fig. 6). However, the process is highly complex and many aspects still need to be understood. Anyhow, the Atg involved in this core autophagic machinery can be subclassified into four main functional groups depending on which stage of autophagsome formation they act: (1) the Atg1–Atg13–Atg17 kinase complex and (2) the class III phosphatidylinositol 3-kinase (PI3K) complex I, consisting of Vps34, Vps15, Atg6/Vps30 and Atg14, are required for the phagophore nucleation; (3) two ubiquitin-like protein conjugation systems (Atg12 and Atg8) and (4) Atg9 and its cycling system, are involved in the expansion steps of the phagophore (Yang & Klionsky, 2010).

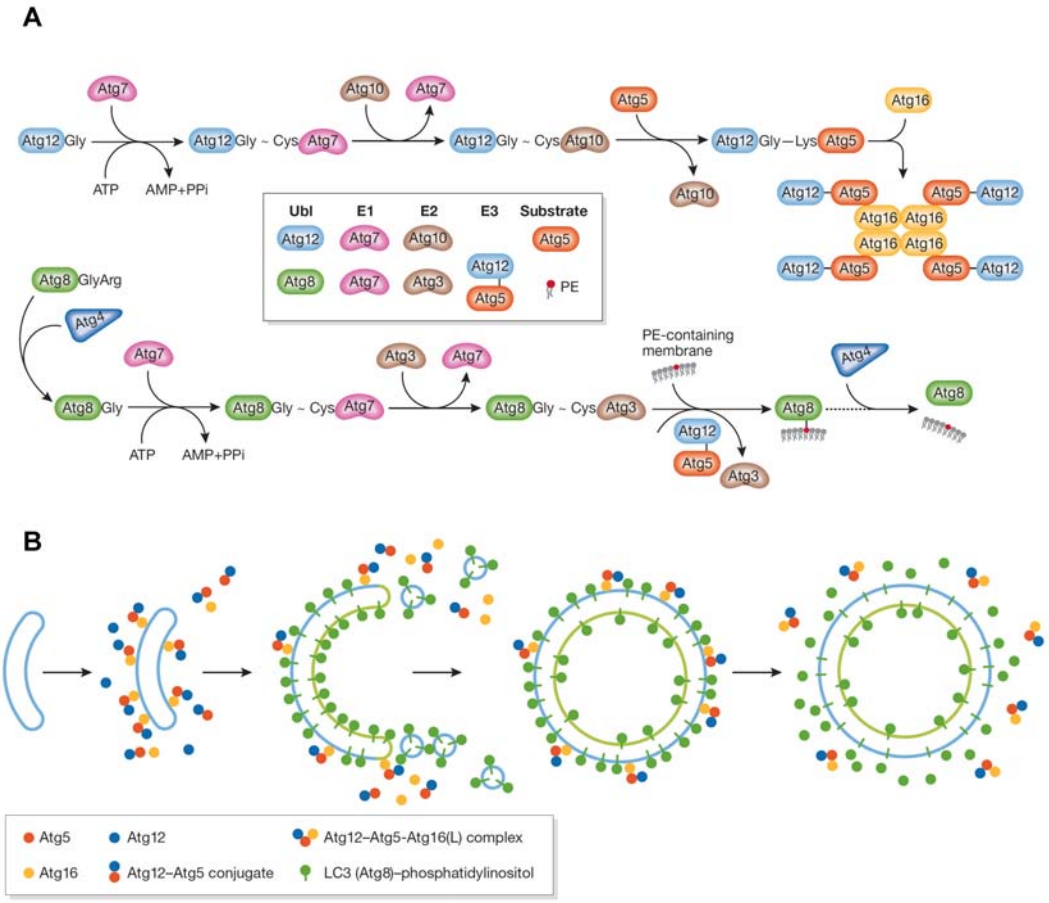

Figure 6.- Atg12 and Atg8 in autophagosome formation. (A) The diagram shows the two ubiquitin-like (Ubl) protein conjugation systems, Atg12 and Atg8, that mediate Atg8 lipidation and recruitment to the autophagosome membrane. (B) The Atg12-Atg5-Atg16L complex is recruited to the phagophore to ensure Atg8 lipidation and recruitment, which supports the phagophore expansion. On completion of the autophagosome, the Atg12–Atg5-Atg16L complex dissociates from the vesicle, and Atg4 proteolytically releases the Atg8 that is present on the external surface. Note that the initiation of the phagophore is initially Atg12-Atg5-Atg16L independent. Figure from Geng & Klionsky, 2008.

The nucleation of the phagophore mainly requires the participation of many Atg proteins, which are recruited to the phagophore-assembly site. Many of these sites can be detected throughout the cytoplasm in mammalian cells (Weidberg *et al.,* 2011). An essential complex for autophagy is the class III PI3K complex 1, composed by the kinase Vps34, Beclin-1 (in yeast Atg6/Vps30), Vps15 and Atg14. This complex produces phosphatidylinositol 3-phosphate (PI3P), which is present at the inner part of the autophagosomes and recruits PI3P-binding proteins important for autophagosome formation. Another important set of Atg important in the autophagosome formation is UNC-51–like kinase (ULK) 1/2 (Atg1 in yeasts), which participates in a cytosolic complex, together with Atg13, Atg101 and Focal adhesion kinase family interacting protein of 200 kDa (FIP200), and is recruited to the phagophore upon autophagy induction in a very early stage (Noda *et al.,* 2010; Weidberg *et al.,* 2011). There is still no data that would link ULK1/2 with the class III PI3K complex 1.

The main molecular regulatory mechanism for the phagohore expansion consists of two ubiquitin-like conjugation systems, Atg12 and Atg8 conjugation systems. They induce LC3 (in yeast Atg8) lipidation and membrane association (Suzuki & Ohsumi, 2007), a crucial event that causes phagophore elongation *in vitro* (Nakatogawa *et al.,* 2007). Like the canonical ubiquitin system, Atg12 is an ubiquitin-like Atg protein that gets activated by Atg7, which is an E1-like enzyme. After activation, Atg12 is transferred to Atg10, an E2-like enzyme, and finally conjugated to the target protein Atg5 through an isopeptide bond, without the participation of an E3 enzyme. Atg5 interacts with Atg16L, which homo-oligomerizes generating an Atg12-Atg5-Atg16L complex. In this second ubiquitin-like conjugation system, LC3 is attached to a phosphatidylethanolamine (PE). LC3 is first proteolytically processed to expose a glycine. This LC3 form is named LC3I. LC3I is activated by Atg7 and transferred to the E2-like enzyme Atg3. The Atg12-Atg5 complex, which binds both Atg3 and PE, is the E3-like complex from the second conjugation system that can transfer LC3 to the PE. This lipidated membrane-bound form of LC3 is called LC3II. LC3 lipidation is reversible and LC3 can be recycled, since Atg4b can release LC3 from the PE (Geng & Klionsky, 2008; Hanada *et al.,* 2007; Ichimura *et al.,* 2004). Since LC3II is essential for the phagophore elongation and it is the only one known protein specifically present at the autophagosome and autolysosome membranes, but at no other vesicles, it is considered to be a good autophagic marker, and its levels correlate with the number and size of autophagosomes and autolysosomes (Rubinsztein *et al.,* 2009). In addition to the ubiquitin-like conjugation systems, Atg9 is an integral membrane protein that cycles between autophagosomes to Golgi and endosomes compartments and vice versa. Although the mechanism and regulation of this cycle has been further analyzed, the function in autophagy is still unknown. However, it has been hypothesized that Atg9 can regulate the autophagosome size, that it can be a carrier of lipids to forming autophagosomes and can regulate LC3 lipidation (Webber & Tooze, 2010).

Once formed, autophagosomes start moving linearly toward the perinuclear region where lysosomes are localized. These dynamics rely on the microtubule network and the dynein/dynactin motor complex (Kimura *et al.,* 2008; Maday *et al.,* 2012). Fusion to lysosomes is what is referred as autophagosome maturation, and the resulting vesicle is called autolysosome. The autophagic body, the structure surrounded by the inner lipid bilayer, is released into the lumen of the vacuole and is consequently degraded by vacuolar hydrolases (Suzuki & Ohsumi, 2007). The resulting products will be released back to the cytosol through permeases (Chen & Klionsky, 2011). Lumenal lysosomal

environment is at pH 4.6-5, which is maintained by proton-pumping vacuolar ATPases (Luzio *et al.,* 2007). Continuous lysosomal turnover of cellular constituents delivered by autophagy and endocytosis is crucial for cellular homeostasis. Lysosomal dysfunction in lysosomal storage disorders, caused by a disruption of the internal environment, can lead to neurodegeneration and present accumulations of autophagosomes that cannot be degraded (Nixon *et al.,* 2008).

2.1.2.3.1 - Selective autophagy

Since its discovery, autophagy has been always defined as a bulk degradation system. However, there are increasing number of evidences that autophagy is rather selective, or at least some autophagy processes require selectivity. Whereas starvation-induced autophagy is a non-selective process, basal autophagy has been hypothesized to be more selective, since it is an important quality control that removes damaged organelles, such as peroxisomes (pexophagy) and mitochondria (mitophagy) and other substrates targeted for lysosomal degradation. These last processes require both cargo targeting and recognition, carried out by specific autophagy receptors that would connect the cargo to the core autophagic mechanism (Knaevelsrud & Simonsen, 2010). In mammalian cells, the selectivity comes from proteins that contain the WXXL motif in their sequence, also named LC3 interaction region (LIR) (Noda *et al.,* 2010). Thus, these proteins can work as autophagy cargo receptors, since they can recognize specifically the cargo that needs to be degraded and LC3 through the LIR motif. The cargo needs to be correctly ubiquitinated to be recognized by the receptors (Knaevelsrud & Simonsen, 2010; Weidberg *et al.,* 2011).

p62, Neighbour of BRCA1 gene 1 (NBR1), Nix and Nuclear dot protein 52 (NDP52) are recently discovered autophagic cargo receptors that have an ubiquitin-associated (UBA) domain and a LIR motif in their sequences (Yamamoto & Simonsen, 2011). To achieve selectivity, a molecular tag or marker is required for recognition of the structures that must be degraded by autophagy. Alternatively to the UPS system, ubiquitin chains linked through lysine 63 may represent a strategy for defining autophagy cargo, since lysine 63-linked ubiquitin chains have been implicated in inclusion biogenesis in many neurodegenerative diseases and these inclusions are preferentially cleared by autophagy (Lim *et al.,* 2005; Tan *et al.,* 2007, 2008).

Both p62 and NBR1 function as autophagic cargo receptors and they are themselves substrates of autophagy, since they are degraded together with the cargo. Autophagy regulates its levels, which do not seem to be altered by proteasomal degradation (Bjorkoy *et al.,* 2005; Kirkin *et al.,* 2009; Pankiv *et al.,* 2007). Both p62 and NBR1 have been shown to interact with protein aggregates and to regulate their clearance (Kirkin *et al.,* 2009). Additionally, p62 also interacts with peroxisomes, mitochondria and even intracellular bacteria (Geisler *et al.,* 2010; Kim *et al.,* 2008; Zheng *et al.,* 2009). Both proteins can even interact together to co-operate (Kirkin *et al.,* 2009).

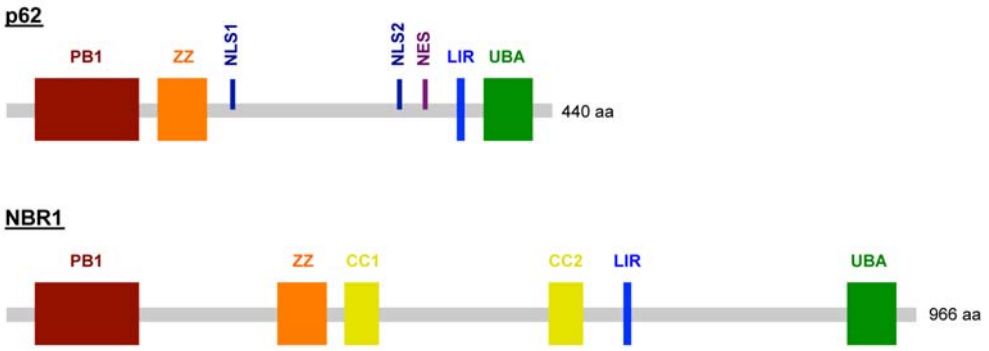

Figure 7.- Schematic diagram of p62 and NBR1 proteins. p62 and NBR1 with their domains and functional motifs are represented on the scheme. Both proteins contain and UBA domain and a LIR motif that are both essential for selective autophagy. Their sequences also contain a PB1 domain that is important for their oligomerization. Figure adapted from Lamark *et al.,* 2009.

p62, a 440 amino acid protein, was the first cargo receptor discovered in mammalian cells for selective autophagy of ubiquitinated proteins. NBR1, on the other hand, is twice larger than p62 with 966 amino acids in its sequence. Although is larger, NBR1 follows a similar domain architecture as p62 and therefore both cargo receptors share key functional features. Both proteins have an N-terminal Phox and Bem1 (PB1) domain important for oligomerization, a LIR motif to interact with LC3, and a C-terminal UBA domain to associate with ubiquitin (Lamark *et al.,* 2009). Moreover, LIR motif has been proved to be important for the localization of p62 into autophagosomes and for its lysosomal degradation (Shvets *et al.,* 2008) (Fig. 7). However, findings from Mizushima's group, who have examined how p62 and NBR1 are loaded into the autophagic vesicles, show that LC3 interaction might be important for the enclosure of p62 into the autophagosomes and for its degradation, but not for the presence of p62 and NBR1 at the autophagosome formation site (Itakura & Mizushima, 2011).

Besides the mechanism by which these receptors interact with LC3 and the autophagsomes, it is also important to take into consideration the way they bind the tagged-cargo. In the brain, the absence of p62 results in the abnormal hyper-accumulation of aggregated insoluble lysine 63-polyubiquitinated proteins, such as Tropomyosin receptor kinase A (TrkA) (Wooten *et al.,* 2008). This suggests that p62 and lysine 63-linked polyubiquitin may be together involved in directing clearance of protein inclusions by autophagy (Tan *et al.,* 2007). On the other hand, NBR1 UBA domain has been reported to bind both lysine 48 and lysine 63-linked polyubiquitin chains (Kirkin *et al.,* 2009).

p62 and NBR1, however, are multifunctional scaffold proteins involved in many other cellular processes such as cell signalling and receptor internalization. This functional diversity is due to the several protein-interacting domains of their sequence (Moscat *et al.,* 2007). p62 signalling has been studied in a broad branch of processes, such as osteoclastogenesis, inflammation, differentiation, neurotrophin biology and obesity, since it can associate to tumour necrosis factor α signalling adaptor, receptor interacting protein, tumour necrosis factor α receptor-associated factor 6 and others. As an example, some mutant alleles of the p62 gene with deleted or non-functional UBA domain have been linked to the adult onset Paget's disease of the bone, causing defects in signalling and aberrant osteoclastogenesis (Moscat *et al.,* 2007). NBR1 has been linked to hereditary muscle diseases, since it interacts with the sarcomeric protein kinase Titin and with p62 in the M line of the sarcomer. Mutations in Titin cause dissociation of the complex and dissolution of the M line (Lange *et al.,* 2005). Moreover, NBR1 loss of function leads to bone remodelling and an increase in bone volume and density, through a hyper-activation of p38 Mitogen-activated protein kinase (MAPK) that favours osteoblastogenesis (Whitehouse *et al.,* 2010). Due to such amount of interacting partners, p62 knockout mice present a complex phenotype. It has been reported that these mice develop mature-onset obesity, leptin resistance, as well as impaired glucose and insulin intolerance (Rodriguez *et al.,* 2006). Moreover, deficiency of p62 leads to a mature-onset (6 months old mice) accumulation of hyper-phosphorylated Tau, neurofibrillary tangles, and neurodegeneration, followed by increased anxiety, depression, loss of working memory, and reduced serum Brain-derived neurotrophic factor (BDNF) levels (Wooten *et al.,* 2008). Younger p62 knockout mice (4-8 weeks of age) lack liver and neuronal abnormalities (Komatsu *et al.,* 2007).

In addition to p62 and NBR1, there are other autophagic receptors such as Nix and NDP52. Nix mediates the damaged mitochondrial clearance. Binding studies have

shown that Nix, as well as p62 and NBR1, also interacts with LC3. Nix function on mitochondrial turnover is LC3 dependent, since an ablation of this interaction retards the mitochondrial clearance (Novak *et al.,* 2010). NDP52 has been studied in the field of removing cytosolic bacteria from the cytoplasm of cells, and share with the others the ability to bind LC3 and ubiquitinated cargo (Mostowy *et al.,* 2011; Thurston *et al.,* 2009).

2.1.2.3.2 - p62 and NBR1 in aggregate formation and clearance

p62 has been found in inclusion bodies in many neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, and also in the R6/2 mouse hippocampus and cortex, and in a cellular model of Huntington's disease (Bjorkoy *et al.,* 2005; Nagaoka *et al.,* 2004; Nakaso *et al.,* 2004; Zatloukal *et al.,* 2002). NBR1 has been detected in Mallory bodies of the liver and in Lewy Bodies (Kirkin *et al.,* 2009; Odagiri *et al.,* 2012). Moreover, both proteins are required for the formation of ubiquitinated protein aggregates, also called p62 bodies, sequestosomes or aggresome-like inducible structures mediated by they self-oligomerization through the PB1 domain and ubiquitin binding (Bjorkoy *et al.,* 2005; Ichimura *et al.,* 2008; Kirkin *et al.,* 2009; Knaevelsrud & Simonsen 2010; Lamark *et al.,* 2009).

As commented before, lack of p62 does not induce protein aggregation, at least in young mice. Interestingly, when autophagy is impaired, a marked p62-positive inclusion accumulation can be observed, that can be reverted by p62 gene depletion (Komatsu *et al.,* 2007). In addition to this, also fewer p62 bodies were formed in cells after knocking down NBR1 when compared to control cells (Kirkin *et al.,* 2009).

These results have lead to the proposal of the following model for the role of NBR1 and p62 in aggregate formation and protein clearance: (1) Under conditions of increased misfolded protein production, soluble and toxic oligomeric proteins accumulate and poly-ubiquitin chains are conjugated to these oligomers; (2) ubiquitinated cargo is recognized by NBR1 and p62 and delivered to the forming autophagosomes via interaction with membrane-bound Atg8 family proteins; (3) if degradation of these soluble complexes is incomplete due to autophagy disruption, NBR1 and p62 promote formation of p62 bodies; (4) both NBR1 and p62 participate in autophagic degradation of p62 bodies (Kirkin *et al.,* 2009).

2.1.2.3.3 - Autophagy regulation

Autophagy is tightly regulated in order to generate a rapid response when needed. The identification of the mammalian target of rapamycin kinase (mTOR) together with the fact that rapamycin (an mTOR inhibitor) could up-regulate autophagy processes, was a key breakthrough to start understanding the complex signalling that lead to autophagy regulation (Blommaart *et al.,* 1995; Heitman *et al.,* 1991). Another important finding was that autophagy was distinctly regulated by class I and class III PI3K. While class III PI3K product, PI3P, promotes autophagy in an mTOR-independent manner, the class I PI3K products, phosphatidylinositol (3,4)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3), have inhibitory effects (Blommaart *et al.,* 1997; Petiot *et al.,* 2000).

Thus, autophagy regulation could be classified in mTOR-dependent and independent signalling pathways (Fig. 8).

A) mTOR-dependent pathway

mTOR was first described after many genetic and biochemical approaches to identify the target of the immunosuppressant rapamycin, a compound produced by a soil bacterium found on Easter Island (Zoncu *et al.,* 2011). mTOR, which is a 289 kDa serine/threonine kinase, is a member of the PI3K-related protein kinases family and is an energetic imbalance sensor that relies at the interface between cellular growth and starvation, regulating, upon activation, the biosynthetic program in contraposition to catabolic pathways.

mTOR, however, does not work alone. It is the catalytic core of two complexes, the mTOR complex (mTORC) 1 and 2, first described as sensitive and insensitive complexes to rapamycin. The complex that has been shown to negatively regulate autophagy, but also to regulate protein synthesis and translation, is mTORC1, by directly phosphorylating the ULK1/2-Atg13-FIP200 complex, 4E-binding protein 1 (4EBP1) and p70 ribosomal S6 protein kinase-1 (S6K1) (Gibbons *et al.,* 2009). mTORC1 generates a convergent pathway integrating information from nutrient, metabolic and hormonal signals to regulate autophagy.

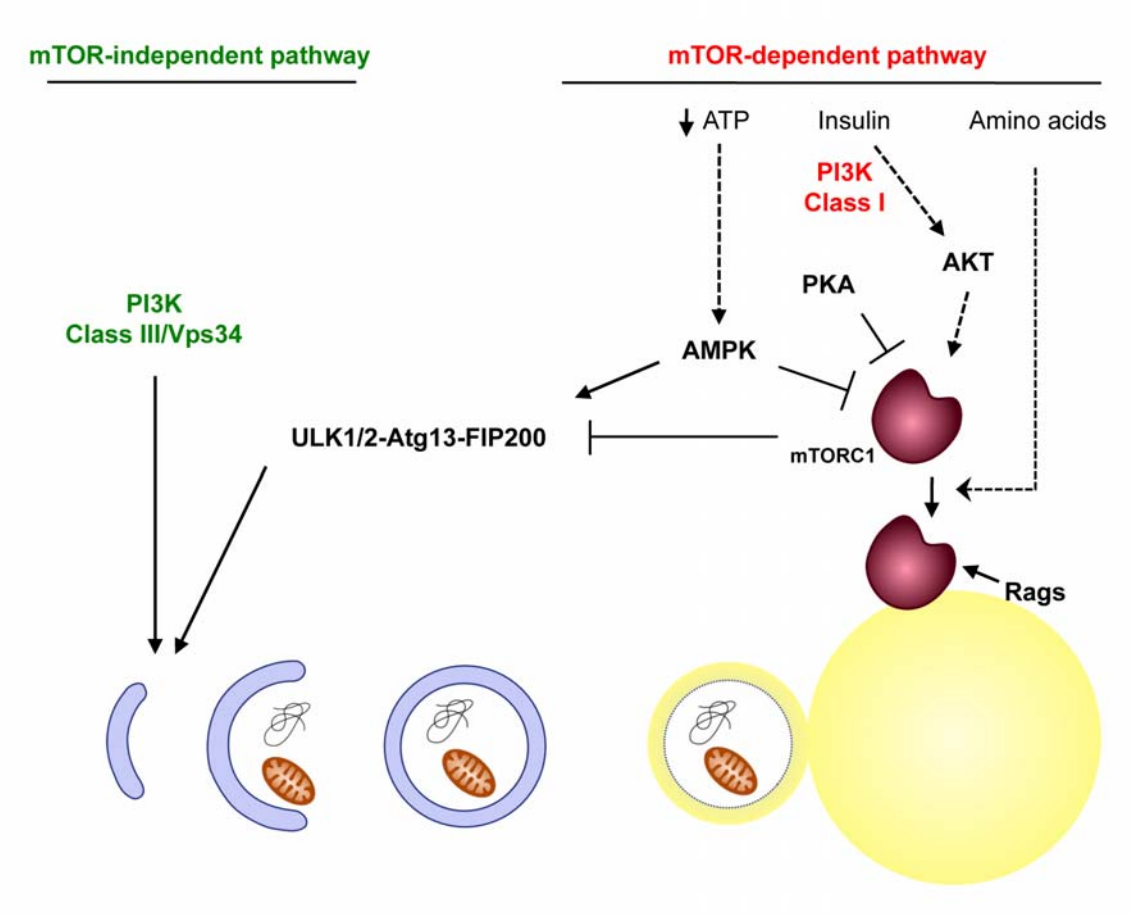

Figure 8.- Autophagy regulation. The diagram represents the two pathways that regulate autophagosome synthesis, the mTOR-independent and the mTOR-dependent pathways. Metabolic impairment, insulin and amino acids converge to regulate the mTOR-dependent pathway. Class I and class III PI3K regulate autophagy in opposite ways. Figure adapted from Duran *et al.,* 2011 and Mizushima *et al.,* 2008.

Insulin and amino acids have been shown to regulate autophagy in an mTOR-dependent manner (Arico *et al.,* 2001; Blommaart *et al.,* 1995; Kanazawa *et al.,* 2004). Insulin signal transduction leads to the activation of the class I PI3K, which is followed by the production of PIP3 that enables AKT to be recruited to the membrane and get activated. Expression of an active form of AKT inhibits autophagy, which can be reverted by rapamycin-inhibition of mTOR (Yang & Klionsky, 2010). Therefore, mTOR is downstream of AKT in the insulin-signalling pathway. Amino acids, on the other hand, have been shown to enhance mTORC1 activity via the Ras-related GTPase (Rag) and thus, to block autophagy (Inoki *et al.,* 2012; Sancak *et al.,* 2010). p62 has also been reported to play a role in the mTORC1 complex activation since it is a binding partner of Raptor and Rag proteins and mediates their interaction as well as Rags activation (Duran *et al.,* 2011). Whereas p62 deletion has no effect on the insulin-activated mTORC1, p62 is selectively required for mTORC1 activation and to signal nutrient sensing.

The first autophagy target downstream of mTORC1 is the ULK1/2-Atg13-FIP200 complex, which is, as commented before, an important mediator of phagophore nucleation. ULK1/2-Atg13-FIP200 complex is regulated upon multiple phosphorylation events (Bach *et al.,* 2011). Under basal conditions mTORC1 is associated to this complex and phosphorylates both ULK1/2 and Atg13, resulting in autophagy inhibition. However, as soon as mTORC1 activity is inhibited, ULK1/2 and Atg13 are rapidly dephosphorylated, leading to the complex activation (Hosokawa *et al.,* 2009; Kamada *et al.,* 2000, 2010; Kim *et al.,* 2011a). ULK1/2 is a serine/threonine protein kinase that once it is dephosphorylated, results enzymatically active. This leads to other phosphorylation events on Atg13, FIP200, and ULK1/2 itself (Mizushima, 2010).

Elevated levels of protein kinase A (PKA) activity are shown to inhibit autophagy, and inactivation of the PKA pathway is sufficient to induce an autophagic response (Stephan *et al.,* 2009). In yeast, the regulation is triggered by directly phosphorylating Atg1 and Atg13 independently of TORC1 (Budovskaya *et al.,* 2005; Stephan *et al.,* 2009). To date, there have been no reported evidences that PKA can regulate ULK1/2 in the mammalian system. However, PKA can regulate mTOR by directly phosphorylation and thus, leading to autophagy inhibition (Blancquaert *et al.,* 2010; Mavrakis *et al.,* 2006).

Finally, 5' AMP-activated protein kinase (AMPK), a key energetic sensor, pathway can also activate autophagy by inhibiting either directly or indirectly mTORC1 (Gwinn *et al.,* 2008; Inoki *et al.,* 2003). Moreover, it has been recently described that under conditions of glucose starvation AMPK can also directly phosphorylate ULK1/2 to activate the complex and promote autophagy in an mTOR-independent manner (Kim *et al.,* 2011a).

Thus, the ULK1/2-Atg13-FIP200 complex is a central regulatory unit in autophagy, which can be modulated by a lot of factors that are mostly integrated at the mTOR pathway.

B) mTOR-independent pathway

The generation of PI3P has been found to trigger a diverse cellular signalling mediated by proteins that are able to recognize it (Burman & Ktistakis, 2010). PI3P is, therefore, able to create platforms that serve to recruit specific effectors for membrane trafficking events. It has been hypothesized that in autophagy, PI3P may form the

platform for autophagosome biogenesis, since it is enriched at the inner membrane of the autophagosomes (Burman & Ktistakis, 2010; Obara & Ohsumi, 2011). Therefore, besides the ULK1/2-Atg13-FIP200 complex, class III PI3K are the second regulatory unit in autophagy. Thus, class III PI3K inhibitors, such as 3-methyladenine, are able to block autophagosome biogenesis. However, these kinases are part of the class III PI3K complex I (composed by Vps34, Beclin-1, Vps15 and Atg14), and any disruption of the complex leads to decreased PI3K activity. B-cell lymphoma-2 (Bcl-2) association to Beclin-1 leads to autophagy down-regulation (Furuya *et al.,* 2005; Pattingre *et al.,* 2005). This Bcl-2-Beclin-1 partnership can be modulated by Bcl-2 proteolysis, phosphorylation of Bcl-2 by c-Jun N-terminal kinase-1 (JNK1) at sites that interfere with binding to Beclin-1, and by other factors that compete for Bcl-2 binding (Weidberg *et al.,* 2011). Furthermore, autophagy/beclin-1 regulator-1 (AMBRA1) and Endophilin B1 are factors that have been associated to the stabilization of Beclin-1 into the class III PI3K complex (Fimia *et al.,* 2007; Takahashi *et al.,* 2007).

Finally, other factors can act both at mTOR-dependent and independent pathways to regulate autophagy. IKKβ is known to regulate autophagy both by enhancing AMPK phosphorylation-dependent mTOR inhibition and JNK1-mediated Bcl-2 phosphorylation (Criollo *et al.,* 2010). Moreover, nitric oxide impairs autophagosome formation as it decreases JNK1 activity, and thus, Bcl-2 phosphorylation, and activates mTORC1 in an IKKβ dependent manner (Sarkar *et al.,* 2011).

2.1.3 - Protein degradation mechanisms in Huntington's disease

Huntingtin inclusions are positive for ubiquitin, indicating that these structures, or the monomers that compose them, are correctly tagged for their clearance (Davies *et al.,* 1997; DiFiglia *et al.,* 1997). However, mutant huntingtin progressively accumulates in affected neurons during the progression of Huntington's disease (Zhou *et al.,* 2003), suggesting that degradation pathways are not that efficient in clearing it. Moreover, the question if soluble or aggregated mutant huntingtin can directly alter the UPS or autophagy efficacy in the degradation of other substrates has also emerged.

Colocalization of proteasome subunits, as well as chaperones, with mutant huntingtin inclusions has been observed in a cellular model of Huntington's disease, suggesting an interaction of mutant huntingtin with the UPS degradation system (Warrick *et al.,* 1999; Wyttenbach *et al.,* 2000). Moreover, bacterial and yeast

chaperones, and even small molecule inducers of chaperones, decrease aggregate formation as well as cell death in Huntington's disease cell culture models (Carmichael *et al.,* 2000). Two hypotheses arise from these findings. Meanwhile the first one implies considering mutant huntingtin as substrate for the proteasome, the second one is about a putative UPS failure induced by mutant huntingtin. As previously commented, mutant huntingtin cannot be degraded by the proteasome, thus, proteasome may participate in its accumulation by inducing the formation of aggregates (Venkatraman *et al.,* 2004). Another issue is if mutant huntingtin can alter the proteasome function leading to accumulation of other proteins that are normally degraded through the UPS. Some groups supported the idea that mutant huntingtin could impair the UPS function in Huntington's disease models and also in post-mortem human tissue (Bence *et al.,* 2001; Bennett *et al.,* 2005; Mitra *et al.,* 2009; Seo *et al.,* 2004), whereas other groups did not find any alteration (Bett *et al.,* 2006; Diaz-Hernandez *et al.,* 2003). Finally, two groups cross-mated mice models of Huntington's disease with transgenic UPS green fluorescent protein (GFP) reporter mice (Bett *et al.,* 2009; Ortega *et al.,* 2010). With this approach, they were able to measure the UPS activity upon Huntington's disease pathology. Bett and collaborators showed no alterations in the degradation of the UPS reporter in the R6/2 mouse model, meanwhile Ortega and collaborators found an early impairment of the UPS immediately after an acute expression of the mutant huntingtin transgene in the conditional HD94 mouse model. However, UPS activity recovered in a time-dependent manner correlating with inclusion bodies formation (Ortega *et al.,* 2010). Such finding could explain the contradictory findings explained above. Confirming this, mutant huntingtin filamentous aggregates inhibit proteasome activity, but only when they are not recruited into inclusion bodies (Diaz-Hernandez *et al.,* 2006; Mitra *et al.,* 2009). This fact strengthens the previously mentioned hypothesis that inclusion body formation is a protective mechanism by neutralizing the toxicity of intermediate mutant huntingtin species.

Increases in components of the chaperone-mediated autophagy, LAMP2A and chaperone HSC70, have been observed in the knock-in HdhQ111 model. The authors suggest that chaperone-mediated autophagy activity is increased at early stages of Huntington's disease contributing to degradation of truncated, but not full-length, mutant huntingtin. However, the efficiency of this compensatory mechanism may decrease with age contributing to cellular dysfunction (Koga *et al.,* 2011). Moreover, *in vivo* attempting to selectively target mutant huntingtin for chaperone-mediated autophagy degradation, induced amelioration of motor symptoms (Bauer *et al.,* 2010).

A blockage of the proteasome and chaperone-mediated autophagy may contribute to protein aggregation. Then, activation of autophagy would be important to degrade protein aggregates and reduce the pool of their soluble cytosolic forms that contribute to aggregate formation (Kon & Cuervo, 2010). Autophagy is one of the primary degradation pathways for various aggregate-prone proteins associated with neurodegenerative diseases (Ravikumar *et al.,* 2004; Webb *et al.,* 2003). An *in vivo* block of autophagy results in the accumulation of ubiquitinated proteins in inclusion bodies, which increase in size and number upon aging (Hara *et al.,* 2006; Komatsu *et al.,* 2006). Moreover, similarly to the UPS, components of the autophagy pathway have been found colocalizing with mutant huntingtin aggregates. mTOR is inactivated in a mouse model of Huntington's disease as it is sequestered into aggregates, and p62 has also been found into these mutant huntingtin inclusions in the R6/2 mouse model (Nagaoka *et al.,* 2004; Ravikumar *et al.,* 2004). These findings suggest that autophagy could participate in mutant huntingtin degradation, and that it could be compromised in Huntington's disease pathology. It is for this reason that distinct pharmacological approaches have been performed to increase the autophagic levels in distinct models of the disease with the aim to degrade mutant huntingtin and ameliorate symptoms (Ravikumar *et al.,* 2004; Sarkar *et al.,* 2007a, 2007b). Although indeed autophagy seems a good therapeutic target, conflicting results exist when studying basal autophagy levels in Huntington's disease. Induction of autophagy has been reported in human Huntington's disease brain, in transgenic mice expressing mutant huntingtin fragments, in the HdHQ200 knock-in mice, and in cellular models of Huntington's disease (Heng *et al.,* 2010; Kegel *et al.,* 2000; Ravikumar *et al.,* 2004). In contrast, the autophagic response is partially impaired in embryonic fibroblasts and in striatal cells derived from the HdHQ111 knock-in mice (Martinez-Vicente *et al.,* 2010). While autophagosomes are able to form and fuse with lysosomes, the authors report that expression of mutant huntingtin results in inefficient cargo loading, primarily affecting organelle sequestration, in particular that of lipid droplets, and mitochondria.

2.2 - ALTERATION IN SIGNALLING PATHWAYS INVOLVED IN SURVIVAL AND APOPTOSIS

Besides aggregate formation, mutant huntingtin can also trigger cellular stress, and even death, inducing alterations at signalling pathways. Among these pathways,

the AKT-mTOR and protein kinase C have been shown to be important in the regulation of cell survival and death.

2.2.1 - AKT-mTOR signalling pathway

AKT is a key regulator of a wide range of cellular processes including growth, proliferation, metabolism and cell survival. Alterations of this signalling pathway have been associated to many diseases such as cancer, diabetes and neurodegeneration.

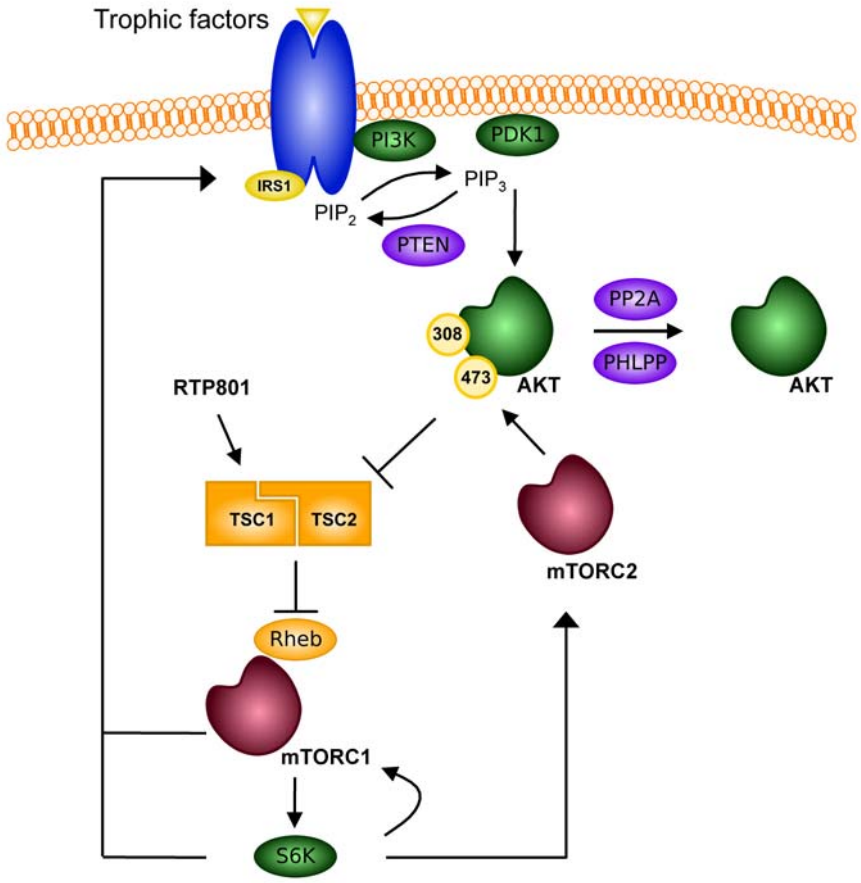

Figure 9.- The AKT-mTOR pathway. Hormone and growth factor signalling trigger AKT phosphorylation by both PDK1 at the Thr308 and mTORC2 at the Ser473, residues that undergo dephosphorylation by the phosphatases PP2A and PHLPP, respectively. Once active, AKT inhibits the inhibitory TSC1-TSC2 complex, leading to mTORC1 activation in a Rheb-dependent manner. mTORC1 is also modulated by other factors such as RTP801 that is induced under stressful conditions. Positive feedback loops do exist, since both mTORC1 and its substrate S6K can phosphorylate IRS-1 and S6K can also phosphorylate the mTORC1 and mTORC2 complex. Figure adapted from the Cell Signaling homepage, http://www.cellsignal.com.

All these functions are triggered through the activation of different downstream targets. Indirect activation of the aforementioned complex mTORC1, links AKT to many intracellular processes such as transcription, translation and autophagy (Liao & Hung, 2010) (Fig. 9).

2.2.1.1 - AKT

The serine/threonine kinase AKT is a 60 KDa protein that was first described by three different groups who reported a high degree of homology with PKA and PKC (Bellacosa *et al.,* 1993; Coffer & Woodgett, 1991; Jones *et al.,* 1991). Mammalian cells express three different AKT isoforms, AKT1, AKT2 and AKT3, who share 80% amino acid sequence homology although being encoded by different genes. *Akt1* is ubiquitously expressed at high levels, whereas *Akt2* is highly expressed in insulin-sensitive tissues including the liver, skeletal muscle and adipose tissue. *Akt3* is mostly expressed in the brain and testis, and in contrast, lower expression levels are found in intestinal organs and muscle tissue (Bellacosa *et al.,* 1993; Coffer & Woodgett, 1991; Jones *et al.,* 1991; Nakatani *et al.,* 1999). All three isoforms have a similar structure that is critical for their activation. A pleckstrin homology (PH) domain responsible for phospholipid binding is found at the N-terminus of AKT, followed by a catalytic domain or activation loop that contains one of the phosphorylation sites important for its activation (threonine 308 in AKT1; Thr308). Finally, the C-terminus of the protein contains a hydrophobic domain with a serine residue important for its full activation (Ser473 in AKT1) (Matheny & Adamo, 2009).

AKT is activated in response to hormone and growth factor signalling that trigger the generation of PIP3 by the class I PI3K (Franke *et al.,* 1997). PIP3 act as docking sites at the membrane that recruit both AKT and PDK1 through binding to its PH domain (Alessi *et al.,* 1997). Once at the membrane, AKT can be phosphorylated at Thr308 by the 3-phosphoinositide dependent protein kinase-1 (PDK1), which results in a conformational change of AKT that is followed by mTORC2-dependent phosphorylation at the Ser473, leading its full activation and detachment from the membrane (Andjelkovic *et al.,* 1997). Phosphorylation at the catalytic domain has been proposed to be sufficient for activation of AKT, however full activation is achieved when both the catalytic and hydrophobic domains are phosphorylated (Alessi *et al.,* 1996). AKT signalling comes to an end with the participation of phosphatases. PH domain leucine-rich repeat protein phosphatase (PHLPP) specifically dephosphorylates the

hydrophobic motif of AKT, resulting in a decrease of its activity, whereas the Thr308 site is dephosphorylated by protein phosphatase 2A (PP2A) (Brognard *et al.,* 2007, Gao *et al.,* 2005). Moreover, levels of PIP3, and thus membrane-recruiting of AKT, are down-regulated specifically by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which is activated upon dephosphorylation (Rahdar *et al.,* 2009).

Once AKT is active, is ready to phosphorylate its downstream targets. Among them, there are pro-apoptotic targets such as Bcl-2–associated X protein, Bcl-2-associated death promoter and members of the Forkhead Box O that become inactive by AKT-mediated phosphorylation (Brunet *et al.,* 1999; Datta *et al.,* 1997; Gardai *et al.,* 2004). Thus, one of the mechanisms by which AKT triggers cell survival is the direct inhibition of pro-apoptotic substrates. Active AKT can also exert a role in the regulation of the cell cycle by phosphorylating and inhibiting $p21^{Cip1/WAP1}$ and $p27^{Cip1/WAP1}$ (Liang *et al.,* 2002). One of the most important targets of AKT due to the high number of pathways that can regulate is the glycogen synthase-3β (GSK3β), which is also inhibited upon phosphorylation (Cross *et al.,* 1995). Phosphorylation of GSK3β by AKT can influence on cellular structure, growth and even apoptosis (Jope & Johnson, 2004). Finally, the main target of AKT in the context of cell growth pathway is the tuberous sclerosis protein (TSC) 2, which is inactivated by AKT-dependent phosphorylation and, when dephosphorylated and active, is able to promote the hydrolysis and inhibition of its downstream target Ras homolog enriched in brain (Rheb) that positively regulates mTORC1 through a still unknown mechanism (Inoki *et al.,* 2002). Thus, activation of mTORC1 can be triggered indirectly by AKT to regulate other processes such as transcription, translation and autophagy.

2.2.1.2 - mTOR

Along the years, mTOR has attracted increasing scientific interest because of its involvement in many human diseases, such as several types of cancer (Zoncu *et al.,* 2011).

The only difference between mTORC1 and mTORC2 complexes is the regulatory accessory protein that mTOR binds, which is a scaffold protein, used to assemble the complex and bind regulatory factors and substrates. Those are the regulatory-associated protein of mTOR (Raptor) and the rapamycin-insensitive companion of

mTOR (Rictor) that define mTORC1 and mTORC2, respectively (Hara *et al.,* 2002; Sarbassov *et al.,* 2004) (Fig. 10). Rictor and Raptor, not only bind mTOR and its substrates, but also regulatory proteins. While Raptor binds the negative regulator 40 kDa Pro-rich AKT substrate (PRAS40), Rictor binds the protein observed with Rictor (Protor), which also helps in assembling the complex, and the mammalian stress-activated MAPK-interacting protein 1 (mSin1) (Zoncu *et al.,* 2011). Besides Rictor and Raptor, both mTORC1 and mTORC2 also assemble positive and negative regulators, to the complexes, the mammalian lethal with SEC13 protein 8 (mLST8/GβL) and the DEP domain-containing mTOR-interacting protein (Deptor), which are positive and negative regulators respectively (Peterson *et al.,* 2009). Thus, mTORC1 contains four associated regulatory proteins, Raptor, PRAS40, Deptor and mLST8/GβL, and mTORC2 contains Rictor, mSin1, Protor, Deptor and mLST8/GβL.

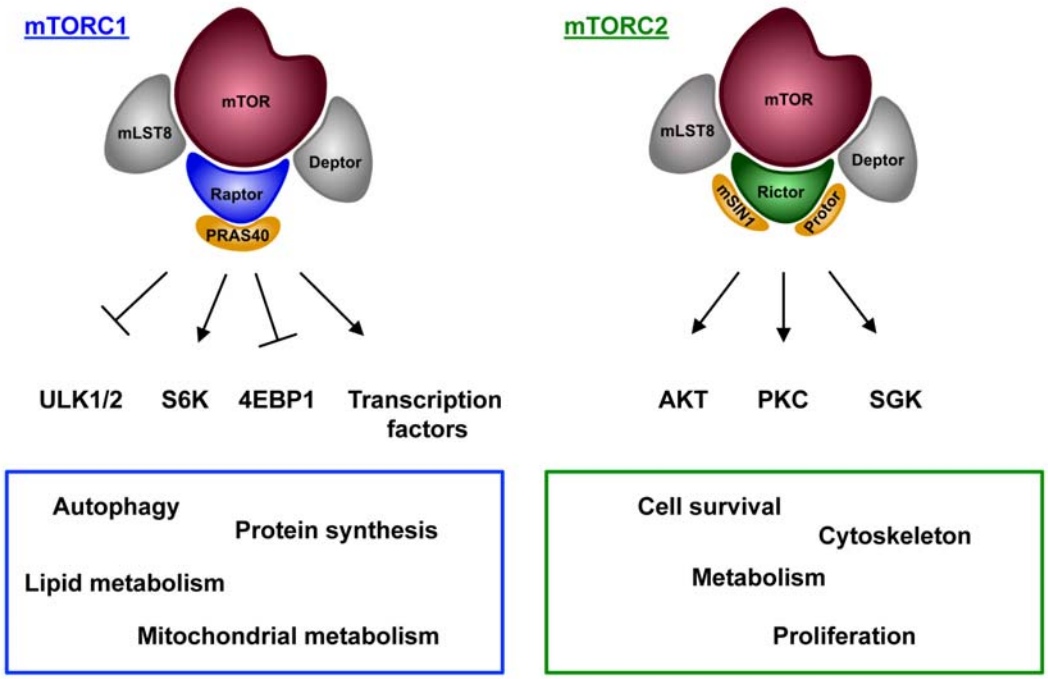

Figure 10.- mTOR complexes, mTORC1 and mTORC2. The diagram shows the two complexes that mTOR generates, mTORC1 and mTORC2, as well as the specific mTOR-interacting partners, substrates and functions of each complex. Figure adapted from Bove *et al.,* 2011 and Zoncu *et al.,* 2011.

All these regulatory proteins bound to mTOR permit specific regulation of both complexes. As an example, rapamycin binds the small protein 12 kDa FK506-binding protein (FKBP12) and, together, bind the FKBP12-rapamycin-binding domain of mTOR. This binding inhibits mTORC1 activity, probably by disrupting the association of Raptor to mTOR, and thus preventing the access of mTOR to its substrates (Bove *et*

al., 2011; Kim *et al.,* 2002). Although rapamycin effects are specific for the complex mTORC1, prolonged treatments can also affect mTCORC2 by impeding the assembly of mSin1 and Rictor with mTOR (Sarbassov *et al.,* 2006). mTORC1 and mTORC2 assemblies occur both in the cytoplasm and in the nucleus. However, while mTORC2 assembly is abundant in both cellular compartments, mTORC1 complex assembly predominantly occurs in the cytoplasm, despite high protein levels of raptor can be found in both the cytoplasm and the nucleus (Rosner & Hengstschlager, 2008).

Up to date, six phosphorylation sites have been described at the mTOR sequence. In order of discovery those are Ser2448, Ser2481, Thr2446, Ser1261, Ser2159 and Thr2164, being the first two the ones far most characterized (Foster & Fingar, 2010) (Fig. 11). While Ser2481 is an autophosphorylation site, and thus serves as a biomarker to analyze intrinsic mTOR activity (Soliman *et al.,* 2010), phosphorylation at Ser2448 is regulated by both AKT and S6K1, and is sensitive to rapamycin treatment (Holz & Blenis, 2005; Nave *et al.,* 1999). AMPK, in response to low nutrient levels, and also S6 kinase 1 (S6K1) have been suggested to phosphorylate mTOR at Thr2446, with less information about its physiological significance (Cheng *et al.,* 2004; Holz & Blenis, 2005). Both Thr2446 and Ser2448 phophorylation sites are found in a negative regulatory domain of mTOR that, at least upon AKT-mediated Ser2448 phosphorylation, gets inactivated leading to increased mTOR activity (Sekulic *et al.,* 2000). Ser1261 phosphorylation event may participate in promoting mTOR catalytic activity, although the kinase responsible of this phosphorylation still remains unknown (Acosta-Jaquez *et al.,* 2009).

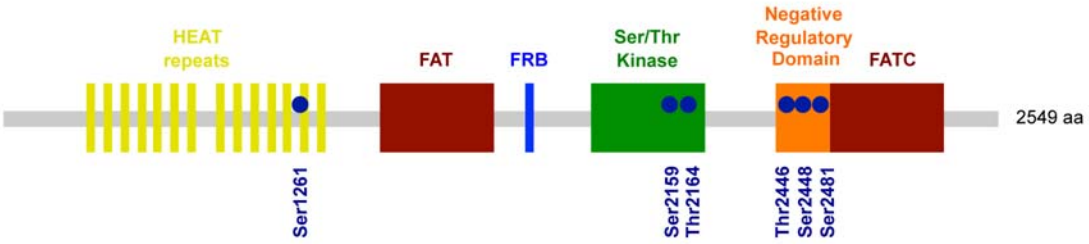

Figure 11.- Schematic diagram of the mTOR protein. The diagram shows the domain architecture of mTOR and its phosphorylation sites. Figure adapted from Acosta-Jaquez *et al.,* 2009; Ekim *et al.,* 2011; Foster & Fingar, 2010; Zoncu *et al.,* 2011.

While phopsphorylation at Ser 2448 is mainly associated to mTORC1, there is higher stoichiometry of mTOR Ser2481 autophosphorylation in mTORC2 (Copp *et al.,* 2009). However, Ser2481 autophosphorylation is also found associated to mTORC1,

and is sensitive or insensitive to rapamycin treatments depending on which mTORC complex is analyzed (Soliman *et al.,* 2010). At last, novel mTOR Ser2159 and Thr2164 phosphorylations promote mTORC1 signalling through weaken the inhibitory raptor-PRAS40 interaction (Ekim *et al.,* 2011).

Finally, all the regulatory proteins present in either mTORC1 and mTORC2 can also run phosphorylation events and thus, finely tune mTORC1 and mTORC2 signalling (Foster & Fingar, 2010).

Regulatory components of mTORC1 and mTORC2 not only permit specific regulation of their functions but also dictate substrate specificity. On one hand, mTORC1 phosphorylates and activates S6K1, and inhibits 4EBP1, thus increasing the rate of protein synthesis by enhancing mRNA translation initiation and progression. mTORC1 also enhances the activity of some transcription factors implicated in the lipid synthesis and mitochondrial metabolism (Zoncu *et al.,* 2011). At the same time, and as previously explained, mTORC1 inhibits autophagosome synthesis by directly phosphorylating ULK1/2 and Atg13 (Kim *et al.,* 2011a). On the other hand, mTORC2 phosphorylates members of the AGC kinase family, such as AKT, serum and glucocorticoid-regulated kinase (SGK) and some members of the PKC family (Garcia-Martinez & Alessi, 2008; Ikenoue *et al.,* 2008; Sarbassov *et al.,* 2005). Moreover, it has also been suggested that mTORC2 could be implicated in the reorganization of actin cytoskeleton (Jacinto *et al.,* 2004).

mTORC1 is not only activated upon growth factor stimulation, but as previously mentioned also senses amino acids, insulin and ATP. Even the Ras-MAPK pathway and the PKC pathway have been suggested to regulate mTORC1 activity by phosphorylating TSC2 and inhibiting the TSC1-TSC2 complex (Ma *et al.,* 2005; Roux *et al.,* 2004). However, very little is known about mTORC2 activation mechanisms, and some suggestions have emerged during the last few years. mTORC2 is activated in response to PI3K, since mTORC2 gets active in response to growth factors and pharmacological inhibition of PI3K reduces mTORC2 kinase activity (Huang *et al.,* 2008). Moreover, and contrary to the inhibitory effect on mTORC1, the TSC1–TSC2 complex is able to activate mTORC2 *in vitro* (Huang *et al.,* 2008). Finally, ribosomes have been suggested to play a role in mTORC2 activation and signalling (Zinzalla *et al.,* 2011).

Downstream mTORC1-effector S6K1 might participate in important feedback loops. On one hand, it is able to phosphorylate mTOR, potentially participating in a positive feedback loop on mTORC1 (Holz & Blenis, 2005). On the other hand, it

participates in a negative regulatory feedback loop, since it is able to phosphorylate the insulin receptor substrate-1 resulting in a decrease in its protein stability and decreased AKT signalling (Tzatsos & Kandror, 2006). mTORC2 would also integrate mTORC1 activity, since S6K1 is also able to phosphorylate and inhibit Rictor (Julien *et al.,* 2010).

Finally, stress conditions such a hypoxia or DNA damage can increase RTP801 levels, a stress-responsive protein who is expressed at very low levels within different tissues being brain, muscle and intestine the tissues that express this protein at a lowest levels (Shoshani *et al.,* 2002). The increase in RTP801 levels, in turn, inhibits mTORC1 activity promoting the TSC1-TSC2 complex activity (Corradetti *et al.,* 2005).

2.2.1.3 - AKT-mTOR pathway in the nervous system and deregulation in Huntington's disease

The AKT-mTOR pathway has been extensively studied in order to find therapies against tumors. Within the nervous system, this pathway has also been involved in pathologies including neurodegenerative diseases, since both AKT and mTOR are linked to neuronal survival and maintenance of synaptic contacts, and rapamycin treatment has been an approach to reverse and ameliorate the symptoms of diverse diseases.

AKT is present at low levels in the adult brain (Owada *et al.,* 1997), but its expression and activation increase dramatically in neurons during cellular stress or injury (Chong *et al.,* 2005). For instance, activated AKT has been proposed as an important neuroprotective pathway in distinct acute (Endo *et al.,* 2007; Owada *et al.,* 1997; Zhang *et al.,* 2006) and chronic models of neurodegeneration (Gines *et al.,* 2003; Saavedra *et al.,* 2010). Moreover, *in vivo* delivery of a dominant negative form of AKT within the substantia nigra reduced the number of dopaminergic neurons and doubled the incidence of apoptotic neurons (Ries *et al.,* 2009). Besides promoting neuronal survival, accumulating data suggest a role for AKT in promoting neurite outgrowth, enhancing the diameter of processes and also increasing axonal branching and regeneration (Gavalda *et al.,* 2004; Kwon *et al.,* 2006; Markus *et al.,* 2002; Namikawa *et al.,* 2000).

On the other hand, mTOR is crucial in the adult brain for numerous physiological processes such as synaptic plasticity, learning, memory, and brain control of food uptake (Garelick & Kennedy, 2011; Swiech *et al.,* 2008). Moreover, the activation of mTOR pathway is involved in neuronal development, dendrite development and spine morphogenesis (Swiech *et al.,* 2008). An induction of RTP801 in both *in vitro* and *in*

vivo models of Parkinson's disease is known to promote neuronal death by suppressing activation and signalling of mTOR (Malagelada *et al.,* 2006). One of the mechanisms that could account for this neuronal death is a suppression of the mTOR-dependent AKT activation (Malagelada *et al.,* 2008).

In Huntington's disease, reduced levels of PHLPP1 were detected in the striatum, cortex and hippocampus of several mouse models of the disease, that were due to reduced PHLPP1 gene expression (Saavedra *et al.,* 2010). In good correlation with this phenomenon, increased phosphorylation levels of striatal AKT (pSer473 AKT) have been observed in different models of Huntington's disease (Gines *et al.,* 2003; Saavedra *et al.,* 2010). Moreover, an increase in the phosphorylation of AKT targets GSK3β (Ser9) and FoxO (Ser256) has been also detected. Although PHLPP1 levels were also deregulated in the cortex and hippocampus of R6/1 mice, this was not the case for pSer473 AKT levels, indicating that other factors, than the reduction of PHLPP1 levels, would contribute to increase pSer473 AKT levels *in vivo* (Saavedra *et al.,* 2010). Interestingly, in the striatum of HD94 mice after shutting-down the expression of mutant huntingtin, PHLPP1 protein levels returned to control levels, while pSer473 AKT levels were only partially reverted (Saavedra *et al.,* 2010). These results suggest that increased levels of pSer473 AKT is a specific mechanism taking place in striatal neurons expressing mutant huntingtin, which could be the sum of increased activation of kinases that phosphorylate AKT and decreased levels of PHLPP1 (Saavedra *et al.,* 2010). Interestingly, in an acute rat model of Huntington's disease showing massive cell death, a decrease in pSer473 AKT levels has been observed during cell dysfunction, just before neurodegeneration occurs (Colin *et al.,* 2005). It has been hypothesized that increased AKT activation could counteract mutant huntingtin toxicity, raising the threshold, above which apoptotic signals can occur (Saavedra *et al.,* 2010).

mTOR has also been studied, but from a more therapeutic point of view, since its inhibition may account for increased autophagy and thus, mutant huntingtin aggregates degradation. Inhibition of mTOR with rapamycin improves behavioural performance and decreases aggregate formation in a mouse model of Huntington's disease (Ravikumar *et al.,* 2004). Interestingly, the same authors have found that mTOR is sequestered in the mutant huntingtin aggregates, which impairs its kinase activity and induces autophagy (Ravikumar *et al.,* 2004).

2.2.2 - PKC signalling pathway

PKC, which is another member of the AGC kinase family, is a serine/threonine kinase that play key roles in many signalling pathways that control from cellular growth, proliferation and differentiation to cell death (Freeley *et al.,* 2011).

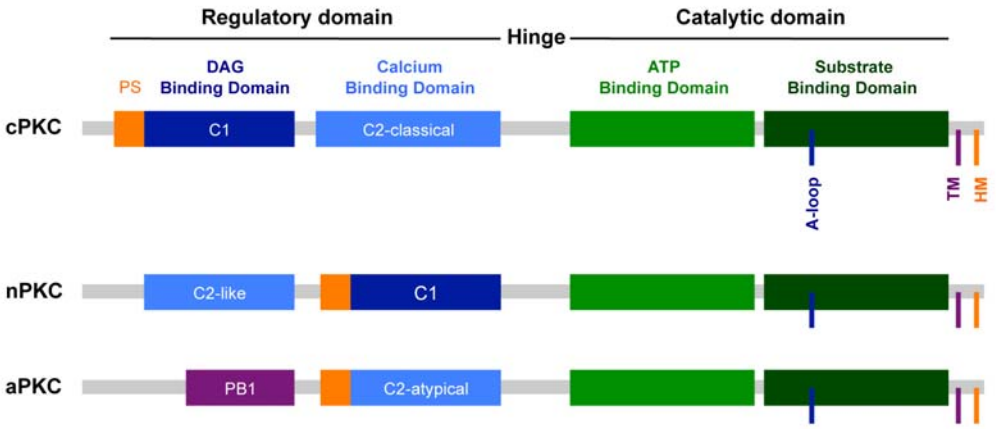

Figure 12.- Schematic diagram of the three main PKC subfamilies. The diagram represents the differences in domain architecture of the three PKC subfamilies: cPKC, nPKC and aPKC. The scheme also represents the phosphorylation sites at the A-loop, TM and HM that occur at the PKC catalytic domain. Figure adapted from Duquesnes *et al.,* 2011.

Up to date, nine different PKC isoforms, transcribed from separate genes, have been described and characterized. All of them can be classified in three main subfamilies, depending on structural features and cofactor requirements, the conventional PKCs (cPKCs; comprise PKC α, βI/βII and γ isoforms), the novel PKCs (nPKCs; comprise δ, ε, η and θ) and the atypical PKCs (aPKCs; comprise PKC ζ and ι) (Freeley *et al.,* 2011) (Fig. 12). All of them, however, share a similar primary structure, which contains an N-terminal regulatory domain and a C-terminal catalytic domain (Duquesnes *et al.,* 2011). The N-terminal domain contains regions where second messengers, cofactors and even scaffold proteins may bind, and also a pseudosubstrate sequence that binds and inhibits the catalytic domain. Upon second messenger and cofactor PKC-binding, the pseudosubstrate sequence undergoes proteolysis and PKC becomes catalytically active (Orr & Newton, 1994).

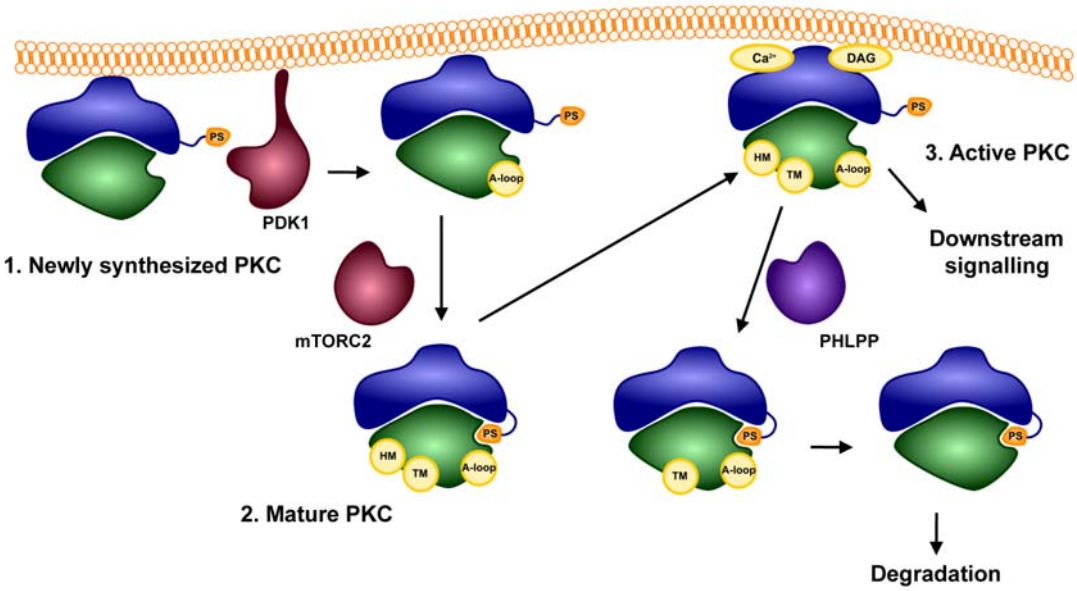

Figure 13.- From cPKC activation to cPKC degradation. Newly synthesized PKC is processed by ordered and coupled phosphorylations. First, PDK1 phosphorylates the A-loop and, then, mTORC2 phosphorylates the TM and the HM, leading to a mature PKC. Upon second messengers, such as Ca2+ and DAG, PKC is recruited to the membranes where it adapts an open conformation allowing downstream signalling. This open conformation is sensitive to PHLPP-mediated dephosphorylation on the HM. Further dephosphorylation on the A-loop, TM and HM, triggers PKC degradation. Figure adapted from Newton, 2010.

Whereas the catalytic domain and the pseudosubstrate sequence are common regions into all PKC isoforms, the regulatory domain is slightly different and, thus, the activation system. cPKCs are activated by a combination of diacylglycerol (DAG) and phospholipid binding to their conserved region 1 (C1) domains and Ca^{2+}-dependent phospholipid binding to their conserved region 2 (C2) domains. nPKCs are similarly activated by DAG and phospholipids, but do not respond directly to Ca^{2+}. Unlike cPKCs and nPKCs, aPKCs do not depend on Ca^{2+} or DAG for activation since they lack C1 and C2, but are instead activated by other means such as interaction with the partitioning defective 6–CDC42 complex through their PB1 domain (Rosse *et al.,* 2010).

Activation of PKCs involves two steps in which PKC first undergoes maturation followed by activation in a second messenger-dependent manner. Maturation of PKC requires ordered phosphorylations at different motifs, the activation loop site, the turn motif and the hydrophobic motif (Keranen *et al.,* 1995) (Fig. 13). PDK1 is the kinase involved in phosphorylating all PKC isozymes at the activation loop site, which is required for both PKC activation and PKC stability (Balendran *et al.,* 2000).

Phosphorylations at the turn motif and hydrophobic motif are triggered by the complex mTORC2 (Facchinetti *et al.,* 2008; Ikenoue *et al.,* 2008). Fully phosphorylated and mature PKCs localize to the cytosol until second messengers, such as DAG and Ca^{2+}, recruit them to membranes. Membrane-bound PKC allows downstream signalling (Newton, 2010). Some exceptions do exist in this classical model for PKC activation. PKCδ contains an acidic glutamate at position 500 that assumes the role of the phosphorylated activation-loop (Steinberg, 2004). Interestingly, phosphorylation at the activation-loop of PKCδ, at Thr505, targets the kinase for degradation (Srivastava *et al.,* 2002). Finally, autophosphorylation might also play a role in phosphorylating the turn motif of PKCδ (Li *et al.,* 1997; Parekh *et al.,* 1999).

Membrane-bound and active PKC, however, is highly sensitive to dephosphorylation and, as previously commented, the phosphatase PHLPP dephosphorylate both cPKCs and nPKCs, but not aPKCs, at the hydrophobic motif (Brognard & Newton, 2008). This event has been suggested to trigger further dephosphorylation, enzyme instability and degradation (Gao *et al.,* 2008). In addition, phosphorylated PKC may also get ubiquitinated and degraded, by the proteasome (Leontieva & Black, 2004; Lu *et al.,* 1998; Srivastava *et al.,* 2002). Finally, a third degradation mechanism has been suggested for PKCα, which might undergo monoubiquitinization at the membrane, thus facilitating its internalization, followed by transport along the endosomal pathway, ubiquitin-independent proteasomal delivery and degradation (Melnikov & Sagi-Eisenberg, 2009).

Spatial and temporal control of PKC signalling is not only regulated by phosphorylation events and second messengers, but also via interaction with scaffolding proteins that anchor the PKCs to various intracellular locations in the cell, thus generating PKC responses in specific cellular microdomains. Among scaffold proteins that have been characterized to influence PKC function there are Receptors for Activated C Kinases (RACKs) (Ron *et al.,* 1994), A-Kinase Anchoring Proteins (AKAPs) (Klauck *et al.,* 1996), 14-3-3 proteins (Van Der Hoeven *et al.,* 2000) and even p62 (Sanchez *et al.,* 1998).

The identification 30 years ago of PKC as one of the targets of the phorbol esters, natural products with tumour-promoting activity, pushed PKC to the cancer research field (Griner & Kazanietz, 2007). Tumour-related processes such as proliferation and cell cycle progression, invasion and angiogenesis, are both positively and negatively regulated by distinct PKC isozymes (Griner & Kazanietz, 2007).

However, PKCs are involved in a broad spectrum of other cellular processes. Isozyme and cell type, are two main factors that define precise PKCs effects and

functions. Different isozymes can trigger overlapping functions or even opposite effects (Mischak *et al.,* 1993). PKCs regulation of actin cytoskeleton dynamics has also been described, inducing further effects, not only on cell migration, but also on neurite outgrowth and cellular morphology (Larsson, 2006). Even autophagy might also be regulated by PKCs (Chen *et al.,* 2008). PKCs are also important in mediating survival and apoptosis. While PKC α, β, γ and ε are mainly considered to induce cell survival, PKCδ classically triggers apoptosis (Reyland, 2007). However, findings that PKCδ can also trigger cell survival in some proliferating cells suggest a more PKC functional complexity, depending on the cell type were they are expressed (Chen *et al.,* 2011b).

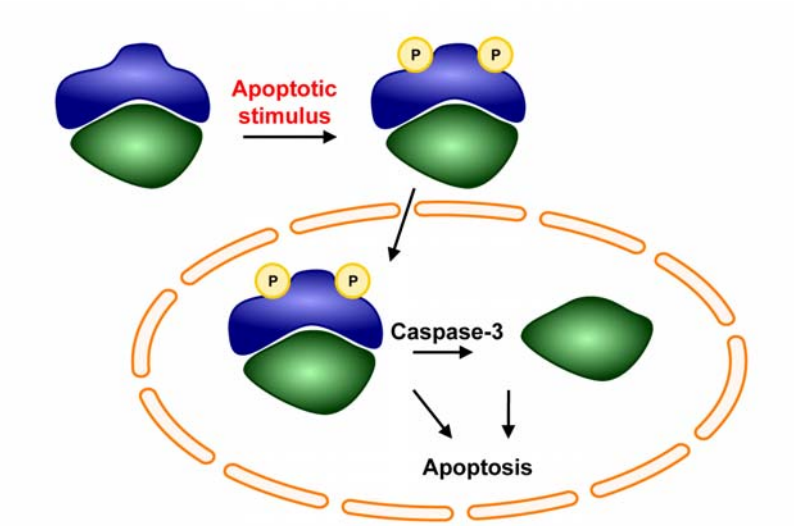

Figure 14.- Model for PKCδ induction of apoptosis. PKCδ undergoes specific phosphorylation events at tyrosines 64 and 155 that are essential for its nuclear translocation. Once in the nucleus, it is cleaved by caspase-3 at the hinge region generating the δCF. Both PKCδ and δCF induce apoptosis. Figure adapted from DeVries-Seimon *et al.,* 2007.

Upon apoptotic stimuli, PKCδ undergoes PKCδ-specific phosphorylation events at tyrosines 64 and 155 that are essential for nuclear translocation and apoptosis induction (Adwan *et al.,* 2011; Humphries *et al.,* 2008) (Fig.14). Once in the nucleus, PKCδ is cleaved by caspase-3 at the hinge region that link the regulatory domain to the catalytic domain, generating a constitutive catalytic fragment (δCF), which is sufficient, but not essential to induce apoptosis (DeVries-Seimon *et al.,* 2007; Emoto *et al.,* 1995; Ghayur *et al.,* 1996). PKCδ can regulate different apoptotic events, including caspase activation and DNA fragmentation, as well as loss of mitochondrial membrane potential (Matassa *et al.,* 2001).

2.2.2.1 - cPKCs and nPKCs in the nervous system and in Huntington's disease

PKC isoforms are involved in a broad range of neuronal functions, and have been implicated in learning and memory, and in long-term potentiation (Bank *et al.,* 1988). PKC activation directly increases the formation of mature synapses during learning procedurals, and regulates changes of pre- and post-synaptic ultra-structure (Hongpaisan & Alkon, 2007). Reduced PKC activity has been observed in post-mortem brains of suicide victims (Pandey *et al.,* 2004), suggesting an involvement of PKCs in cognition and mood regulation. However, different results are obtained when studying PKCs under pathological conditions since sustained activation of PKCs might be also detrimental to spine morphology, as seen in *in vitro* studies of hippocampal pyramidal cells that have shown that sustained activation of PKC induces spine loss and collapse of the spine's actin cytoskeleton (Calabrese & Halpain, 2005). Similar results have been observed *in vivo*, in the rat pre-frontal cortex, where inhibition of PKC signalling prevents spine loss and protects working memory performance during chronic stress exposure (Hains *et al.,* 2009). The authors suggest that PKC inhibitors may be neuroprotective in disorders with deregulated PKC signalling, such as bipolar disorder, schizophrenia and post-traumatic stress disorder.

All these studies suggest that PKCs must be tightly regulated within the nervous system, and that any alteration in their signalling can impair neuronal function.

PKCs have also been implicated in neurodegenerative diseases. Reduced PKC activity has been observed within Alzheimer's disease (Cole *et al.,* 1988). Administration of non-specific PKC activator is capable of lowering β-amyloid protein levels in neurons (Hung *et al.,* 1993), an effect that is triggered by both PKCα and PKCε (Hongpaisan *et al.,* 2011; Kim *et al.,* 2011b). Chronic administration of bryostatin-1, a PKCα and PKCε activator, into a mouse model of Alzheimer's disease, restored the levels of PKCα and PKCε, reduced the level of soluble β-amyloid, prevented the loss of hippocampal synapses and memory impairment, and even prevented the reduction in BDNF expression in the CA1 pyramidal neurons (Hongpaisan *et al.,* 2011). In Parkinson's disease, PKCδ has been suggested to play a role in the dopaminergic neuronal apoptosis. An irreversible and competitive peptide inhibitor that mimic the caspase-3 cleavage site of PKCδ, rescued the MPP+ and 6-hydroxydopamine-induced toxicity in mouse primary mesencephalic cultures (Kanthasamy *et al.,* 2006). PKCδ is also increased during kainate-induced neuronal death, and the authors suggest this isoform could contribute to the apoptotic responses that occur after excitotoxic insults (Kaasinen *et al.,* 2002).

In Huntington's disease, less published data do exist about PKC isozymes. However, PKCβII immunoreactivity was found decreased in both caudate nucleus and putamen from Huntington's disease patients (Hashimoto *et al.,* 1992). Moreover, PKCβII expression levels were also decreased in the striatum and cortex in the R6/2 mouse model of the disease (Harris *et al.,* 2001). Finally, also alterations in PKCδ were found, since this isozyme was detected in mutant huntingtin inclusions in cerebellar mouse-tissue sections (Zemskov *et al.,* 2003). In the same group, however, the authors failed to find differences in the pro-apoptotic δCF, despite the previously reported activation of caspase-3 in the same mouse model (Chen *et al.,* 2000).

II. AIMS

Huntington's disease is a progressive neurodegenerative disorder with an onset that depends on the CAG repeat length. Mutant huntingtin induces a large amount of toxic effects that trigger cell dysfunction. However, before the symptoms start to emerge gradually, individuals are healthy. Thus, it is plausible that compensatory mechanisms may be activated to regulate a balance between cell death and survival (Butler *et al.,* 2006). These compensatory mechanisms might be responsible for the gradual nature of Huntington's disease progression. We have studied three different mechanisms, which could enhance cell survival and delay cell dysfunction, along the progression of the disease in R6/1 mouse model brains. We first studied selective autophagy, since it could represent one of those mechanisms, participating in the degradation of the toxic protein, thus releasing cells from the toxic insult (Fig. 15).

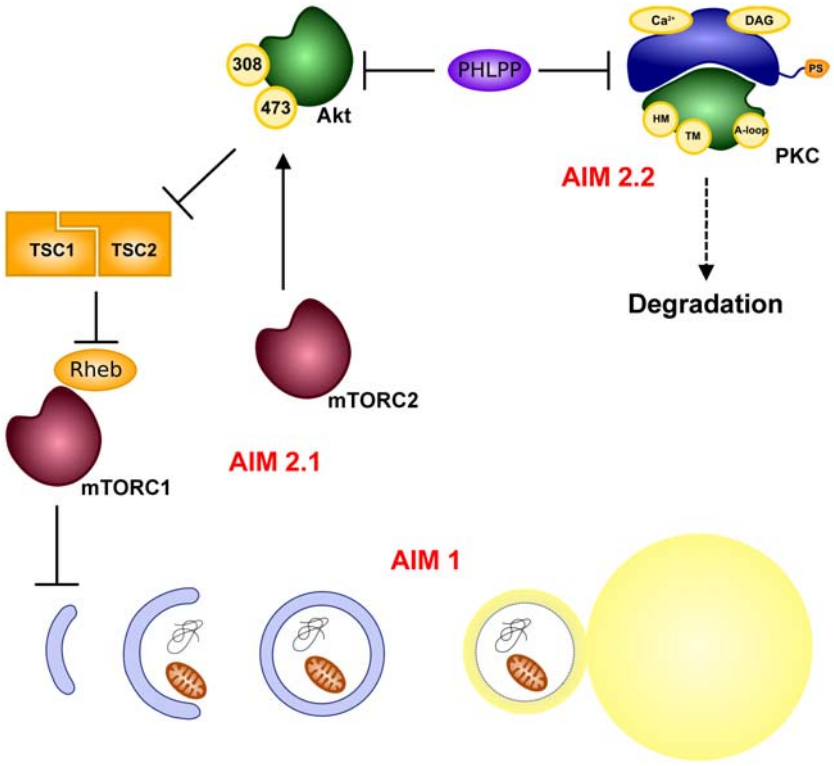

Figure 15. Scheme indicating the different aims developed in this work. On one hand, we have analyzed the selective autophagic flux, since maintained levels of this process are important to degrade mutant huntingtin and release the cells from its toxicity. On the other hand, we have analyzed the role of mTOR in the regulation of autophagy and also the pro-survival kinase AKT. Finally, we have analyzed the levels of some members of the PKC protein family since previous results suggest their deregulation, due to decreased levels of the phosphatase PHLPP that shunts PKC for degradation.

Interestingly, autophagy-deficient mice develop neurodegenerative decline even in the absence of harmful gene products such as mutant huntingtin (Komatsu *et al.,* 2006). Moreover, pro-survival signalling pathways can also counteract cell death.

Previous results from our group have revealed an interesting imbalance between the pro-survival kinase AKT and PHLPP1. They observed an AKT over-activation that could be partially mediated by a decrease in PHLPP1 protein levels (Saavedra *et al.,* 2010). Thus, we have further studied the role of pro-survival and pro-apoptotic kinases in Huntington's disease. On one hand, we have examined the mTOR pathway, since mTOR regulates both autophagy and AKT-mediated cell survival (Fig. 15). On the other hand, we have also focused our interest in both pro-survival and pro-apoptotic PKC isoforms, since their levels are regulated by PHLPP-mediated dephosphorylation (Gao *et al.,* 2008) (Fig. 15).

AIM 1. – To study the regulation of selective autophagy along the progression of Huntington's disease.

1.1. To analyze p62 and NBR1 protein levels along the progression of the disease in different brain regions affected in Huntington's disease, as an indicator of selective autophagic activity.

1.2. To analyze p62 and NBR1 intracellular localization in cells expressing mutant huntingtin in a mouse model and in human Huntington's disease brains.

1.3. To analyze age-dependent mechanisms that could regulate autophagy.

AIM 2. – To characterize the regulation and the role of pro-survival and pro-apoptotic protein kinases in Huntington's disease.

2.1. To define the role of mTOR in the regulation of both autophagy and the AKT pro-survival pathway in Huntington's disease.

2.2. To study the regulation of different members of the PKC protein family in Huntington's disease.

III. METHODS

1 - HUNTINGTON'S DISEASE MOUSE MODELS

For the development of the objectives of this Thesis, we have used the R6/1 mouse model of Huntington's disease that was originally obtained from Jackson Laboratory (Bar Harbor, ME, USA). R6/1 mice express an N-terminal exon 1 fragment of mutant huntingtin, originally containing a stretch of 115 CAG repeats (Mangiarini *et al.,* 1996). However, the CAG repeat sequence is highly unstable, and CAG repeat-length determination in the mice used here revealed a longer CAG stretch (Giralt *et al.,* 2011b). Thus, our R6/1 mouse colony, with a B6CBA background, express the exon 1 of mutant huntingtin with 145 CAG repeats. Mice were genotyped and CAG repeat-length was determined by PCR amplification of the repeat using HD1 and HD2 fluorescently labelled primers as previously described by the Huntington's Disease Collaborative Research Group (HDCRG, 1993), and subsequent size determination in an ABI 3100 analyzer. Results were double checked by Laragen, Inc. (Los Angeles, CA, USA). Male R6/1 mice were used for all the experiments, and results were compared to non-transgenic wild-type mice. Transgenic and non-transgenic mice were littermates.

Animals were housed with access to food and water ad libitum in a colony room kept at a constant temperature in between 19-22ºC and at 40-60% humidity, under a 12:12 hours light/dark cycle. Upon birth, litters containing distinct genotypes were being housed together. Microchips were implanted on the back subcutaneously, and were used to number all mice and have access to recorded genotype data.

All animal-related procedures were performed in compliance with the National Institute of Health Guide for the care and use of laboratory animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01), and the Generalitat de Catalunya (99/1094), in accordance with the Directive 86/609/EU of the European Commission.

2 - HUMAN BRAIN TISSUE

All human brain samples were obtained from the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS, Barcelona (NTB-Biobanc-HC-IDIBAPS) (Table 2).

Case nº	Pathological diagnosis	CAG repeats	Gender	Age (years)
1	Normal	-	Female	81
2	Normal	-	Male	31
3	Normal	-	Female	68
4	Normal	-	Male	56
5	Normal	-	Male	64
6	Normal	-	Female	71
7	Normal	-	Female	60
8	Normal	-	Male	39
9	HD, Vonsattel grade 4	62	Female	28
10	HD, Vonsattel grade 4	44	Male	59
11	HD, Vonsattel grade 4	43	Male	60
12	HD, Vonsattel grade 3-4	-	Male	55
13	HD, Vonsattel grade 3	45	Male	53
14	HD, Vonsattel grade 1	40	Male	73

Table 2. Human post-mortem Huntington's disease (HD) brains. Among the post-mortem brains that were provided by the NTB-Biobanc-HC-IDIBAPS, 8 were histopathologically non-related to Huntington's disease, 5 were histopathologically diagnosed with Vonsattel grade 3-4 and 1 with Vonsattel grade 1.

Half brain was fixed in 10% buffered formaldehyde solution for 4 weeks and then, 25 brain areas were selected for histopathological evaluation, including frontolateral and orbitofrontal, temporal, parietal and occipital cortices, anterior cingulate, anterior and posterior basal ganglia, anterior, medial and posterior thalamic nuclei, hippocampus, amygdala, midbrain, pons, medulla oblongata, olfactory bulb, cerebellar vermis, hemisphere and dentate nucleus, and cervical spinal cord.

3 - CELL CULTURE

A conditionally immortalized striatal neuronal progenitor cell line ($STHdh^{Q7/Q7}$) was used, which was derived from striatal precursors isolated from HdhQ7 mice at embryonic day 14 and immortalized with the SV40 Large T antigen (Trettel *et al.,* 2000). Cells were grown at 33°C in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 1% streptomycin-penicillin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 400 µg/ml G418 (Geneticin; Invitrogen, Carlsbad, CA, USA).

4 - PHARMACOLOGICAL TREATMENTS

Cells were treated with different drugs in a different time-course and concentration depending on the planned experiment. Xpo-1 was inhibited with the irreversible inhibitor Leptomycin B (LMB; 10 nM; Sigma Chemical Co, St. Louis, MO, USA), which inactivates Xpo-1 by alkylation at one single cysteine residue (Kudo *et al.,* 1999). To inhibit the vacuolar ATPase and thus lysosomal acidification and lysosomal-dependent degradation, we used Bafilomycin A1 (BafA1; 100 nM; Merck, Darmstadt, Germany) (Bowman *et al.,* 1988; Yoshimori *et al.,* 1991). A combination of NH_4Cl (20 mM; Merck, Darmstadt, Germany) and leupeptin (0.1 mM; Sigma Chemical Co, St. Louis, MO, USA) was used to more effectively block lysosomal-dependent degradation without affecting other proteolytic systems (Fuertes *et al.,* 2003). Protease and phosphatase inhibitors were used to avoid protein degradation and dephosphorylation events in protein extracts.

5 - PLASMIDS AND TRANSFECTION

The Tandem Fluorescent-tagged LC3 construct (tf-LC3) was a kind gift from Dr. Yoshimori (Department of Cellular Regulation, Research Institute for Microbial Diseases, Osaka, Japan). This plasmid contains a bacterial resistance to kanamycin and expresses rat LC3 together with mRFP and GFP as tags, and was generated by inserting mRFP cDNA to a pEGFP-LC3 plasmid with kanamycin resistance (Kimura *et al.,* 2007).

Exon-1 huntingtin plasmids, expressing either 16 or 94 polyQ repeats and tagged with the cyan-fluorescent protein (CFP), were a kind gift from Dr. Lucas (Centre for Molecular Biology 'Severo Ochoa', Madrid, Spain). They were generated with ampicillin resistance. It is important to note that the 94 polyQ stretch is interrupted by an arguinine residue (40Q+1R+54Q).

A plasmid construct to over-express PKCδ with a C-terminal Haemmagglutinin (HA) epitope tag was kindly supplied by Dr. Weinstein through the Addgene platform (Addgene plasmid 16386; Cambridge MA, USA). The plasmid was generated at the Dr. Weinstein laboratory by ligating full-length PKCδ open reading frame into the mammalian expression vector pHACE, which contains a bacterial resistance to ampicillin (Soh & Weinstein, 2003).

To expand the plasmid, E. Coli (Subcloning Efficiency^{TM} DH5α^{TM} Competent Cells; Invitrogen, Carlsbad, CA, USA) were transformed by thermal shock. Plasmid (250 ng) was added to 50 µl-cultured bacteria and left incubating on ice for 15 min. To induce transformation, the mixture was incubated for 1.5 min at 42ºC and quickly placed again on ice for 2 min. Lysogeny broth media (LB; 800 µl) was added, and was incubated for 1h at 37ºC at 250 rpm to let transformed E. Coli grow. Finally, the transformation (200 µl) was spread on a pre-warmed agar plate containing 20 µg/ml kanamycin or 500 µg/ml ampicillin. Colonies were left growing overnight at 37ºC.

Plasmid isolation was performed from 5 ml bacterial culture using the Wizard Plus SV Minipreps and Maxipreps DNA purification system from Promega (Madison, WI, USA).

Transfection procedures were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as instructed by the manufacturer. Cells at 50% confluence were transfected with 1µg of plasmid in Opti-MEM media. Media was replaced 6 hours later by serum-supplemented DMEM media.

6 - PROTEIN EXTRACTION

6.1 - Mouse and human brain tissue

Animals were killed by decapitation at different steps of the disease. The brain was quickly removed and the striatum, cortex and hippocampus were quickly dissected out.

All mouse and human samples were homogenized by sonication in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 150 mM NaCl, protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 µg/µl aprotinin, 1 µg/µl leupeptin) and phosphatase inhibitors (2 mM Na_3VO_4, 100 mM NaF).

6.2 - Cell culture

STHdh$^{Q7/Q7}$ cells were washed once with PBS (phosphate-buffered saline) and total cellular proteins were extracted by incubating cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 150 mM NaCl, protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 µg/µl aprotinin, 1 µg/µl leupeptin) and phosphatase inhibitors (2 mM Na_3VO_4).

Samples were centrifuged at 16,100 x g for 20 min at 4ºC, and supernatants were collected. Protein concentration was determined using the Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

7 - SUBCELLULAR FRACTIONATION

7.1 - Mouse tissue

For subcellular fractionation, fresh tissue was homogenized in a teflon-glass potter in lysis buffer containing 4 mM HEPES, 0.32 M sucrose, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 2 mM sodium orthovanadate, 0.1 mg/ml benzamidine. Homogenates were centrifuged at 3,000 x g for 10 min to obtain cytosolic (supernatant) and nuclear (pellet) enriched fractions. Nuclear enriched fraction was resuspended in lysis buffer containing 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM PMSF, 10 mg/µl leupeptin, 2 mM Na_3VO_4 and was sonicated afterwards.

7.2 - Cell culture

STHdh$^{Q7/Q7}$ cells were rinsed once and pelleted with PBS. Pellets were homogenized in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, and protease and phosphatase inhibitors as above. Samples were centrifuged 10 min at

3,000 x g to obtain cytosolic (supernatant) and nuclear (pellet) fractions. Nuclear fractions were resuspended in lysis buffer, sonicated and centrifuged 20 min at 16,100 x g. Supernatant was kept as a nuclear enriched fraction and pellet was discarded.

8 - WESTERN BLOT ANALYSIS

Western blot analysis was performed following a standard protocol. Proteins were denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue and heated at 100ºC for 5 min. They were resolved in denaturing polyacrylamide gels (SDS-PAGE) at different polyacrylamide concentrations; at 35 mA during 1 h. Proteins were then transferred to a nitrocellulose membrane (Whatman Schleicher&Schuell; Dassel, Germany) during 1.5 hours at 90 V and at 4ºC to avoid excessive warming. Nitrocellulose membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) solution plus 5% bovine serum albumin and 5% skimmed milk during 1h at room temperature. Membranes were washed twice in TBS-T and blotted overnight at 4ºC with the following primary antibodies (Table 3):

Antigen	Molecular weight	Host specie	Dilution	Source
Catalase	60 KDa	Rabbit	1:6,000	Millipore (Massachusetts, CA, USA)
4EBP1	15-20 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
4EBP1 (pThr37/46)	15-20 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
Exportin-1	112 KDa	Mouse	1:1000	BD Biosciences (Franklin Lakes, NJ, USA)
Huntingtin (181-810 aa)	350 KDa	Mouse	1:1000	Millipore (Massachusetts, CA, USA)
Huntingtin (EM48)	over 350 KDa	Mouse	1:1000	Millipore (Massachusetts, CA, USA)
LC3B	14, 16 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
mTOR	289 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
mTOR (pSer2481)	289 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)

mTOR (pSer2448)	289 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
NBR1	150 KDa	Mouse	1:1000	Abnova (Taipei, Taiwan)
PKCα	80 KDa	Mouse	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
PKCα (pSer422)	80 KDa	Goat	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
PKCβII	80 KDa	Rabbit	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
PKCδ	78 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
PKCδ	78 KDa	Mouse	1:1000	BD Biosciences (Franklin Lakes, NJ, USA)
PKCδ (pThr505)	78 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
p62	62 KDa	Guinea Pig	1:1000	Progen (Heidelberg, Germany)
Raptor	150 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
Rictor	200 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
S6K1 (pSer371)	70-85 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
S6 Ribosomal Protein (pSer235/236)	32 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
SGK	48 KDa	Mouse	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
SGK (pSer657)	48 KDa	Goat	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
TFEB	53 KDa	Goat	1:1000	Abcam (Cambridge, UK)
ULK1	140 KDa	Rabbit	1:1000	Abcam (Cambridge, UK)
ULK1 (pSer757)	140 KDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)

Table 3. Primary antibodies for western blot. A list of primary antibodies is provided, as well as their source and the dilution that was used for western blot.

After primary antibody incubation, membranes were washed three times for 10 min with TBS-T and incubated for 1h at room temperature with the appropriated horseradish peroxidase-conjugated secondary antibody (Table 4).

Secondary antibody	Dilution	Source
Guinea Pig IgG	1:2,000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Donkey Anti-Goat IgG	1:2,000	Promega (Madison, WI, USA)
Anti-Mouse IgG	1:2,000	Promega (Madison, WI, USA)
Anti-Rabbit IgG	1:2,000	Promega (Madison, WI, USA)

Table 4. Secondary antibodies for western blot. A list of secondary antibodies is provided, as well as their source and the dilution that was used for western blot. All antibodies are conjugated to the horseradish peroxidase.

Membranes were washed again three times for 10 min to remove secondary antibody remains and the reaction was finally visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blot replicates were scanned and densitometries were quantified using the GelPro analyzer program version 4.0.

For protein loading control, membranes were incubated 15 min with an antibody against α-tubulin or α-actin. Anti-Lamin B and NeuN antibodies were used for loading control in nuclear fractions (Table 5).

Antigen	Molecular weight	Host specie	Dilution	Source
α-Actin	45 KDa	Mouse	1:20,000	MP Biomedicals (Aurora, OH, USA)
Lamin B	67 KDa	Goat	1:500	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
NeuN	46-48, 66 KDa	Mouse	1:1000	Millipore (Massachusetts, CA, USA)
α-Tubulin	55 KDa	Mouse	1:50,000	Sigma Chemical Co (St. Louis, MO, USA)

Table 5. Primary antibodies used for loading controls in western blot. A list of primary antibodies is provided, as well as their source and the dilution that was used for western blot. All these antibodies were used to detect loading control proteins.

9 - GENE EXPRESSION ASSAY

Total RNA was extracted from fresh striata and cortex of 8-, 12- and 30-week-old wild-type and R6/1 mice using the Nucleospin RNA II kit from Macherey-Nagel (Düren, Germany) following the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA with random primers by using the StrataScript First Strand cDNA Synthesis System (Stratagene, La Jolla, CA). To provide negative controls and exclude contamination by genomic DNA, we omitted the reverse transcriptase in the negative control condition.

cDNA was then analyzed by Q-PCR using the TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA, USA) or PrimeTime Mini qPCR Assays from Integrated DNA Technologies (Coralville, IA, USA. Q-PCR was performed in a 25 µl reaction buffer containing 12.5 µl Brilliant Q-PCR Master Mix, 1.25 µl TaqMan Gene Expression Assays and 10 ng of cDNA (Table 6). Both control and experimental reactions were placed in a thermal-cycler to undergo the following thermal-cycling program: 40 cycles of a two-step PCR; 95°C for 30 s and 60°C for 1 min, after initial denaturing at 95°C for 10 min. PrimeTime Mini qPCRs were performed in a 12.5 µl reaction buffer containing 6.25 µl Mater Mix from Takara Bio Inc. (Shiga, Japan) and 0.625 µl PrimeTime Mini qPCR Assays and 5 ng of cDNA (Table 6).

Gene	Assay	Source
18S	Hs99999901_s1	Applied Biosystems (Foster City, CA, USA)
18S	PrimeTime Std qPCR Assay	Integrated DNA Technologies (Coralville, IA, USA)
NBR1	Mm.PT.45.6651111	Integrated DNA Technologies (Coralville, IA, USA)
p62/SQSTM1	Mm00448091_m1	Applied Biosystems (Foster City, CA, USA)
PKCα	Mm00440852_m1	Applied Biosystems (Foster City, CA, USA)
PKCδ	Mm00440891_m1	Applied Biosystems (Foster City, CA, USA)

Table 6. Gene expression probes. A list of probes, together with their source, is provided.

Thermal-cycling program was slightly different from the TaqMan Gene Expression Assays and was the following: 40 cycles of a two-step PCR; 95°C for 5 s and 60°C for 20 s, after initial denaturing at 95°C for 30 s. All Q-PCR assays were performed in

duplicate and samples were subjected to the PCR reaction in the same way for each TaqMan Gene Expression.

Data were analyzed and quantified using the Comparative Quantitation Analysis program of the MxProTM Q-PCR analysis software version 3.0 (Stratagene) with the 18S gene expression as internal loading control. Results were normalized to cDNAs of wild-type mouse striata and expressed as a percentage of these data.

10 - MORPHOLOGICAL ANALYSIS

10.1 - Immunocytofluorescence

Cells were grown on cover glasses and fixed in 4% paraformaldehyde in PBS for 10 min after different treatments. Cells were incubated with 0.2 M glycine during 20 min to block paraformaldehyde. After that, cells were permeated in PBS with 1% bovine serum albumin and 0.1% saponin for 10 min. Blocking was performed with 1% bovine serum albumin in PBS for 1 hour before incubating specimens overnight at 4ºC with the primary antibody diluted in the same blocking buffer (Table 7). Remaining primary antibody was removed in three consecutive washes with PBS and specimens were incubated with the subtype-specific fluorescent secondary antibody (Table 8). To stain nuclei, cells were incubated during 5 min at room temperature with Hoechst 33258 (1:10,000; Invitrogen, Carlsbad, CA, USA) and washed afterwards with PBS. Finally cover glasses were mounted with Mowiol-mounting media (Merck, Darmstadt, Germany).

STHdh$^{Q7/Q7}$ cells were transfected with the tf-LC3 were fixed as described above and nuclei were stained with Hoechst 33258. STHdh$^{Q7/Q7}$ cells transfected with CFP-16Q, CFP-94Q, HA, and HA-PKCδ, were fixed 72h after transfection. Immunocytofluorescence was performed against HA and nuclei were stained with Hoechst 33258. All cover glasses were mounted with Mowiol-mounting media (Merck, Darmstadt, Germany).

10.2 - Immunohistofluorescence

Animals were deeply anesthetized with pentobarbital (60 mg/kg) and intracardially perfused with a 4% paraformaldehyde in 0.1mM phosphate buffer. Brains were

removed and post-fixed for 2h in the same solution, cryoprotected with 30% sucrose in PBS with 0.02% sodium azide and frozen in dry-ice cooled isopentane. Serial coronal cryostate 30 µm-thick sections were collected in PBS as free-floating sections and processed for immunohistofluorescence.

Antigen	Host specie	Dilution	Source
poly-Glutamine (clone 5TF1-1C2)	Mouse	1:10,000	Millipore (Massachusetts, CA, USA)
Huntingtin (EM48)	Mouse	1:150	Millipore (Massachusetts, CA, USA)
mTOR	Rabbit	1:100	Cell Signaling (Beverly, MA, USA)
pSer2481 mTOR	Rabbit	1:100	Cell Signaling (Beverly, MA, USA)
pSer2448 mTOR	Rabbit	1:100	Cell Signaling (Beverly, MA, USA)
NBR1	Mouse	1:100	Abnova (Taipei, Taiwan)
p62	Guinea Pig	1:100	Progen (Heidelberg, Germany)
p62	Mouse	1:500	BD Biosciences (Franklin Lakes, NJ, USA)
PKCδ	Mouse	1:500	BD Biosciences (Franklin Lakes, NJ, USA)
Ubiquitin	Rabbit	1:400	DAKO (Glostrup, Denmark)
HA	Rabbit	1:100	Sigma Chemical Co, (St. Louis, MO, USA)

Table 7. Primary antibodies for immunofluorescence and immunohistochemistry. A list of primary antibodies is provided, as well as their source and the dilution that was used for immunofluoresce and immunohistochemistry.

Sections were washed twice with PBS and incubated with NH_4Cl 50 mM during 30 min to block free fixation-remaining aldehydes and stop aldehyde-induced fluorescence. Tissue was permeated along 20 min by treatment with PBS containing 0.5% Triton X-100 and blocked during 1h at room temperature in PBS plus 0.2% bovine serum albumin, 0.2% lysine, 0.2% glycine, 0.2% sodium azide, 0.5% Triton X-100 and 5% normal horse serum (Pierce Biotechnology, Rockford, IL, USA). Slices were incubated overnight at 4°C with the corresponding primary antibodies in a buffer containing PBS plus 0.3% Triton X-100, 0.2% bovine serum albumin and 0.2% sodium azide (Table 7). After primary antibody incubation, slices were washed in PBS twice for 10 min and incubated 2h at room temperature with subtype-specific fluorescent

secondary antibodies (Table 8). After two consecutive 10 min-washes, slices were incubated 10 min at room temperature with Hoechst 33258 (1:4000; Invitrogen, Carlsbad, CA, USA) for nuclear staining and washed again twice with PBS before being mounted with Mowiol (Merck, Darmstadt, Germany) on silane-coated slides. No signal was detected in controls incubated in the absence of the primary antibody.

The protocol used for mTOR and phospho-mTOR immunohistofluorescence stains was slightly different. Washes were performed in TBS and slices were blocked in TBS containing 0.3% Triton X-100 and 5% normal goat serum (Pierce Biotechnology, Rockford, IL, USA). Primary and secondary antibodies were diluted in the same buffer and Hoechst 33258 was prepared in TBS. Incubation times were the same as the standard protocol already described.

Antibody	Dilution	Source
DAKO Envision System HRP Labelled Polymer anti-mouse	Ready-to-use system	DAKO (Glostrup, Denmark)
Alexa Fluor 647 Goat Anti-Mouse IgG (H+L)	1:500	Invitrogen (Carlsbad, CA, USA)
Alexa Fluor 647 Goat Anti-Rabbit IgG (H+L)	1:500	Invitrogen (Carlsbad, CA, USA)
Cy2 AffiniPure Goat Anti-Mouse IgG (H+L)	1:200	Jackson ImmunoResearch (West Grove, PA, USA)
Cy3 AffiniPure F(ab')2 Fragment Goat Anti-Rabbit IgG, F(ab')2 Fragment Specific	1:200	Jackson ImmunoResearch (West Grove, PA, USA)
Cy3 AffiniPure F(ab')2 Fragment Rabbit Anti-Mouse IgG, F(ab')2 Fragment Specific	1:200	Jackson ImmunoResearch (West Grove, PA, USA)
DyLight 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	1.200	Jackson ImmunoResearch (West Grove, PA, USA)
DyLight 488 AffiniPure Goat Anti-Guinea Pig IgG (H+L)	1:200	Jackson ImmunoResearch (West Grove, PA, USA)
DyLight 549 AffiniPure F(ab')2 Fragment Goat Anti-Guinea Pig IgG	1.200	Jackson ImmunoResearch (West Grove, PA, USA)

Table 8. Secondary antibodies for immunofluorescence and immunohistochemistry. A list of secondary antibodies is provided, as well as their source and the dilution that was used for immunofluoresce and immunohistochemistry.

10.3 - Immunohistochemistry

Five µm-thick paraffin-embedded sections were obtained from frontal cortex and caudate nucleus of human post-mortem brains and mounted on slides. To deparaffinize and rehydrate the tissue, slides were heated at 56ºC for 1h and washed sequentially with xylene, absolute ethanol, 96% ethanol and milliQ H_2O. For antigen retrieval slides were then incubated in 98% formic acid (5 min) at room temperature, followed by 20 min incubation in 10 mM sodium citrate buffer at pH 6.0 at 95ºC. Slides were then incubated (15 min) at room temperature in a wash buffer from DAKO (Glostrup, Denmark) with 3% normal goat serum. Just after this blocking step, slides were washed three times in wash buffer and incubated 30 min with the primary antibody, diluted in DAKO Real TM antibody diluent (Glostrup, Denmark) (Table 7). Three washes were needed to remove the remaining antibody and slides were incubated 30 min with the DAKO Envision System HRP Labelled Polymer anti-mouse secondary antibody (Glostrup, Denmark) (Table 8).

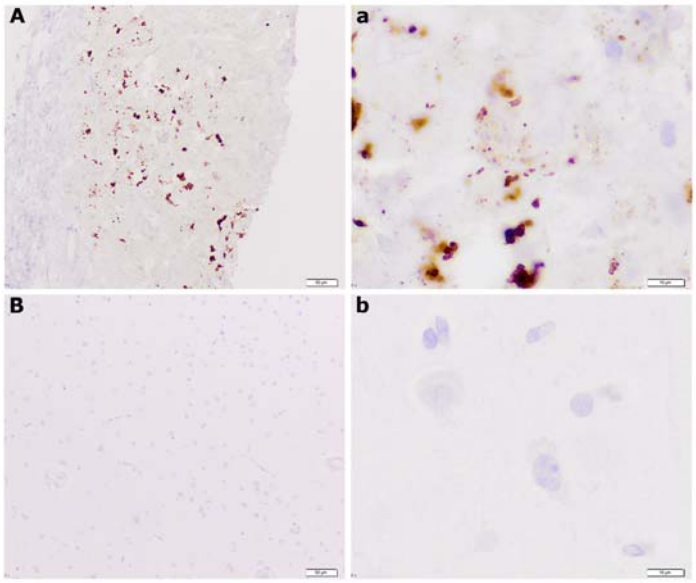

Figure 16. Control of NBR1 immunoreactivity. Images (A and a) illustrate human liver with abundant NBR1 immunoreactive Mallory bodies. Note the diffuse cytoplasmic granular staining pattern in a higher magnification (a). (B and b) No immunoreactivity is observed after omitting the primary antibody in human frontal cortex of a Huntington's disease case with pathological severity Vonsattel grade 3.

Finally, detection of immunostaining was performed using the Envision kit (DAKO, Glostrup, Denmark), diaminobenzidine (DAB) was used as chromogen and sections

were counterstained with hematoxilin for 1 min with the DAKO automation Hematoxylin Histologial Staining Reagent (Glostrup, Denmark).

To perform p62 immunohistochemistry, antigen retrieval was performed incubating slides directly in citrate buffer, avoiding treatment with formic acid.

A section of cirrhotic human liver tissue with abundant Mallory bodies was used as positive control for anti-NBR1 immunohistochemistry (Fig. 16A). The primary antibody was omitted in brain samples to evaluate staining specificity (Fig. 16B).

11 - IMAGE UPTAKE AND ANALYSIS

Stained tissue sections and cells were examined by using an Olympus BX60 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with an Orca-ER cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) or Leica TCS SP5 laser scanning spectral confocal microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany) with Argon and HeNe lasers attached to a Leica DMI6000 inverted microscope. Confocal images were taken using a HCX PL APO lambda blue 63.0x 1.40 OIL UV objective with a standard (one Airy disk) pinhole.

Images for the tf-LC3 experiment were obtained by using the confocal microscope, with a 63x objective with a 2.0 digital zoom and standard (one Airy disk) pinhole. For each cell, the entire three-dimensional stack of images from the ventral surface to the top of the cell was obtained by using the Z drive in the Leica TCS SP5 microscope. The size of the optical image was 0.5 µm. Co-localization was measured by using the 'colocalization' plug-in of the freeware ImageJ v1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA). Briefly, for each cell stack, the cell area was delineated and the total number of double-positive pixels for mRFP and GFP for each cell was summed. This value was divided by the number of total positive pixels for mRFP in the stack, and multiplied by 100 (Kimura *et al.,* 2007).

Images of CFP-16Q or CFP-94Q and HA or HA-PKCδ double-transfected STHdh$^{7Q/7Q}$ cells, were obtained by using the Olympus BX60 epifluorescence microscope with the 40x objective. Only double-transfected cells were examined, and those with condensed or fragmented nuclei were considered apoptotic (Xifro *et al.,* 2011). The percentage of cell death was calculated by normalizing the amount of

condensed nuclei to the total nuclei examined. At least 200 cells were evaluated for each condition in each independent experiment.

12 - HUNTINGTIN IMMUNOPRECIPITATION

Striatal and cortical tissue were sonicated in immunoprecipitation buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL, 2 mM PMSF, 2.5 mM NaF, 1 mM $NaVO_4$ and 1:1000 protease inhibitor cocktail and centrifuged at 16,100 x g for 20 min at 4ºC. Supernatant protein-concentration was determined and 300 µg protein was incubated overnight at 4ºC on a rotary mixer with anti-huntingtin antibody (MAB2166 or EM48, 1 µg; both from Millipore, Massachusetts, CA, USA) in immunoprecipitation buffer. The immune complexes were precipitated overnight at 4ºC with the addition of 5% protein A-Sepharose Cl-4B (Sigma Chemical Co, St. Louis, MO, USA). Beads were collected by centrifugation, and washed sequentially with immunoprecipitation buffer, immunoprecipitation buffer-PBS (1:1) and PBS. Finally the immune complexes were denatured by 10 min-boiling in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue. The Immune complexes were finally resolved on a 6% SDS-PAGE, and western blot analyzes were carried out as described above. Negative controls were obtained by incubating protein lysates with AffiniPure Mouse Anti-Human IgG or AffiniPure Rabbit Anti-Mouse IgG from Jackson Laboratory (Bar Harbor, ME, USA).

13 - PULSE AND CHASE

This method was used to determine long-lived protein proteolysis, and it is based on the incorporation of a radiolabelled amino acid in the proteins synthesized during the labelling period (pulse), followed by tracking the released radiolabelled amino acids into the medium that are the labelled proteins that have undergone degradation (chase).

$STHdh^{Q7/Q7}$ cells were plated at an approximately 40% confluence in 12-well plates with serum-supplemented DMEM medium. Pulse was performed as soon as cells reached 60-70% confluence with 2 µCi/ml per well of [2,3,4-H3]-L-valine (1 mCi/ml; American Radiolabeled Chemicals, St. Louis, MO, USA). Pulse was 48h long to maximize long-lived proteins labelling, and was performed at 33ºC, the same

temperature at which cells normally grow. After 48 h, pulse medium was washed twice with 1x HBSS (Gibco, Paisley, UK) and replaced by chase medium, containing serum-supplemented DMEM with 2.8 mM non-radioactive valine. Cells were again incubated at 33ºC and chase-aliquots were taken at different time-points (0, 6, 18, 24 h).

Chase-aliquots contain non-degraded proteins as well as small peptides and amino acids. To discriminate between degraded protein radioactivity (peptides and small amino acids) and non-degraded protein radioactivity, 20% trichloroacetic acid was added to chase-aliquots to a final concentration of 10%. Precipitation in trichloroacetic acid was facilitated by addition of bovine serum albumin at a final concentration of 0.5 mg/ml. Samples were placed at 4ºC for at least 1h and then centrifuged 10 min at maximum speed. Supernatants were transferred into individual scintillation liquid vials with 5 ml scintillation liquid. Counts per minute (CPM) were measured during 3 min in a Wallac 1409 liquid scintillation counter (Wallac Oy, Turku, Finland).

To calculate the total amount of radioactivity incorporated by the cells during the labelling time, cells were washed twice with Hanks's solution and solubilised at 33ºC during 2h in 2 ml of buffer containing 0.1 N NaOH and 0.1% sodium deoxycholate, after taking the last chase-aliquot. Once cells were dissolved, 15 µl of 1 M HCl were added to neutralize the lysate. The whole amount of lysate was transferred to scintillation liquid vials with 5 ml scintillation liquid and counted together with the chase-aliquots.

Proteolysis was considered as the amount of radioactivity incorporated by the cells during the labelling time transformed in acid soluble radioactivity (peptides and amino acids) at each time-point during the incubation time. It was calculated as the acid soluble radioactivity divided by the total amount of radioactivity (acid soluble radioactivity plus radioactivity incorporated by the cells during the labelling time) and multiplied by 100 (Martinez-Vicente *et al.,* 2010).

Pulse and chase was performed equally and at the same time for control and Leptomycin B-treated cells. Leptomycin B treatment was first added to the pulse medium 6h prior to the chase, to reach the time point 0 of the chase with exportin-1 already inhibited. Cells were exposed to Leptomycin B treatment during all the rest of the experiment time, since it was again added when pulse medium was replaced for chase medium.

Moreover, the experiment with or without Leptomycin B was also performed in cells grown in serum-deprived medium and in cells grown in complete medium with NH_4Cl and leupeptin, conditions in which protein degradation is enhanced or

repressed, respectively. Serum deprivation and NH_4Cl and leupeptin treatments started at the same time of the chase.

14 - STATISTICAL ANALYSIS

All the results were expressed as the mean ± SEM. Statistical analysis was performed using the Student's t test or the one- or two-way ANOVA, followed by Bonferroni's post hoc test as appropriate and indicated in the figure legends. A 95% confidence interval was used and values of $p < 0.05$ were considered as statistically significant.

IV. RESULTS

1 - SELECTIVE AUTOPHAGY REGULATION ALONG THE PROGRESSION OF HUNTINGTON'S DISEASE

Huntington's disease is characterized by the formation of protein aggregates, which can be degraded by selective autophagy. Autophagic response has been found increased upon mutant huntingtin exposure, but has been mainly studied *in vitro*. We wondered here whether selective autophagy is altered by mutant huntingtin expression *in vivo*, in brain regions that degenerate in Huntington's disease, by analyzing the R6/1 mouse brain. To do so, we used two autophagy cargo receptors, p62 and NBR1, as autophagic activity reporters. We examined the protein levels and intracellular distribution of these two cargo receptors in the striatum, cortex and hippocampus of R6/1 mice along the progression of the disease. Their intracellular distribution was also analyzed in human Huntington's disease caudate nucleus and frontal cortex.

1.1 - p62 AND NBR1 PROTEIN LEVELS IN THE STRIATUM, CORTEX AND HIPPOCAMPUS ALONG THE PROGRESSION OF THE DISEASE

1.1.1 - p62 and NBR1 protein levels are deregulated in the R6/1 mouse brain

Protein levels of p62 and NBR1 were analyzed by western blot in the striatum, cortex and hippocampus of R6/1 mice at different ages. Protein levels of p62 and NBR1 were similarly deregulated along the progression of the disease, but differed between brain regions. Compared to wild-type mice striatum, both p62 and NBR1 protein levels were decreased at 12 weeks of age (Fig. 17A and B), whereas at older ages p62 was increased (Fig. 17A) and NBR1 protein levels were not changed (Fig. 17B). In the R6/1 mice cortex, p62 and NBR1 protein levels were significantly reduced from 12 and 8 weeks of age onwards, respectively (Fig. 17C and D), with a higher reduction of p62 (by about 50% at both ages; Fig. 17C) than of NBR1 (by about 30% at all the ages analyzed; Fig. 17D). In the R6/1 hippocampus, both p62 and NBR1 protein levels decreased at 12 weeks of age whereas both were up-regulated at 30 weeks of age (Fig. 17E and F).

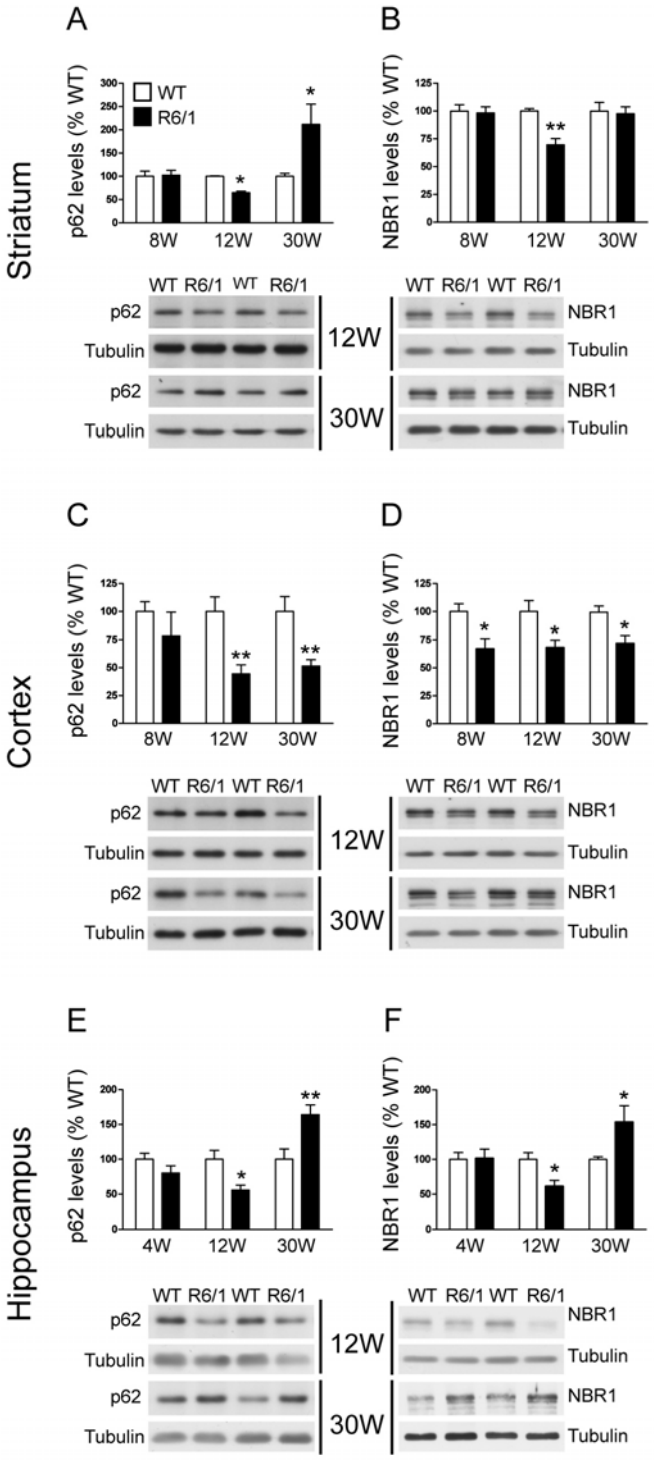

Fig. 17. Regulation of p62 and NBR1 in R6/1 mice brain regions during the progression of the disease. (A, C, E) Protein levels of p62 and (B, D, F) NBR1 were analyzed by western blot in protein extracts obtained from (A, B) striatum, (C, D) cortex and (E, F) hippocampus of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. Representative immunoblots show the protein levels of p62, NBR1 and α-tubulin in WT and R6/1 mice at different ages. The graphs show p62 and NBR1 protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (p62 or NBR1/α-tubulin ratio), and shown as mean ± s.e.m (n = 4-7). Data were analyzed by Student's t-test. * P< 0.05 and ** P< 0.01 as compared with WT mice.

In summary, both proteins were similarly deregulated in the R6/1 mouse, being p62 and NBR1 protein levels reduced early in the disease in the three brain regions examined, meanwhile they accumulated in the striatum and hippocampus, but not in the cortex, at late stages of the disease.

1.1.2 - p62, but not NBR1, gene expression is enhanced in the R6/1 mouse brain

To check whether changes at the protein level could be due to transcriptional deregulation, p62 and NBR1 mRNA levels were analyzed by Q-PCR in both striatal and cortical samples from 8-, 12- and 30-week-old R6/1 mice. When compared to wild-type levels, R6/1 mice striatum and cortex displayed enhanced p62 gene expression at all ages analyzed (Fig. 18A and B) whereas NBR1 mRNA levels were unchanged (Fig. 18C and D).

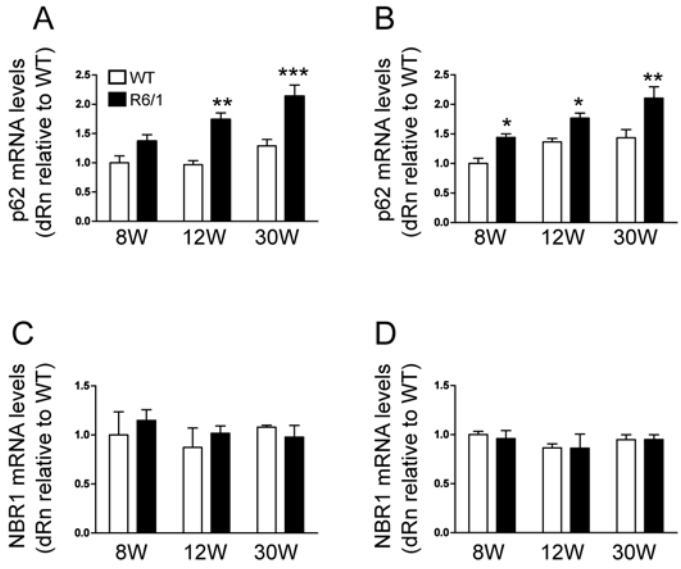

Figure 18. Regulation of p62 and NBR1 mRNA levels in R6/1 mice striatum and cortex at different stages of the disease. The graphs show (A, B) p62 and (C, D) NBR1 mRNA levels analyzed by Q-PCR in the (A, C) striatum and (B, D) cortex of 8-, 12- and 30-week-old (W) wild-type (WT) and R6/1 mice. Results were normalized to the 18S gene expression levels and are expressed as percentage of 8-week-old WT values. Data are the mean ± s.e.m (n = 4-7) and were analyzed by Student's t-test. * P< 0.05, ** P< 0.01 and *** P< 0.001 as compared with WT mice.

The transcription factor EB (TFEB) has been recently described as an important regulator of autophagy by driving the expression of both autophagic and lysosomal genes (Settembre *et al.,* 2011). Upon ERK-2 dependent phosphorylation, TFEB

translocates from the cytosol to the nucleus to regulate gene expression (Settembre *et al.,* 2011). The p62 gene is among its targets (Settembre *et al.,* 2011). Thus, we analyzed whether TFEB protein levels were altered in 30-week-old R6/1 brain. We detected increased TFEB protein levels in both striatum (Fig. 19A) and cortex (Fig. 19B) from 30-week-old R6/1 mice in comparison to wild-type mice. In addition, TFEB protein levels were increased in cortical nuclear enriched fractions of 30-week-old R6/1 mice when compared to those in wild-type mice (Fig. 19C).

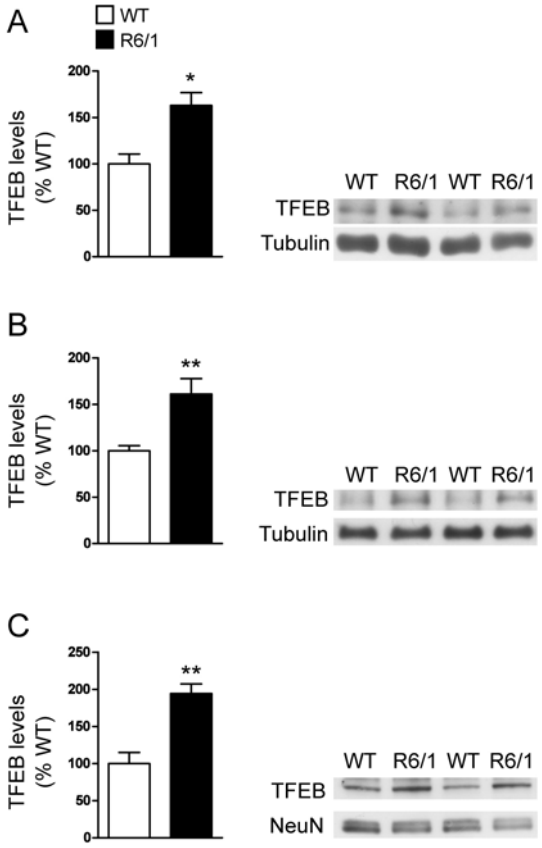

Figure 19. Transcription factor TFEB protein levels are increased in the striatum and cortex of 30-week-old R6/1 mice. Protein levels of TFEB were analyzed by western blot in protein extracts obtained from (A) striatum and (B) cortex. Moreover, TFEB protein levels were also analyzed in (C) nuclear-enriched fractions of 30-week-old mice cortex. Values obtained by densitometric analysis of western blot data and normalized to α-tubulin (for total extracts) or NeuN (for the nuclear-enriched extracts), are expressed as percentage of WT values. Data are the mean ± s.e.m (n= 3-6), and were analyzed by using the Student's t-test. * P< 0.05, and ** P< 0.01 as compared with WT mice.

These results show a transcriptional deregulation of p62, but not of NBR1, that could be in part due to an increase in the TFEB transcription factor protein levels and to its nuclear localization. In addition, they also indicate that the brain region-dependent

deregulation of p62 and NBR1 observed at a protein level is not triggered by means of gene expression.

1.1.3 - p62 and NBR1 interact with mutant, but not with wild-type, huntingtin

The interaction of p62 and NBR1 with mutant huntingtin might hamper their detection by western blot. Thus, we analyzed the interaction of p62 and NBR1 with huntingtin in 30-week-old wild-type and R6/1 mice striatum by immunoprecipitation.

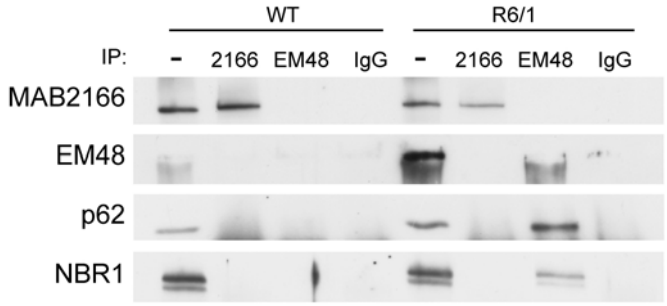

Figure 20. Both p62 and NBR1 interact with mutant huntingtin. Interaction of p62 and NBR1 with huntingtin was analyzed in protein extracts from the striatum of WT and R6/1 mice at 30 weeks of age by immunoprecipitation (IP). Wild-type huntingtin was immunoprecipitated with the 2166 antibody, mutant huntingtin with the EM48 antibody and mouse IgGs antibodies were used as control. Membranes were then subjected to immunoblotting with different antibodies as indicated in the representative immunoblot. – indicates protein extract without immunoprecipitation.

Both proteins co-immunoprecipitated together with mutant, but not with wild-type, huntingtin in R6/1 mice striatum (Fig. 20). To be sure that p62 and NBR1 sequestration into mutant huntingtin aggregates would not alter their detection by western blot (Landles *et al.,* 2010), we analyzed the stacking gels corresponding to samples of wild-type and R6/1 mice striatum at different ages. We only detected mutant huntingtin in the stacking gels corresponding to samples of 30-week-old R6/1 mice striatum (Fig. 21A). Thus, we analyzed p62 and NBR1 in the same conditions. Neither p62 nor NBR1 were detected in the stacking gels (Fig. 21B), suggesting a non-covalent interaction between these proteins and ubiquitinated- mutant huntingtin, as has been previously shown for the interaction between p62 and ubiquitin (Vadlamudi *et al.,* 1996).

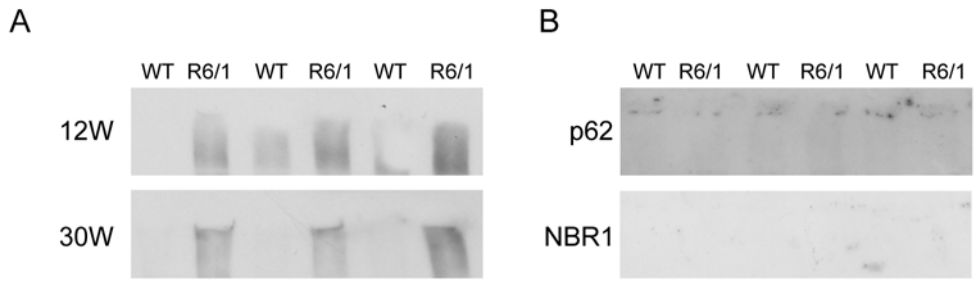

Figure 21. p62 and NBR1 are not retained in the stacking gels. (A) The presence of mutant huntingtin was analyzed by western blot by using EM48 antibody in stacking gels corresponding to samples obtained from 12- and 30-week-old wild-type (WT) and R6/1 mice striatum. (B) The presence of p62 and NBR1 was also analyzed by western blot in stacking gels corresponding to samples obtained from WT and R6/1 mice striatum at 30 weeks of age.

Thus, both p62 and NBR1 interact with mutant huntingtin, but this interaction does not interfere in their detection in the western blot.

1.1.4 - The peroxisome marker catalase is deregulated in the R6/1 mouse brain

All these results suggested that differences in p62 and NBR1 protein levels could be due to alterations in selective autophagic activity. The pathway involving ubiquitin and p62 is used by mammalian cells for targeting diverse types of substrates for autophagic degradation. Peroxisomes get monoubiquitinated and recognized by p62 to get degraded by selective autophagy (Kim *et al.,* 2008). Thus, we wondered if at late stages of the disease, were we found distinct p62 and NBR1 protein levels within striatal and cortical tissue, we would find also differences in the degradation efficiency of this organelle. To do that, we analyzed by western blot the levels of the peroxisome marker catalase in 30 week-old mice cortex and striatum. In good correlation with p62 and NBR1 protein levels, we detected unchanged and decreased protein levels of catalase in striatum (Fig. 22A) and cortex (Fig. 22B), respectively.

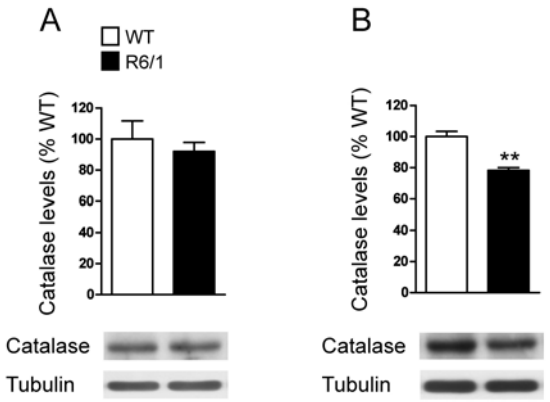

Figure 22. Catalase levels are decreased in the cortex, but not in the striatum, of 30-week-old R6/1 mice. Catalase protein levels were analyzed by western blot in the (A) striatum and (B) cortex of wild-type (WT) and R6/1 mice at 30 weeks of age. Representative immunoblots show catalase and α-tubulin in 30-week-old WT and R6/1 mice. The graphs show catalase protein levels in R6/1 mice with respect to their littermate controls. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (catalase/α-tubulin ratio), and shown as mean ± s.e.m. (n = 6). Data were analyzed by Student's t-test. ** P< 0.01 as compared with WT mice.

1.2 - p62 AND NBR1 INTRACELLULAR LOCALIZATION IN CELLS EXPRESSING MUTANT HUNTINGTIN

1.2.1 - p62 is increased in the nuclear fraction of R6/1 mice brain

The autophagic proteins, p62, Alfy, Beclin-1 and diabetes- and obesity-regulated gene shuttle between the cytoplasm and the nucleus (Knaevelsrud & Simonsen, 2010). However, autophagy is restricted to the cytoplasm since the nucleus is lack of membrane-limited proteolytic organelles. Thus, we wondered if the previously observed alterations in whole amount of p62 protein (Fig. 17) could be restricted to any of these two compartments. We analyzed by western blot p62 protein levels in cytoplasmic and nuclear enriched fractions obtained from wild-type and R6/1 mice striatum, cortex and hippocampus at different stages of the disease. At 12 weeks of age, p62 protein levels were decreased in cytoplasmic enriched fractions and unchanged in nuclear enriched fractions with respect to wild-type values (Fig. 23A). Interestingly, in 30-week-old R6/1 mice striatum, we detected increased p62 protein levels in nuclear enriched fractions, whereas its levels were reduced in cytoplasmic enriched fractions (Fig. 23B). A similar regulation of p62 protein levels was observed in nuclear and cytoplasmic enriched fractions from 30-week-old R6/1 cortex (Fig. 23C) and hippocampus (Fig. 23D), although the increase in cortical nuclear p62 levels was much lower than in the other

regions (striatum: 406 ± 62%; hippocampus: 403 ± 25%; cortex: 191 ± 45% with respect to wild-type values).

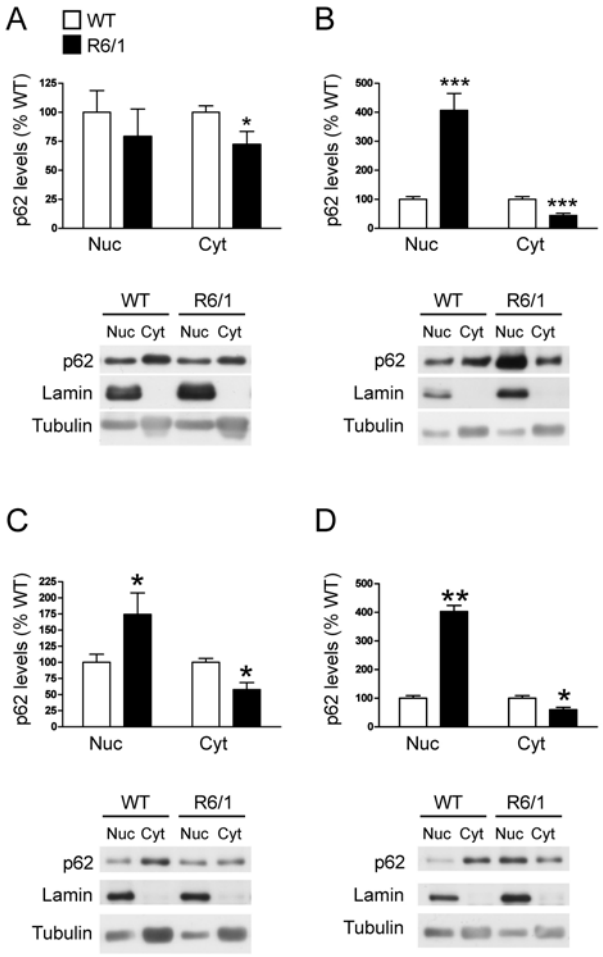

Figure 23. p62 is localized in the nucleus of R6/1 striatal, cortical and hippocampal cells at late stages of the disease. Protein levels of p62 were analyzed by western blot in nuclear (Nuc) and cytoplasmic (Cyt) fractions prepared from the striatum of wild-type (WT) and R6/1 mice at (A) 12 and (B) 30 weeks of age. Nuclear and cytoplasmic p62 protein levels were also analyzed in 30-week-old WT and R6/1 (C) cortex and (D) hippocampus. Representative immunoblots show protein levels of p62 in cytoplasmic (loading control α-tubulin) and nuclear (loading control lamin) fractions from wild-type and R6/1 mice. Values, obtained by densitometric analysis of western blot data and normalized to α-tubulin or lamin, are expressed as percentage of WT values. Data are the mean ± s.e.m. (n= 4-8), and were analyzed using the Student's t-test. * P< 0.05, ** P< 0.01 and *** P< 0.001 as compared with WT mice.

These results indicate that only at late stages of the disease p62 accumulates in the nucleus. Among the regions examined, striatum and hippocampus present the highest accumulation of nuclear p62.

1.2.2 - p62 localizes in mutant huntingtin nuclear inclusions

To further characterize p62 intracellular distribution in wild-type and R6/1 mice brain, we performed immunohistofluorescence stains on 30-week-old mice brain slices. In wild-type mice, p62 was mainly localized in the cytoplasm (Fig. 24A). In contrast, in R6/1 striatum and hippocampus, p62 was mainly detected in the nucleus where it colocalized with mutant huntingtin inclusions (Fig. 24B and C). In the cortex, p62 also colocalized with nuclear mutant huntingtin but to a lesser extent than in the striatum and hippocampus (Fig. 24C).

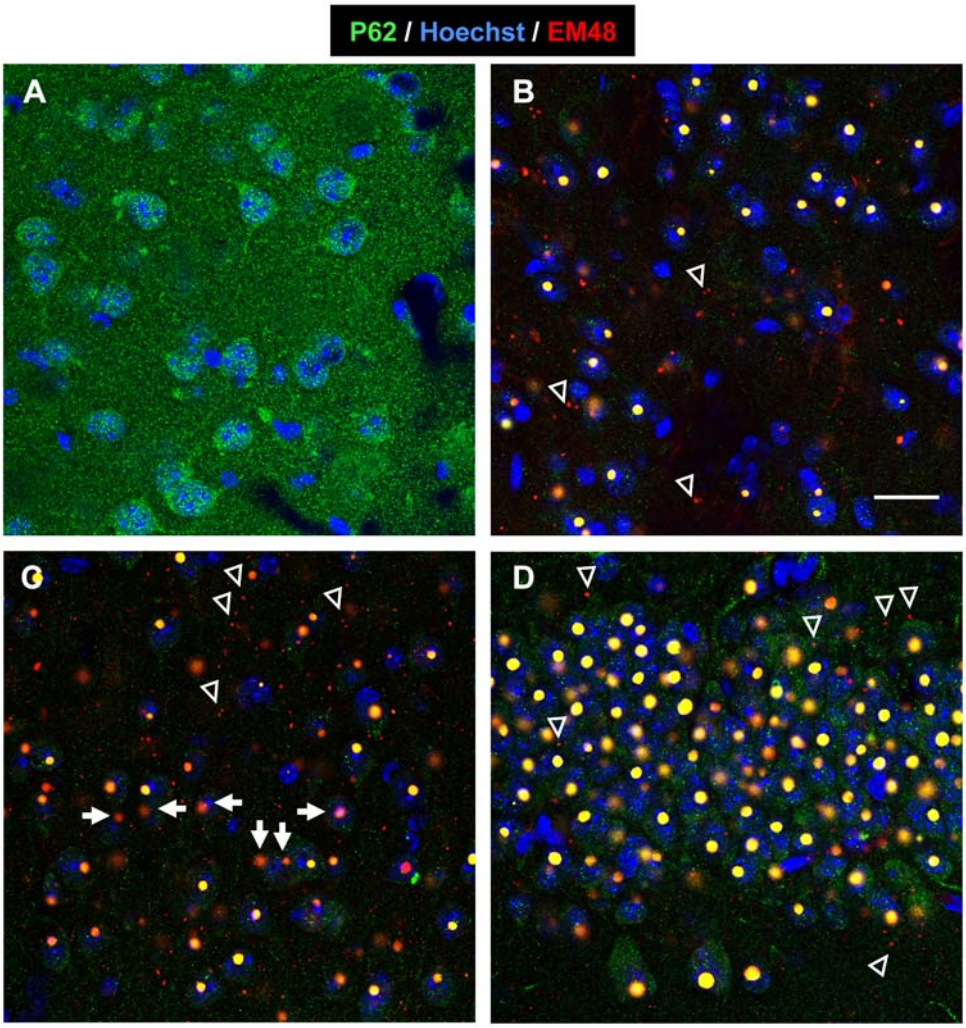

Figure 24. p62 immunostaining in wild-type and R6/1 mouse brain. p62 was analyzed by immunohistochemistry in the striatum of (A) wild-type and (C) R6/1 30-week-old mice. p62 distribution was also analyzed in 30-week-old R6/1 mice (B) cortex and (C) hippocampus. Nuclei were stained with Hoechst 33258 and mutant huntingtin with the EM48 antibody. Merging (yellow) illustrates colocalization of p62 and EM48 in the nuclear inclusions. In the striatum and hippocampus, almost all EM48-positive inclusions are also positive for p62 staining whereas in the cortex there are EM48-positive inclusions that do not colocalize with p62 (arrows). Most of the cytoplasmic mutant huntingtin aggregates do not colocalize with p62 staining (arrow heads).Scale bar 20 µm.

Next, we analyzed whether changes in p62 intracellular distribution observed in R6/1 mouse brain also occur in the brain of Huntington's disease patients.

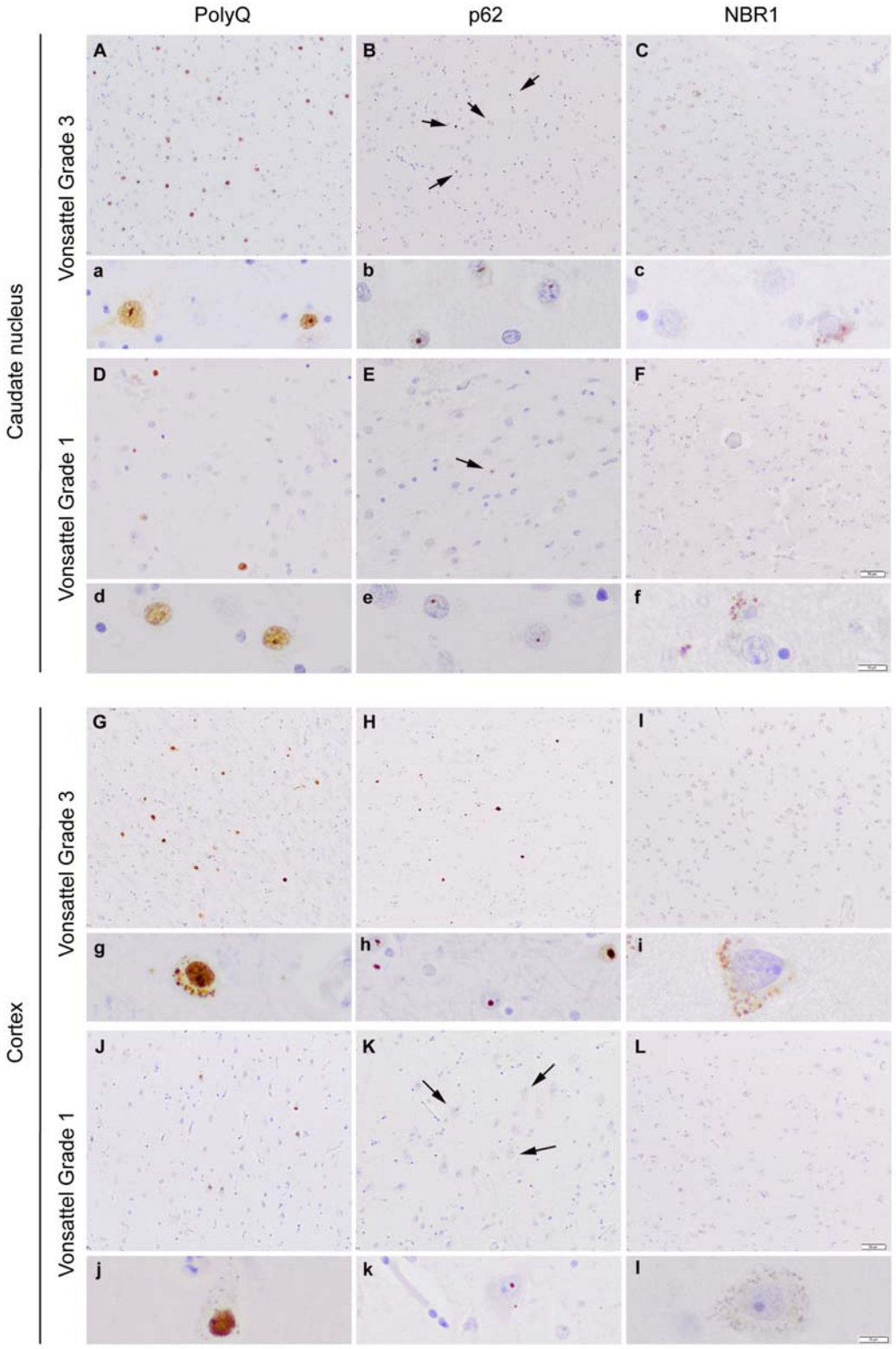

Figure 25. p62, but not NBR1, associates with mutant huntingtin nuclear inclusions in human Huntington's disease brains. PolyQ and p62 were analyzed by immunohistochemistry in the (A-F) caudate nucleus and (G-L) frontal

cortex of two Huntington's disease cases with different pathology: (A-C, G-I) case 1, Vonsattel grade 3, and (D-F, J-L) case 2, Vonsattel grade 1. Representative photomicrographs show (A, D, G, J) polyQ, (B, E, H, K) p62 and (C, F, I, L) NBR1 immunostaining in the caudate nucleus and frontal cortex of case 1 and case 2. (A) High amount of polyQ-immunoreactive nuclei are detected in the most preserved areas of caudate nucleus, which also show compact nuclear aggregates (a). (B, b) p62 immunoreactivity (arrows in B) is mainly observed in compact intranuclear inclusions in a lower density when compared to polyQ immunostaining. (D, d, E, e) Fewer polyQ and less p62-immunoreactive diffuse and compact nuclear inclusions are detected in medium spiny neurons of case 2 compared to case 1. (C, c, F, f) NBR1 immunohistochemistry shows a cytoplasmic granular pattern mainly in astrocytes, but not in neurons (note unstained lipofuscin granules). No nuclear inclusions are detected. (G) High and (J) moderate amount of polyQ-immunoreactive nuclei are detected in frontal cortex neurons, which show, in addition to compact nuclear inclusion bodies, abundant granular cytoplasmic aggregates (g, j). (H, h, K, k) p62 immunoreactivity is mainly observed in compact intranuclear inclusions in a lower density when compared to polyQ immunostaining (arrows in H). (I, i, L, l) NBR1 immunoreactivity shows a granular cytoplasmic staining pattern and no nuclear inclusion bodies are observed. Case 2 shows less polyQ- and p62-immunoreactive diffuse and compact nuclear inclusions and less NBR1 cytoplasmic granular immunoreactivity compared to case 1. Scale bars 50 mm for (A-L) and 10 mm for (a-l).

To this end, we analyzed by immunohistochemistry different brain regions of two representative Huntington's disease cases: (1) a 53 year old man with 45 CAG repeats and Vonsattel grade 3 severity of pathology, and (2) a 73 year old man with late onset disease, 40 CAG repeats and Vonsattel severity grade 1. In case 1, the maximal immunoreactivity was observed using the anti-polyQ-antibody (Fig. 25A and G), which showed a diffuse labelling of the nucleus of affected neurons, most of them harbouring smaller compact, rounded or elongated intranuclear inclusion bodies, varying in size and shape. In addition, a coarse granular cytoplasmic immunoreactivity was observed and few thin and thick neurites were present in the neuropil (Fig. 25A and G). Compact nuclear inclusions containing p62 were observed in all brain regions analyzed (Fig. 25B and H). NBR1 displayed a granular cytoplasmic staining pattern in frontal cortex, following in most, but not in all neurons, the distribution of lipofuscin granules (Fig. 25I). In the striatum, an irregular cytoplasmic granular pattern was mainly observed in astrocytes (Fig. 25C). In case 2, polyQ-immunoreactive nuclear and cytoplasmic inclusions were observed in the same anatomical areas as in case 1, but in a much lower density (Fig. 25D and J). A fraction of nuclear inclusions in the same brain areas was also stained with p62 (Fig. 25E and K), but not with NBR1, antibody (Fig. 25F and L).

p62 contains NES and NLS signals in its sequence that enables it to shuttle between the nucleus and the cytoplasm (Pankiv *et al.,* 2010). While nuclear import is usually mediated by importin-α, nuclear export is facilitated by exportin-1/CRM1 (Xpo-1) binding to the NES (Kutay & Guttinger, 2005). It has been recently shown that Xpo-1 decreases with age in whole brain lysates obtained from both wild-type and R6/2 mice (Chan *et al.,* 2011). Thus, we analyzed whether changes in Xpo-1 levels could

participate in the nuclear accumulation of p62 in R6/1 mice neurons at late stages of the disease.

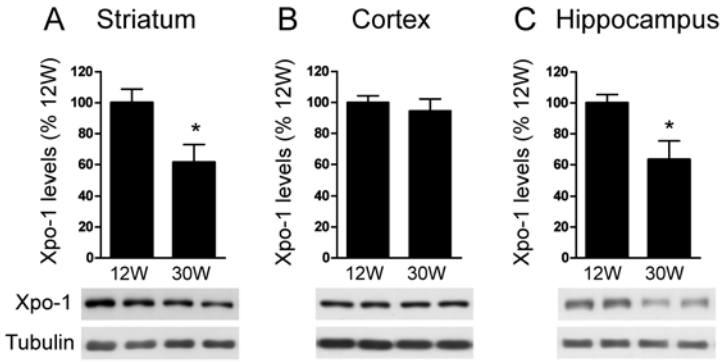

Figure 26. Xpo-1 is decreased with age in the striatum and hippocampus, but not in the cortex, of R6/1 mice. Protein levels of Xpo1 were analyzed by western blot in the (A) striatum, (B) cortex and (C) hippocampus of R6/1 mice at 12 and 30 weeks (W) of age. Values in the graphs (obtained by densitometric analysis of western blot data) are expressed as percentage of values obtained in 12-week-old R6/1 mice (Xpo1/α-tubulin ratio), and shown as mean ± s.e.m (n = 5-10). Representative immunoblots show protein levels of Xpo1 and α-tubulin in 12- and 30-week-old R6/1 mice (A) striatum, (B) cortex and (C) hippocampus. Data were analyzed by Student's t-test. * P< 0.05 as compared with 12-week-old R6/1 mice.

Western blot analysis revealed that Xpo-1 protein levels were similarly decreased with age by about 40% in the striatum and hippocampus of R6/1 mice (Fig. 26A and C). Interestingly, cortical Xpo-1 protein levels were not different between 12- and 30-week-old R6/1 mice (Fig. 26B).

1.3 - CHANGES IN XPO-1 PROTEIN LEVELS MAY CONTRIBUTE TO ALTERED AUTOPHAGY

Since Xpo-1 decreases with age in both wild-type and R6 mouse models (Chan *et al.,* 2011; present results) we wondered whether this reduction would affect autophagy by changing the localization of important proteins for the process. To simulate this situation, a striatal cell line was treated with LMB, an irreversible Xpo-1 inhibitor (Kudo *et al.,* 1999). To be sure of the LMB treatment efficiency we analyzed nuclear and cytoplasmic levels of p62 after 24h of treatment. We detected increased nuclear p62 levels in these conditions (Fig. 27).

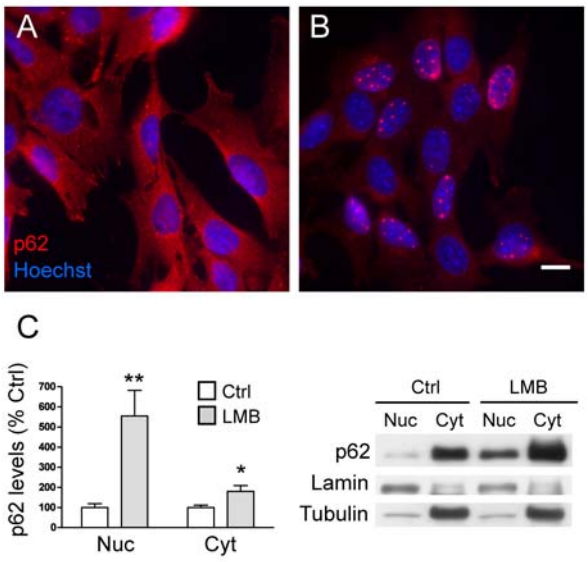

Figure 27. p62 is retained in the nucleus after LMB treatment. A striatal cell line (STHdh$^{Q7/Q7}$) was treated with LMB (10 nM) for 24h. Intracellular distribution of p62 was analyzed by immunocytochemistry in (A) control (Ctrl) and (B) LMB-treated cells. Scale bar 10 μm. (C) Protein levels of p62 were analyzed by western blot in nuclear (Nuc) and cytosolic (Cyt) fractions prepared from control (Ctrl) and LMB-treated cells. Graph shows the results obtained by densitometric analysis of western blot data (normalized to lamin for nuclear extracts and to α-tubulin for cytoplasmic extracts). Data are expressed as percentage of control (Ctrl) values. (C) A representative immunoblot shows protein levels of p62 in cytosolic (loading control α-tubulin) and nuclear (loading control lamin) fractions. Data are the mean ± s.e.m (n = 5-10), and were analyzed by the Student's t-test. * P< 0.05, and ** P< 0.01 as compared with values obtained in control (Ctrl) cells.

Interestingly, LMB treatment for 24h increased LC3II protein levels (Fig. 28A). However, LC3II levels only correlate with the number of autophagosomes within cells at a snapshot time, and this can result from either increased autophagosome formation or reduced autophagosome degradation (Rubinsztein *et al.,* 2009). To discern between these possibilities, we treated striatal cells with BafA1, a vacuolar-ATPase inhibitor, which impairs lysosomes and autophagosome degradation (Rubinsztein *et al*., 2009). This leaded to an accumulation of autophagosomal structures. As expected, BafA1 treatment enhanced LC3II levels (Fig. 28A). Interestingly, when we treated the cells with BafA1 together with LMB, LC3II levels increased to the same extent as BafA1 treatment alone (Fig. 28A). This result suggests that LMB inhibits autophagy at some point in a late step of the process, although not as efficiently as BafA1 does. To further analyze this hypothesis, we measured the rate of autophagosome degradation by transfecting striatal cells with a mRFP-GFP-LC3 (tfLC3) plasmid (Kimura *et al.,* 2007).

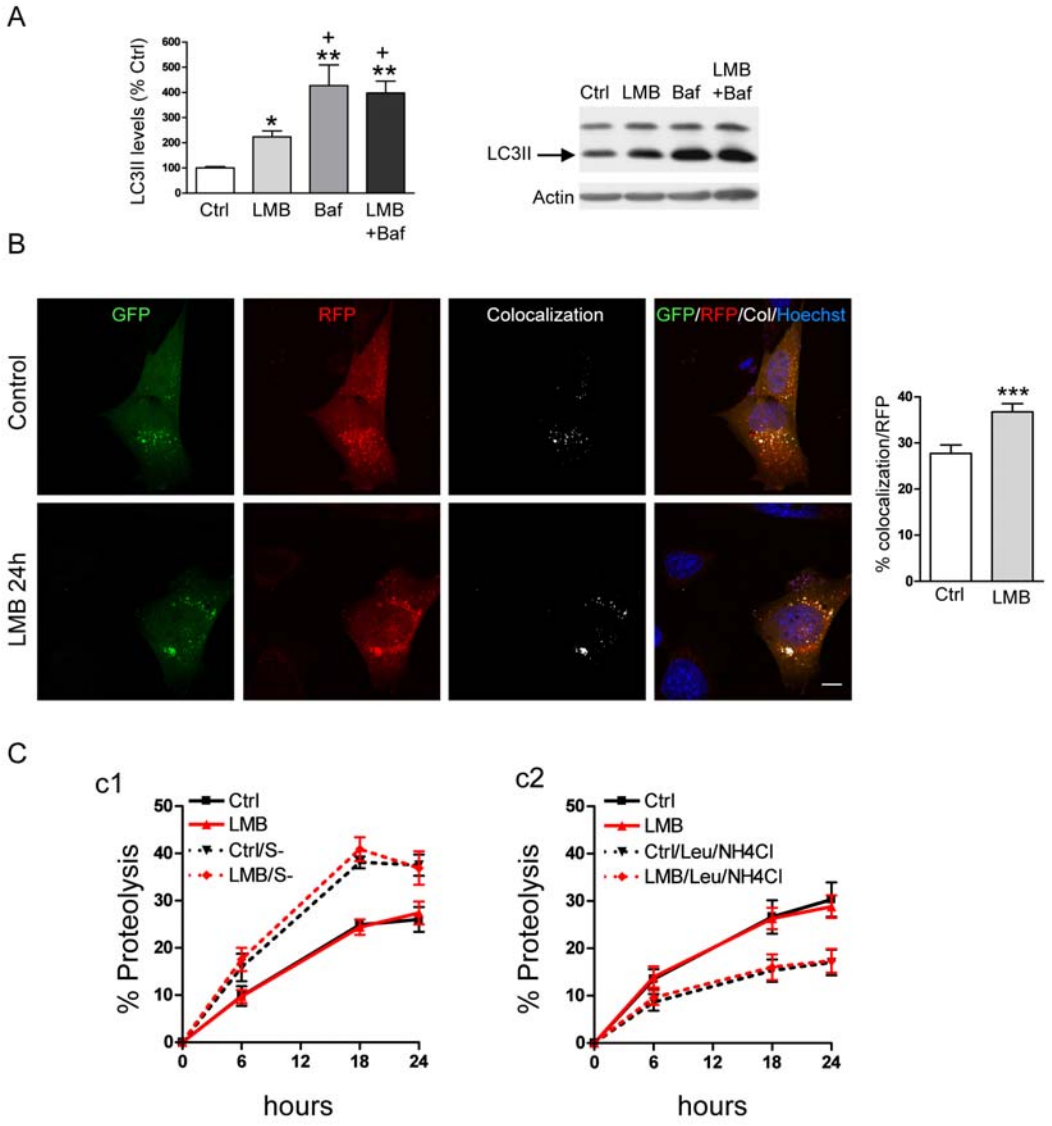

Figure 28. LMB treatment impairs autophagosome degradation but does not modify long-lived protein proteolysis. (A) Graph showing LC3II protein levels in STHdh$^{Q7/Q7}$ cells after LMB (10 nM; 24h), BafA1 (100 nM, 6h) and LMB+BafA1 treatment. Values, obtained by densitometric analysis of western blot data and normalized to α-actin, are expressed as percentage of control (Ctrl) and shown as mean ± s.e.m. (n = 4-5). Data were analyzed by one-way Anova followed by Bonferroni's post-hoc test. * P< 0.05, ** P< 0.01 as compared with control; + P< 0.05 as compared with LMB treatment. Immunoblot shows a representative experiment. (B) Representative images illustrating STHdh$^{Q7/Q7}$ transfected with mRFP-GFP-LC3 plasmid in control conditions and after LMB treatment. Graph shows the percentage of colocalization in control (Ctrl) and LMB-treated (LMB) cells. Data are represented as mean ± s.e.m. (n = 103 ± 3 cells from three independent experiments). Data were analyzed by Student's t test. *** P< 0.001 as compared with % of colocalization in control (Ctrl) cells. Scale bar, 10 μm. (C) Degradation of long-lived proteins was analyzed in confluent STHdh$^{Q7/Q7}$ cells that were labelled with [2,3,4-H3]L-valine (1 mCi/ml) for 48h at 33°C and then maintained in complete (10% FBS) medium with or without LMB treatment for 24h. Proteolysis was measured as the percentage of the initially acid-insoluble radioactivity (protein) transformed into acid-soluble radioactivity (amino acids and small peptides) at each time point. The experiment was performed in (c1) complete (Ctrl) or serum-deprived (S-) medium to enhance proteolysis, and in (c2) complete medium (Ctrl) with or without NH_4Cl (20 mM) and leupeptin (0.1 mM) (Leu/NH_4Cl) to inhibit lysosomal degradation. Data are the mean ± s.e.m of three independent experiments.

Briefly, GFP fluorescence is attenuated by acidic conditions and degraded by lysosomal hydrolases, whereas mRFP fluorescence remains relatively stable (Kimura *et al.*, 2007). While autophagosomes correlated with puncta double positive for mRFP and GFP, autophagolysosomes were mRFP only puncta. Thus, using this approach, we were able to analyze the levels of non-degraded autophagosomes. LMB-treated striatal cells displayed an increase in the number of autophagosomes compared to non-treated cells (Fig. 28B), reinforcing the idea that autophagosome degradation was slightly impaired upon treatment.

Most of the proteins that are degraded by lysosomes have long half-lives. Thus, the degradation rate of long-lived proteins correlates with lysosomal function (Kaushik & Cuervo, 2009). To analyze whether the alterations observed upon Xpo-1 inhibition could lead to alterations in the degradation of long-lived proteins, we performed metabolic labelling in pulse and chase assays and measured whole cellular proteolysis. The experiment was performed in complete or serum-deprived medium to enhance proteolysis, and complete medium with or without NH_4Cl and leupeptin to inhibit lysosomal degradation. We observed that LMB treatment did not alter the degradation of long-lived proteins in any of the conditions analyzed (Fig. 28C).

2 - REGULATION OF PRO-SURVIVAL AND PRO-APOPTOTIC PROTEIN KINASES IN HUNTINGTON'S DISEASE

2.1 - ROLE OF mTOR IN THE REGULATION OF BOTH AUTOPHAGY AND THE AKT PRO-SURVIVAL PATHWAY IN HUNTINGTON'S DISEASE

In the previous aim, we wondered whether autophagy was impaired along the progression of the disease. The results that we obtained suggest that at early stages of the disease autophagy was enhanced, whereas at late stages of the disease autophagy was deregulated in a brain region-dependent manner. While in the cortex autophagy remained enhanced, in the striatum returned to control levels and in the hippocampus even seemed to collapse. One of the regulators of autophagy is mTOR, which in addition regulates AKT phosphorylation at Ser473. Previous results from our group have shown increased levels of pSer473 AKT (Saavedra *et al.,* 2010). This increase in AKT activity occurred specifically in striatal neurons and was suggested to be a compensatory pro-survival mechanism in Huntington's disease that was over-activated, at least in part through PHLPP down-regulation (Saavedra *et al.*, 2010). With this background, we wondered whether mTOR activity would be de-regulated in striatal neurons expressing mutant huntingtin.

2.1.1 - mTOR phosphorylation is increased in the striatum of R6/1 mice

One of the mechanisms to regulate mTOR is phosphorylation. Thus, to analyze mTOR activity, we examined the levels of mTOR phosphorylated at two different sites, Ser2448 (pSer2448 mTOR) and Ser2481 (pSer2481 mTOR). While the first site is phosphorylated by AKT and S6K1 to increase mTOR activity (Holz & Blenis, 2005; Nave *et al.,* 1999), the second is an autophoshorylation event that monitors mTOR catalytic activity (Soliman *et al.,* 2010). Protein levels of non-phosphorylated mTOR and both pSer2448 mTOR and pSer2481 mTOR were analyzed by western blot in homogenates obtained from the striatum of R6/1 mice at 8, 12, 20 and 30 weeks of age. While we found no changes in mTOR total protein levels in R6/1 mice striatum compared to values in control mice, levels of both phosphorylated forms of mTOR increased progressively from 12 weeks of age onwards. Both phosphorylated mTOR forms increased similarly, and doubled the levels detected in control mice striatum at 30 weeks of age.

Our results suggest that mTOR might be catalytically more active in these models of the disease from 12 weeks ahead.

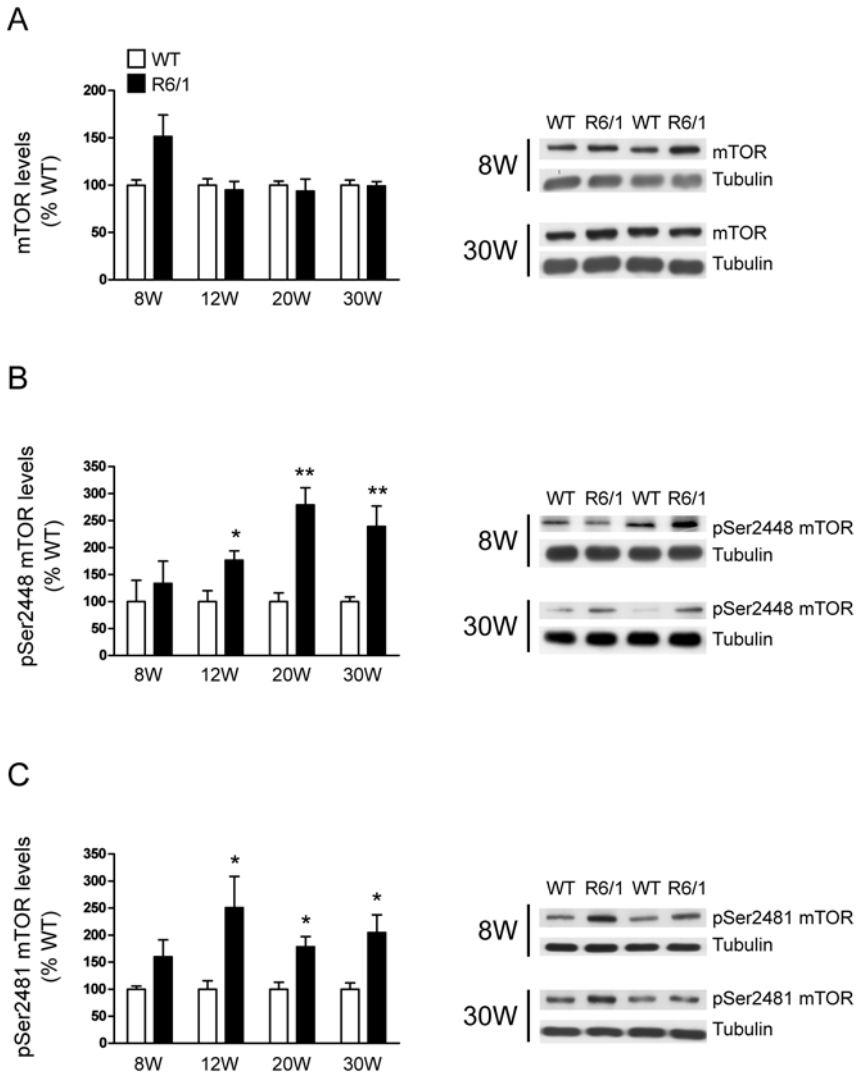

Figure 29. Regulation of mTOR and its phosphorylation levels in the striatum of R6/1 mice along the progression of the disease. Protein levels of (A) mTOR, (B) pSer2448 and (C) pSer2481 mTOR were analyzed by western blot in protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages of the disease progression (W, weeks). Representative immunoblots show the protein levels of mTOR, pSer2448 mTOR, pSer2481 mTOR and α-tubulin in WT and R6/1 mice at different ages. The graphs show mTOR, pSer2448 mTOR and pSer2481 mTOR protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (mTOR, pSer2448 mTOR or pSer2481 mTOR /α-tubulin ratio), and shown as mean ± s.e.m (n = 4-6). Data were analyzed by Student's t-test. * P< 0.05 and ** P< 0.01 as compared with WT mice.

2.1.2 - mTOR is not sequestered in mutant huntingtin aggregates

Contradictory data does exist about mTOR intracellular localization, which seems to vary between different cell types. While mTOR has been found in the nucleus of many types of cancer cells (Zhang *et al.,* 2002), in HeK293 cells mTOR distributes predominantly in the cytoplasm (Kim & Chen, 2000; Zhang *et al.,* 2002).

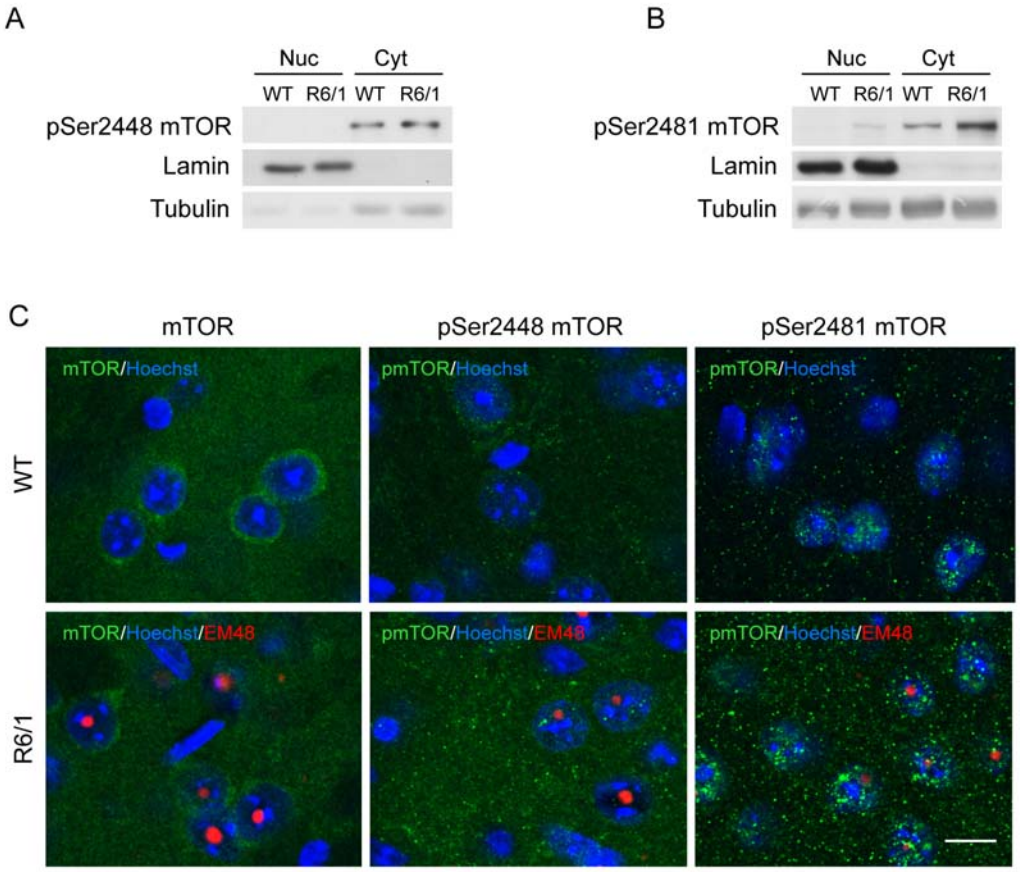

Figure 30. Phosphorylated mTOR is mainly localized in the cytoplasm of striatal cells. pSer2448 mTOR and pSer2481 mTOR localization was determined by (A and B) western blot in nuclear and cytopasmic enriched fractions and by (C) immunohistofluorescence. Protein levels of (A) pSer2448 mTOR and (B) pSer2481 mTOR were analyzed by western blot in nuclear (Nuc) and cytoplasmic (Cyt) fractions of wild-type (WT) and R6/1 mice striatum at 30 weeks of age. Representative immunoblots show protein levels of pSer2448 mTOR and pSer2481 mTOR in cytoplasmic (loading control α-tubulin) and nuclear (loading control lamin) fractions from WT and R6/1 mice. mTOR, pSer2448 mTOR and pSer2481 mTOR were analyzed by immunohistochemistry in the striatum of 30-week-old (C) WT and (D) R6/1 mice. Nuclei were stained with Hoechst 33258 and mutant huntingtin with the EM48 antibody. Images illustrate mTOR, pSer2448 mTOR and pSer2481 mTOR, which do not colocalize (yellow) with EM48 in the nuclear inclusions. Scale bar 10 μm.

Interestingly, previous results from David Rubinsztein's group revealed in 2004 that mTOR was getting sequestered into mutant huntingtin nuclear aggregates in the brain of N171-82Q mouse model of the disease and in human Huntington's disease brains (Ravikumar *et al.,* 2004). Since sequestration of mTOR into the mutant huntingtin aggregates could lead to decrease in its activity, we next wondered whether this could also take place in the striatum of R6/1 mice.

We first analyzed whether intracellular distribution of phosphorylated mTOR was altered by mutant huntingtin. We analyzed both pSer2448 mTOR and pSer2481 mTOR by western blot in cytoplasmic and nuclear enriched fractions obtained from wild-type and R6/1 mice striatum. We found that both phosphorylated forms of mTOR were mainly cytoplasmic (Fig. 30A and B). While we failed to observe pSer2448 mTOR in the nuclear-enriched fractions (Fig. 30A), we detected pSer2481 mTOR in this fraction only in R6/1 mice brains (Fig. 30B), suggesting that at least pSer2448 mTOR was not getting sequestered into mutant huntingtin aggregates. As already observed in whole striatal lysates, R6/1 cytoplasmic enriched fractions presented more phosphorylated mTOR than in wild-type mice (Fig. 30A and B). To further examine if mTOR was getting sequestered into mutant huntingtin aggregates, brain slices from 30 week-old wild-type and R6/1 mice, were processed for immunohistofluorescence against non-phosphorylated mTOR, pSer2448 mTOR and pSer2481 mTOR. R6/1 mice brain slices were also co-stained with EM48 antibody to detect mutant huntingtin and Hoechst 33258 to highlight cellular nuclei. The staining of wild-type mice brain slices with pSer2448 mTOR, pSer2481 mTOR or mTOR, revealed that the three mTOR forms display different distribution. While mTOR and phosphorylated mTOR in Ser2448 presented a more diffuse staining, and mainly cytoplasmic, pSer2481 mTOR formed bigger puncta that were distributed in the cytoplasm and also within the nucleus (Fig. 30C). R6/1 mice striatum displayed a similar pattern of staining (Fig. 30C). We failed to detect any colocalization of mTOR with EM48 positive puncta in R6/1 mice striatum, indicating that mTOR is not sequestered into the mutant huntingtin aggregates, at least in the R6/1 mouse model (Fig. 30C).

2.1.3 - Rictor, but not Raptor, is increased in the striatum of R6/1 mice

Association of mTOR with protein partners define its affinity for different substrates and regulates its activity. Thus, we analyzed the levels of two different protein partners of mTOR, Raptor and Rictor. While Raptor gives substrate specificity to the mTORC1

complex, Rictor is specific for the mTORC2 complex. We examined both protein levels by western blot in the striatum of R6/1 mice along the progression of the disease. While we found and increase in Rictor levels at all ages analyzed, Raptor protein levels remained unaltered (Fig. 31A).

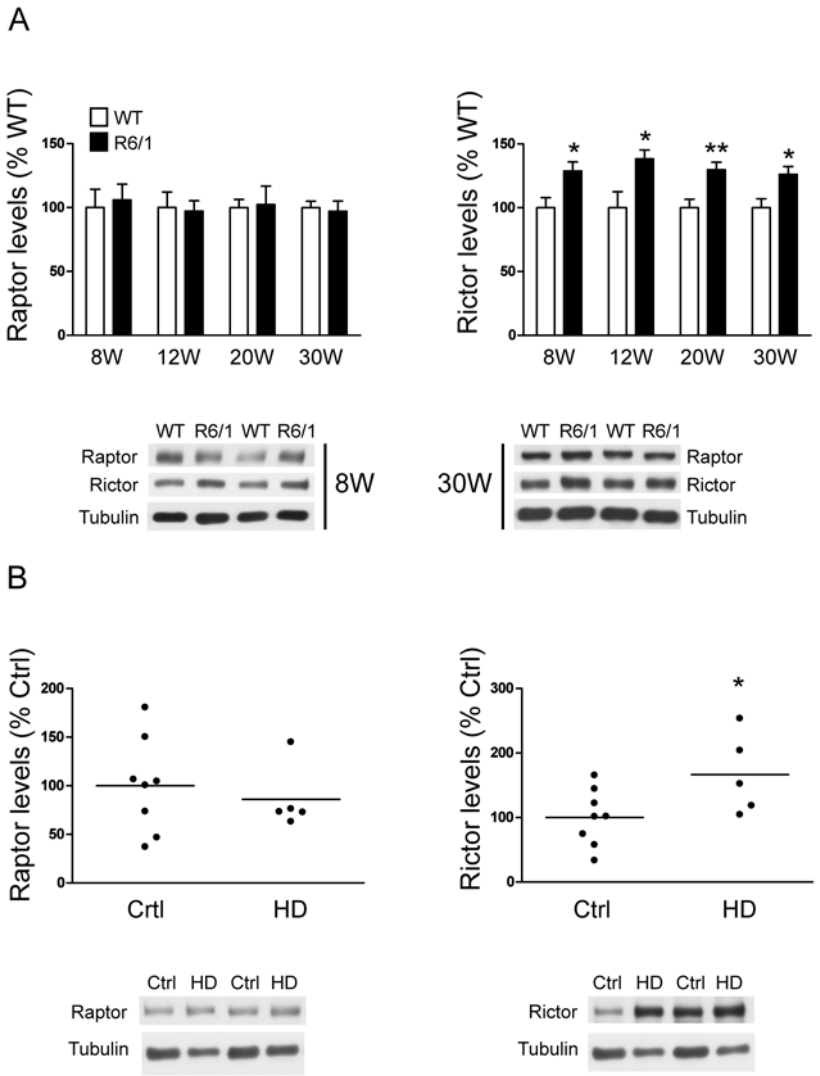

Figure 31. Raptor and Rictor protein levels in R6/1 mice and in human Huntington's disease putamen. (A) Raptor and Rictor protein levels were analyzed by western blot in protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. (B) Raptor and Rictor protein levels were also analyzed by western blot in protein extracts obtained from the putamen of Huntington's disease brains. Representative immunoblots show the protein levels of Raptor, Rictor and α-tubulin in WT and R6/1 mice at different ages and in control (Ctrl) and Huntington's disease (HD) brains. The graphs show (A) Raptor and Rictor protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression and (B) in HD brains with respect to Ctrl. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice or Ctrl brains (Raptor or Rictor/α-tubulin ratio), and shown as mean ± s.e.m (A, n = 4-13; B, n=5-8). Data were analyzed by Student's t-test. * P< 0.05 as compared with WT mice or Ctrl brains.

Furthermore, we analyzed the protein levels of these mTOR-partners in the putamen of Huntington's disease patients. According to the results obtained in mice, Raptor protein levels were unaltered and, interestingly, Rictor protein levels increased in the putamen of Huntington's disease patients when compared with levels in the putamen of non-affected brains (Fig. 31B).

Increased Rictor protein levels together with increased phosphorylated mTOR could lead to increased activity of the mTORC2 complex.

2.1.4 - mTORC1 and mTORC2-specific substrates are deregulated in the striatum of R6/1 mice

To further confirm our hypothesis that increases in phosphorylated mTOR could result into enhanced mTOR activity, we examined mTORC1 and mTORC2-specific targets. On one hand, mTORC1 inhibits autophagy by specifically phoshorylating ULK1, which results in its inhibition (Kim *et al.,* 2011a), and also regulates translation, activating S6K1 and inhibiting 4EBP1 (Fingar *et al.,* 2002). On the other hand, mTORC2 regulates survival and cytoskeleton reorganization through dependent phosphorylation and activation of AKT, PKCα and SGK (Dazert & Hall, 2011). Thus, we analyzed mTOR-specific phosphorylation sites of all these targets by western blot in the same wild-type and R6/1 mice striatal samples, where phosphorylated mTOR protein levels were increased. No differences were observed in the levels of pSer757 ULK1 and ULK1 (Fig. 32A). pSer371 S6K1 was neither altered in any of the studied time points (Fig. 32C), and, consistently, S6 ribosomal protein, which is specifically phosphorylated by S6K1, was unaltered along the disease progression (Fig. 32D). In contrast, pThr37/46 4EBP1 levels were significantly increased in R6/1 mice at all the ages examined (from 8 to 30 weeks of age), while 4EBP1 total levels remained unchanged (Fig. 32B). Besides AKT, phosphorylation levels of other mTORC2 substrates were not altered (Fig. 33A and B). Protein levels of both PKCα and SGK were down-regulated upon the disease progression, and so did their phosphorylated forms, pSer422 PKCα and pSer657 SGK (Fig. 33A and B). No alterations were observed when the phosphorylated levels of these two proteins were normalized to their total protein levels, to measure the relative phosphorylation amount (Fig. 33A and B).

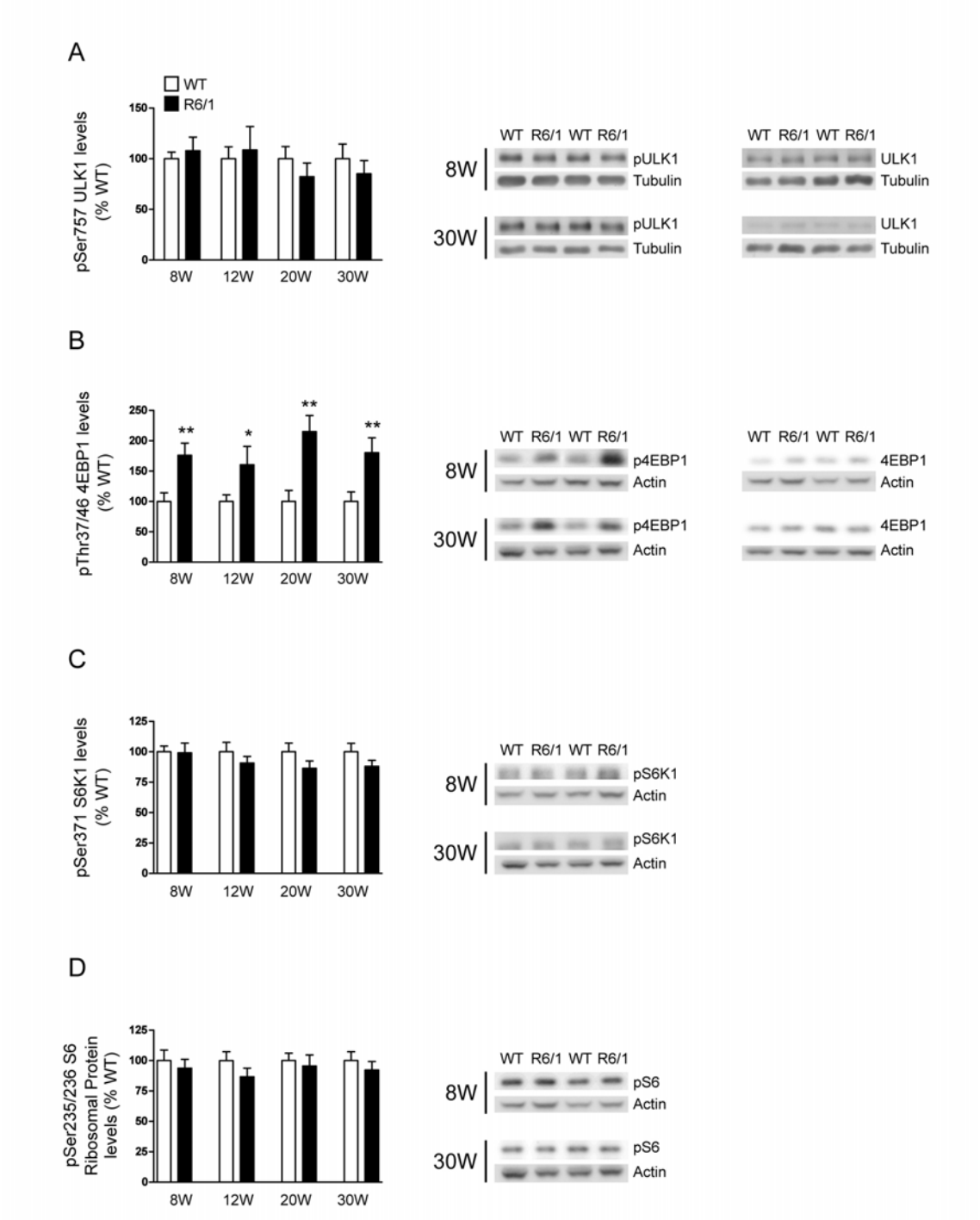

Figure 32. Phosphorylation of mTORC1 substrates in R6/1 mice striatum. (A) pSer757 ULK1, (B) pThr37/46 4EBP1 and (C) pSer371 S6K1 protein levels were analyzed by western blot in protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. (D) Protein levels of a S6K1 substrate, pSer235/236 S6 ribosomal protein, were also analyzed by western blot in protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages of the disease progression. Representative immunoblots show the protein levels of pSer757 ULK1, pThr37/46 4EBP1, pSer371 S6K1, pSer235/236 S6, actin and α-tubulin in WT and R6/1 mice at different ages. Representative immunoblots of (A) ULK1 and (B) 4EBP1 are also shown. The graphs show pSer757 ULK1, pThr37/46 4EBP1, pSer371 S6K1 and pSer235/236 S6 protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (pSer757 ULK1/α-tubulin and

pThr37/46 4EBP1, pSer371 S6K1 or pSer235/236 S6/actin ratio), and shown as mean ± s.e.m (n = 5-12). Data were analyzed by Student's t-test. * P< 0.05 and ** P< 0.01 as compared with WT mice.

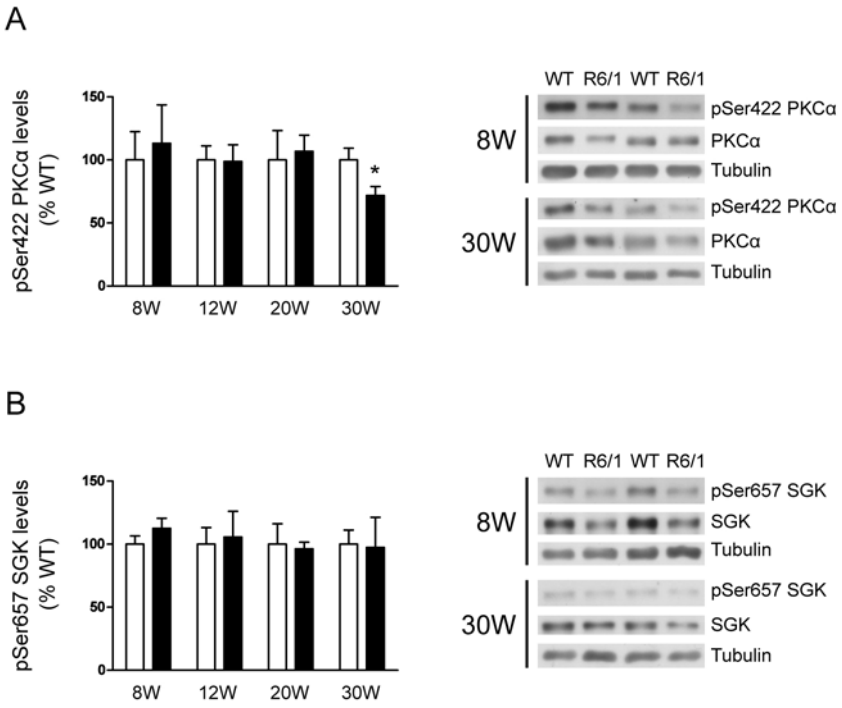

Figure 33. Phosphorylation of mTORC2 substrates. (A) pSer422 PKCα and (B) pSer657 SGK protein levels were analyzed by western blot in protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. Representative immunoblots show the protein levels of pSer422 PKCα, pSer657 SGK and α-tubulin in WT and R6/1 mice at different ages. Representative immunoblots of PKCα and SGK are also shown. The graphs show pSer422 PKCα and pSer657 SGK protein levels in R6/1 mice normalized to total protein (PKCα and SGK) with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (pSer422 PKCα/PKCα and pSer657 SGK/SGK ratio), and shown as mean ± s.e.m (n = 5-6). Data were analyzed by Student's t-test. * P< 0.05 as compared with WT mice.

These results show that mTORC1 and mTORC2 specific substrates are distinctly regulated along the progression of the disease in the striatum of R6/1 mouse model of Huntington's disease.

2.2 - REGULATION OF DIFFERENT MEMBERS OF THE PKC PROTEIN FAMILY IN HUNTINGTON'S DISEASE

PKC family can trigger many functions within the cell, from actin cytoskeleton reorganization, to apoptosis. Results from other groups already show a down-regulation of protein levels of several members of this protein family in Huntington's disease mouse models and human samples. Contrary to this, previous results from our group have shown decreased levels of PHLPP1, a phosphatase that besides AKT also regulates PKC signalling termination and degradation, thus suggesting an increase in the PKC levels. Therefore, in this last aim, we studied whether the levels of different PKC isoforms could be altered in the striatum, cortex and hippocampus of R6/1 mouse model of Huntington's disease at different stages of the disease progression.

2.2.1 - Distinct PKC isoforms levels are decreased in R6/1 mice brains

To determine whether there was a deregulation within the PKC protein family, we chose to analyze the protein levels of some PKC isoforms by western blot in R6/1 mice striatum, cortex and hippocampus at different stages of the disease. We examined two conventional PKCs, PKCα and PKCβII, and a novel PKC, PKCδ. As shown in figures 34-36, the levels of all these isoforms decreased with the disease progression in all the R6/1 mouse brain regions analyzed, when compared with their littermate controls. PKCα protein levels decreased in the striatum and cortex of R6/1 mice from 12 weeks onwards, reaching a 30% reduction in 30-week-old R6/1 mice when compared to their littermate controls (Fig. 34A and B). In the hippocampus, PKCα protein levels also started to decrease at 12 weeks, but reached a 65% reduction in 30-week-old R6/1 mice (Fig. 34C). PKCβII protein levels also decreased in the three brain regions, but at different stages of the disease. While in the striatum PKCβII protein levels started to decrease at 12 weeks of age (Fig. 35A), in the cortex they started to decrease at 20 weeks of age (Fig. 35B), reaching a similar reduction in both regions at 30 weeks of age (of about 50%). In the hippocampus PKCβII protein levels decreased only slightly (20%) at 30 weeks of age (Fig. 35C). However, while PKCα and PKCβII presented a moderate decrease in their levels, PKCδ protein levels were markedly decreased from early stages of the disease (8 weeks of age) when compared to wild-type animals. We observed a marked and similar decrease of PKCδ protein levels (of about 50%) in the striatum and cortex of 8-week-old R6/1 mice compared to their wild-type littermates (Fig. 36A and B). In both regions, this decrease was more accentuated at later stages

with a reduction of more than 75% of total PKCδ protein levels (Fig. 36A and B). In the R6/1 mouse hippocampus we observed a drastic decrease (by about 80%) of PKCδ protein levels at 12 and 30 weeks of age (Fig. 36C).

In the same blots, we analyzed δCF, the 40kDa proteolitically cleaved fragment of PKCδ, but we did not observe any alteration in its protein levels in R6/1 mouse striatum, cortex and hippocampus when compared to control littermates (Fig. 37).

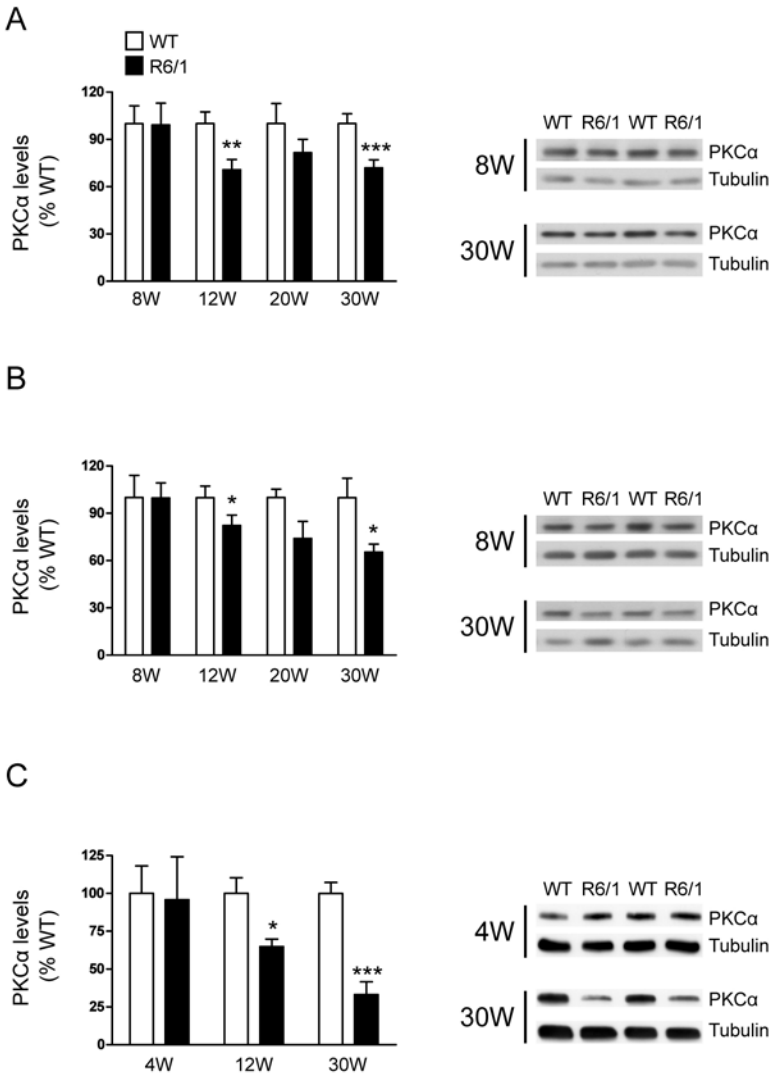

Figure 34. Regulation of PKCα in R6/1 mice brain regions during the progression of the disease. PKCα protein levels were analyzed by western blot in protein extracts obtained from (A) striatum, (B) cortex and (C) hippocampus of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. Representative immunoblots show protein levels of PKCα and α-tubulin in WT and R6/1 mice at different ages. The graphs show PKCα protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (PKCα/α-tubulin ratio), and shown as mean ± s.e.m (n = 4-12). Data were analyzed by Student's t-test. * P< 0.05, ** P< 0.01 and *** P< 0.001 as compared with WT mice.

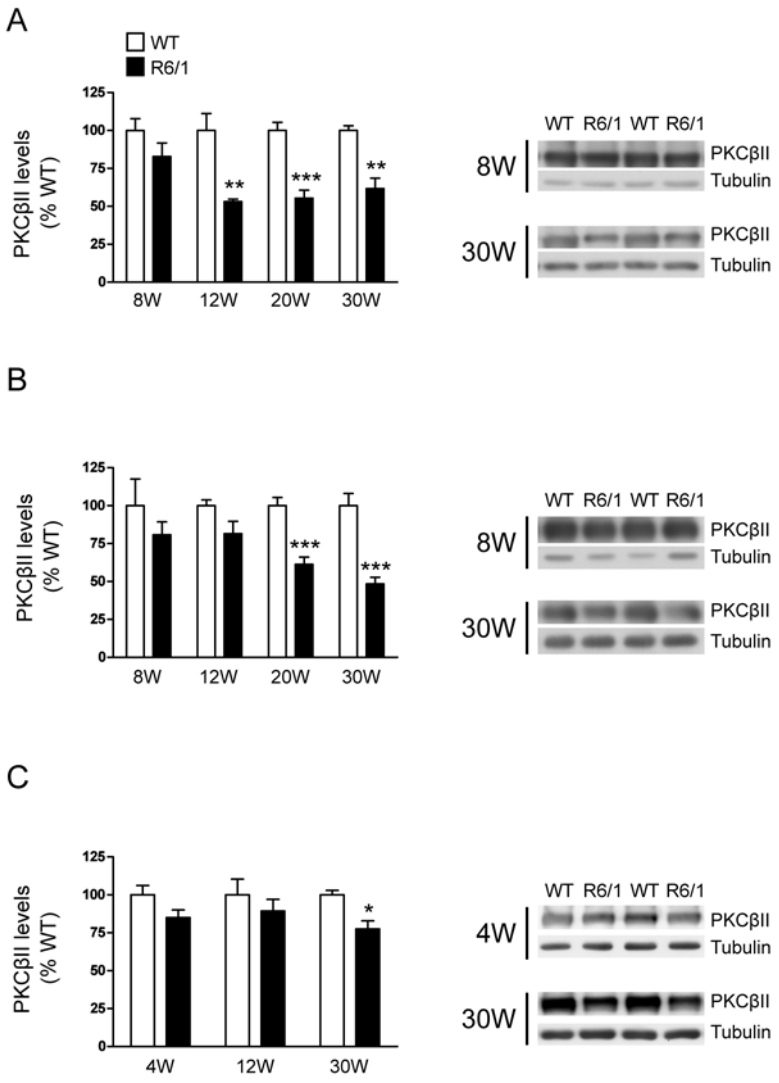

Figure 35. Regulation of PKCβII in R6/1 mice brain regions during the progression of the disease. PKCβII protein levels were analyzed by western blot in protein extracts obtained from (A) striatum, (B) cortex and (C) hippocampus of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. Representative immunoblots show protein levels of PKCβII and α-tubulin in WT and R6/1 mice at different ages. The graphs show PKCβII protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (PKCβII/α-tubulin ratio), and shown as mean ± s.e.m (n = 4-7). Data were analyzed by Student's t-test. * P< 0.05, ** P< 0.01 and *** P< 0.001 as compared with WT mice.

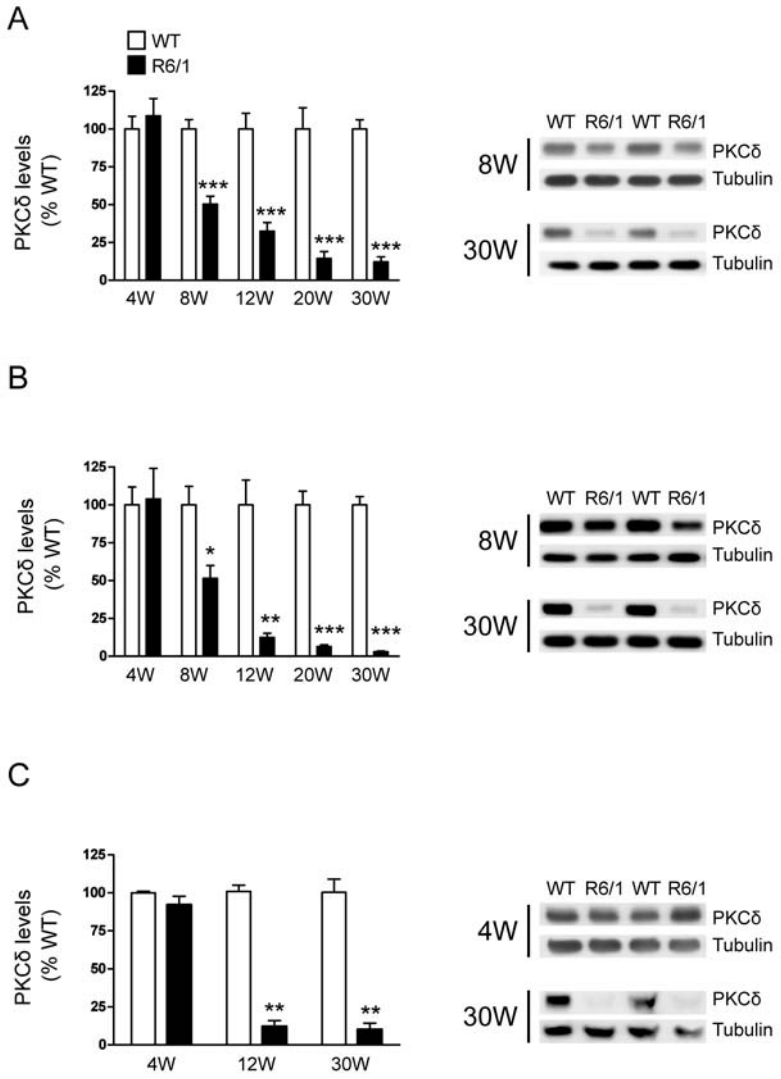

Figure 36. Regulation of PKCδ in R6/1 mice brain regions during the progression of the disease. PKCδ protein levels were analyzed by western blot in protein extracts obtained from (A) striatum, (B) cortex and (C) hippocampus of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. Representative immunoblots show protein levels of PKCδ and α-tubulin in WT and R6/1 mice at different ages. The graphs show PKCδ protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (PKCδ/α-tubulin ratio), and shown as mean ± s.e.m (n = 4-7). Data were analyzed by Student's t-test. * P< 0.05, ** P< 0.01 and *** P< 0.001 as compared with WT mice.

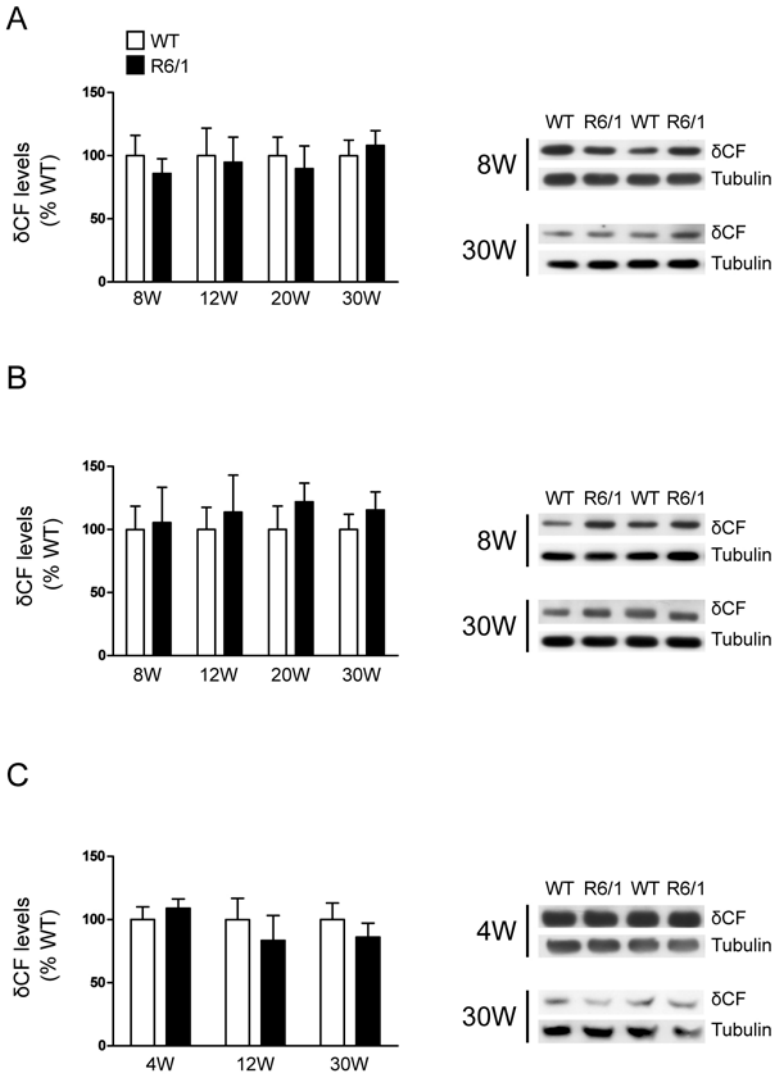

Figure 37. Regulation of the PKCδ cleaved fragment δCF in R6/1 mice brain regions during the progression of the disease. δCF protein levels were analyzed by western blot in protein extracts obtained from (A) striatum, (B) cortex and (C) hippocampus of wild-type (WT) and R6/1 mice at different stages (W, weeks) of the disease progression. Representative immunoblots show protein levels of δCF and α-tubulin in WT and R6/1 mice at different ages. The graphs show δCF protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (δCF/α-tubulin ratio), and shown as mean ± s.e.m (n = 4-7). Data were analyzed by Student's t-test.

2.2.2 - PKCα and δ mRNA levels are not altered in R6/1 mice brains

Decreased PKCβII gene expression levels has been previously described in brains of R6/2 mice (Harris *et al.,* 2001).

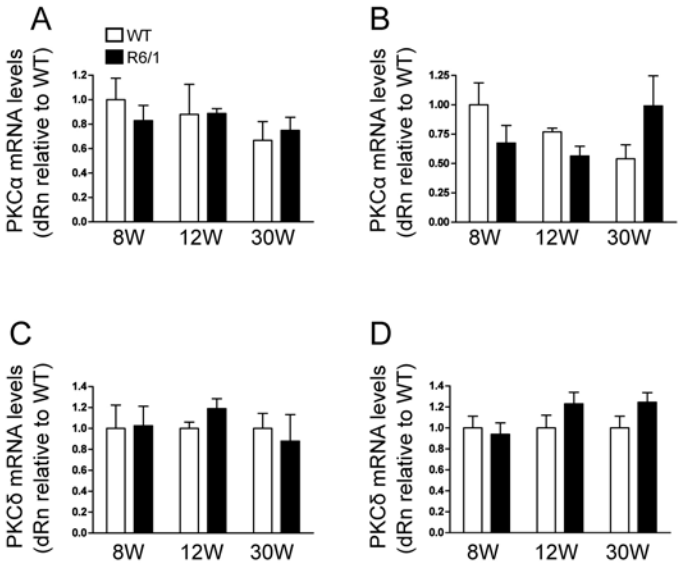

Figure 38. Regulation of PKCα and PKCδ mRNA levels in R6/1 mice striatum and cortex at different stages of the disease. The graphs show (A, B) PKCα and (C, D) PKCδ mRNA levels analyzed by Q-PCR in the (A, C) striatum and (B, D) cortex of 8-, 12- and 30-week-old (W) wild-type (WT) and R6/1 mice. Results were normalized to the 18S gene expression levels and are expressed as percentage of 8-week-old WT values. Data are the mean ± s.e.m (n = 3-6) and were analyzed by Student's t-test.

Thus, to determine whether the decrease in PKCα and PKCδ levels was also associated with a down-regulation of mRNA expression in the presence of mutant huntingtin, we analyzed by Q-PCR the PKCα and PKCδ mRNA levels that were extracted from 8-, 12- and 30-week-old wild-type and R6/1 mice brains. Our results revealed that PKCα and PKCδ mRNA levels were unaltered in R6/1 mice compared to wild-type mice values in both striatum (Fig. 38A and C) and cortex (Fig. 38B and D) at all the ages analyzed. These data suggest that the mechanism involved in this acute down-regulation of PKCs protein levels is not at a transcriptional level, at least for PKCα and PKCδ.

2.2.3 - R6/1 mouse striatum displays decreased PKCδ levels both in the cytoplasm and in the nucleus

For its pro-apoptotic induction, PKCδ undergoes phosphorylation events that trigger its nuclear translocation (Humphries *et al.,* 2008). Thus, we analyzed whether the decrease in PKCδ protein levels could be restricted to any of the two compartments.

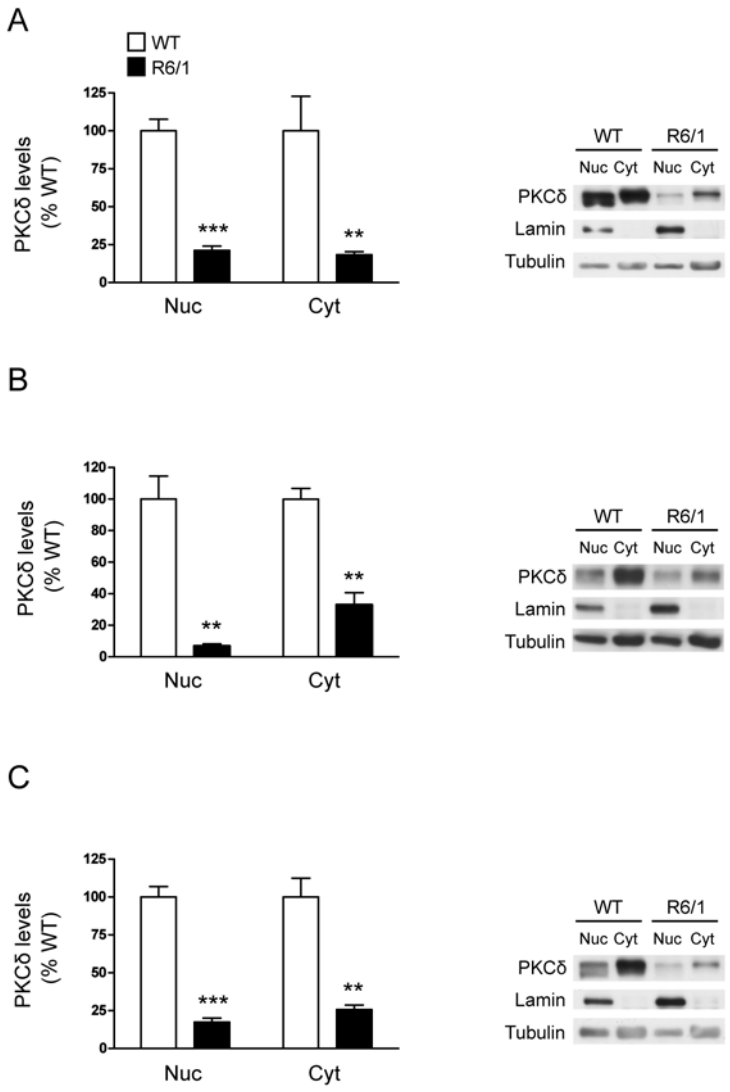

Figure 39. PKCδ protein levels are reduced in both nuclear and cytoplasmic enriched fractions of R6/1 mice striatum, cortex and hippocampus at 30 weeks of age. Protein levels of PKCδ were analyzed by western blot in nuclear (Nuc) and cytoplasmic (Cyt) fractions prepared from (A) striatum, (B) cortex and (C) hippocampus of wild-type (WT) and R6/1 mice at 30 weeks of age. Representative immunoblots show protein levels of PKCδ in cytoplasmic (loading control α-tubulin) and nuclear (loading control lamin) fractions from WT and R6/1 mice. Values were obtained by densitometric analysis of western blot data and normalized to α-tubulin or lamin, and are expressed as percentage of WT values. Data are the mean ± s.e.m. (n= 3-6), and were analyzed using the Student's t-test. * P< 0.05, ** P< 0.01 and *** P< 0.001 as compared with WT mice.

Moreover, PKCδ could get sequestered into the intranuclear aggregates containing mutant huntingtin, as previously observed in cerebellar sections of R6/2 mouse brains (Zemskov *et al.,* 2003).

To address these two issues, we analyzed PKCδ levels in cytoplasmic and nuclear enriched fractions obtained from the striatum, cortex and hippocampus of 30-week old mice. As figure 39 shows, PKCδ levels were significantly decreased in both nuclear and cytoplasmic-enriched fractions of 30-week-old R6/1 mice striatum, cortex and hippocampus compared to protein levels in wild-type mice (Fig. 39). In the nuclear-enriched fractions, PKCδ levels displayed more than a 75% fall in the three brain regions (Fig. 39), indicating that it was not retained into the mutant huntingtin aggregates. The cytoplasmic compartment showed a similar pattern, with a decrease of about 75% in the striatum and hippocampus (Fig. 39A and C), whereas the cortical cytoplasmic-enriched fraction exhibited a 50% reduction of PKCδ protein levels respect to the wild-type animals (Fig. 39B).

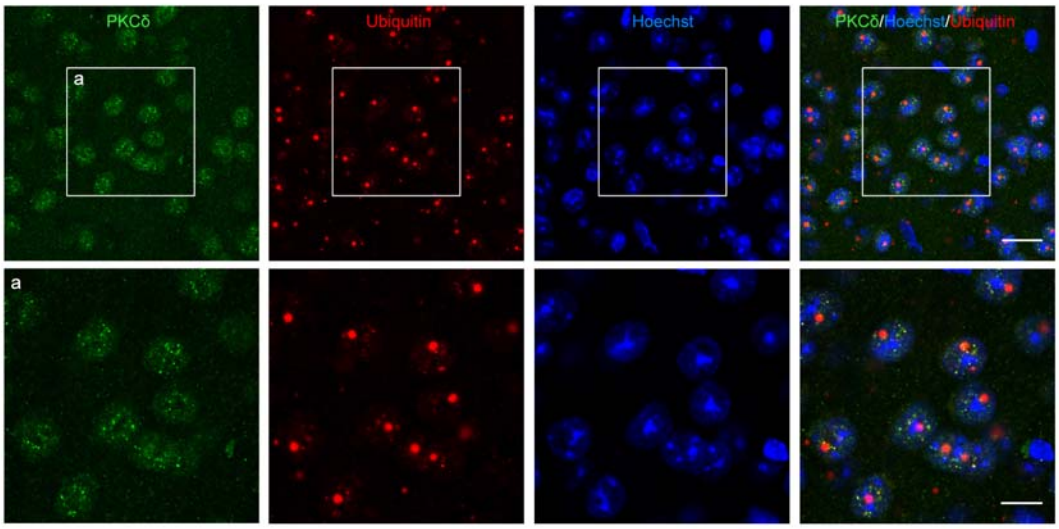

Figure 40. PKCδ does not colocalize with ubiquitin-positive nuclear aggregates in the striatum of R6/1 mice. PKCδ was analyzed by immunohistochemistry in the striatum of 30-week-old R6/1 mice. Nuclei were stained with Hoechst 33258 and nuclear aggregates were stained with an anti-ubiquitin antibody. Images show that PKCδ does not colocalize (yellow) with ubiquitinated nuclear inclusions. Scale bar 20 μm. (a) Higher magnification of the images is marked with a square. Scale bar 10 μm.

We further evaluated the intracellular distribution of PKCδ in 30-week-old R6/1 mouse brain slices by immunohistofluorescence in order to confirm that PKCδ was not retained into the mutant huntingtin aggregates in the R6/1 mouse model. As shown in

figure 40, although PKCδ was detected in the nucleus, it did not colocalize with ubiquitinated mutant huntingtin aggregates in R6/1 brains.

2.2.4 - pThr505 PKCδ levels are increased in the striatum, cortex and hippocampus of R6/1 mice at late stages of the disease

Although phosphorylation at the activation loop in PKCs is an essential event for their maturation (Stempka *et al.,* 1997), the same phosphorylation in PKCδ at Thr505 is a tag for degradation (Srivastava *et al.,* 2002).

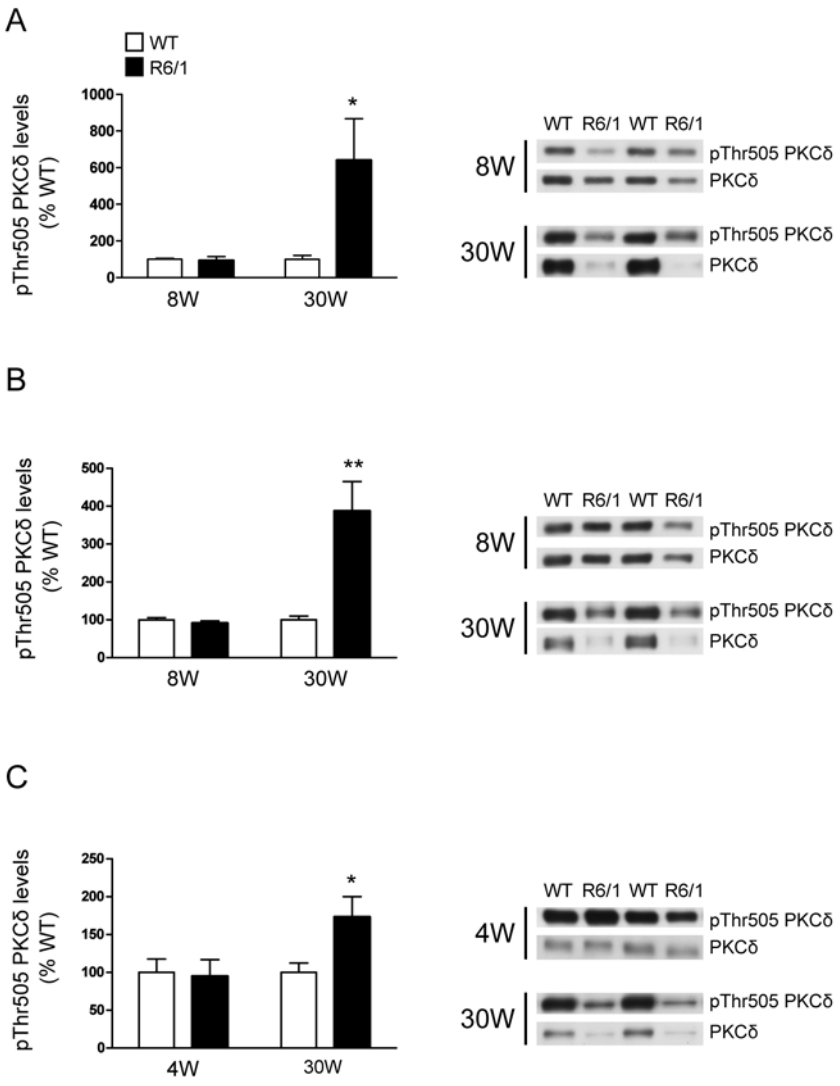

Figure 41. Regulation of pThr505 PKCδ in R6/1 mice brain regions at early and late stages of the disease. pThr505 PKCδ protein levels were analyzed by western blot in protein extracts obtained from (A) striatum, (B) cortex and (C) hippocampus of wild-type (WT) and R6/1 mice at 8 and 30 weeks (W) of age. Representative immunoblots show the protein levels of pThr505 PKCδ and PKCδ in WT and R6/1 mice at 8 and 30W. The graphs show pThr505 PKCδ protein levels normalized to PKCδ in R6/1 mice with respect to their littermate controls at two different stages of

the disease progression. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of WT mice (pThr505 PKCδ/PKCδ ratio), and shown as mean ± s.e.m (n = 3-6). Data were analyzed by Student's t-test. * P< 0.05 and ** P< 0.01 as compared with WT mice.

Thus, in order to know whether decreased levels of PKCδ could be related to changes in its phosphorylation, we examined the pThr505 PKCδ protein levels at different stages of disease progression. We observed an increase of pThr505 PKCδ/PKCδ ratio in the striatum, cortex and hippocampus, of 30-week-old R6/1 mice (Fig. 41). However, at early stages of the disease, phosphorylated levels of PKCδ were not modified in any of the regions analyzed (Fig. 41). This phosphorylation might account for PKCδ degradation at late, but not early, stages of the disease, suggesting that in addition to phosphorylation at Thr505, other mechanisms could account for the down-regulation of PKCδ.

2.2.5 - PKCδ protein levels are also decreased in the putamen of Huntington's disease patients

To know whether these changes in PKCδ also occur in Huntington's disease patients, we analyzed its protein levels in the putamen of human Huntington's disease brains.

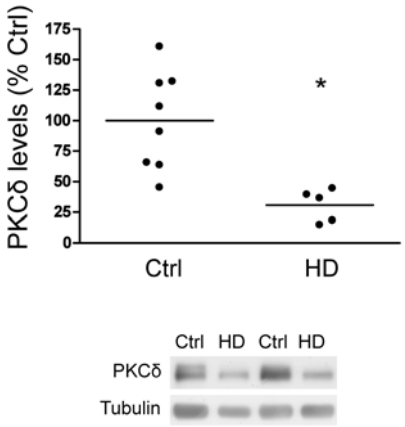

Figure 42. PKCδ protein levels are reduced in the putamen of Huntington's disease human brain. Protein levels of PKCδ were analyzed by western blot in protein extracts obtained from the putamen of Huntington's disease human brains. Representative immunoblots show the protein levels of PKCδ and α-tubulin in control (Ctrl) and Huntington's disease (HD) brain patients. The graph shows PKCδ in HD human brains with respect to Ctrl. Values (obtained by densitometric analysis of western blot data) are expressed as percentage of Ctrl brains (PKCδ/α-tubulin ratio), and shown as mean ± s.e.m (n=5-8). Data were analyzed by Student's t-test. * P< 0.05 as compared with Ctrl brains.

As observed in the striatum of R6/1 mice, PKCδ protein levels were significantly decreased in human Huntington's disease putamen when compared to control cases (by about 60% decrease; Fig. 42).

2.2.6 - Over-expression of PKCδ induces cell death only in cells expressing the exon 1 of mutant huntingtin

We hypothesized that a general decrease in PKCδ protein levels could represent a compensatory pro-survival mechanism generated to delay mutant huntingtin-induced toxicity.

Thus, we asked if over-expression of PKCδ in striatal cells expressing the exon 1 of the mutant huntingtin could enhance their cell death. The striatal cell line STHdh$^{7Q/7Q}$ was transfected with the huntingtin exon 1 with 16Q or 94Q plus HA-PKCδ or HA-only. Immunocytofluorescence to detect HA and nuclei staining with Hoechst 33258 were performed 72h after transfection, and apoptotic nuclei were counted. Mutant huntingtin expression induced a significant increase in cell death (Fig. 43B). We observed that cells expressing mutant huntingtin, but not cells expressing wild-type huntingtin, presented a 5% increase in cell death after over-expression of PKCδ, when compared to cells transfected with the HA-empty vector (Fig. 43B).

This result reinforces the hypothesis that a general decrease in PKCδ in brains of R6/1 mice can represent a compensatory pro-survival mechanism, which is induced from early stages of the disease to protect neurons from mutant huntingtin toxicity.

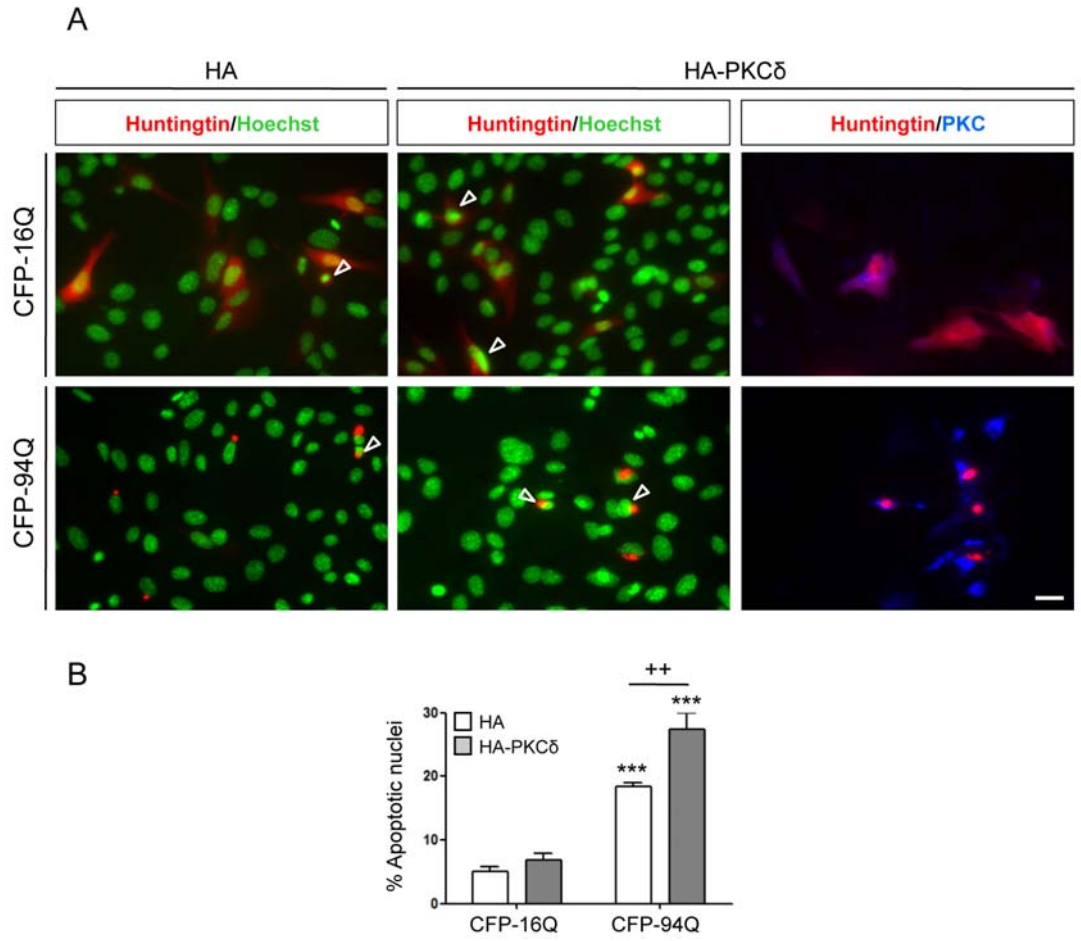

Figure 43. Over-expression of PKCδ increases cell death in striatal cells expressing the exon 1 of the mutant huntingtin. STHdh$^{Q7/Q7}$ cells were transfected with CFP-16Q or CFP-94Q plasmid in combination with HA-only or HA-PKCδ plasmid. Cells were fixed 72h after transfection and condensed nuclei from cells that were transfected with both huntingtin and HA were counted. (A) Representative images illustrate STHdh$^{Q7/Q7}$ cells transfected with either CFP-16Q or CFP-94Q plasmid in combination with HA-PKCδ plasmid. Nuclei were stained with Hoechst 33258. (B) Graph shows the percentage of apoptotic nuclei in CFP-16Q or CFP-94Q-transfected cells, co-transfected with either HA-only or HA-PKCδ plasmid, normalized to the whole number of double-transfected cells. Data are represented as mean ± s.e.m from five independent experiments (an amount of 200-400 nuclei were examined in each condition of every experiment). Data were analyzed by two-way Anova followed by Bonferroni's post-hoc test. *** P< 0.001 as compared with the percentage of apoptotic nuclei in CFP-16Q. ++ P< 0.01 as compared with the percentage of apoptotic nuclei in CFP-94Q co-transfected with HA-only plasmid. Scale bar, 20 μm.

V. DISCUSSION

Selective autophagy contributes to the clearance of aggregate-prone proteins, and pharmacological up-regulation of this pathway could lead to symptomathic amelioration in protein aggregation diseases (Ravikumar *et al.,* 2004; Sarkar *et al.,* 2009; Webb *et al.,* 2003). The first aim of this Thesis consisted in studying the selective autophagic response along the progression of Huntington's disease, and to put this aim into practice, we analyzed the levels of p62 and NBR1, two cargo receptors for selective autophagy, in the brains of R6/1 mice at different ages, which covered the whole pathology. An accumulation of p62 has been used as a marker for autophagy inhibition or defects in autophagic degradation (Bjorkoy *et al.,* 2005; Hara *et al.,* 2008; Komatsu *et al.,* 2007; Settembre *et al.,* 2008), and a decrease in p62 levels as a marker for autophagy induction (Mizushima *et al.,* 2008; Tasdemir *et al.,* 2008), p62 has been suggested to be a proper tool to study autophagic activity (Bjorkoy *et al.,* 2009). Since p62 and NBR1 are both cargo receptors for selective autophagy, both could be used as reporters for monitoring autophagic flux (Larsen *et al.,* 2010).Thus, we considered the alterations in the protein levels of these two receptors as endogenous reporters of the selective autophagic flux. We have found that both proteins levels were similarly altered along the progression of the disease in a region- and age-dependent manner. At an early stage of the disease, both proteins levels were decreased in all regions examined, suggesting an over-activation of selective autophagy. At later stages of the disease this over-activation was still occurring in the cortex of R6/1 mice. However, this was not the case in the striatum and hippocampus, were p62 and NBR1 protein levels returned to control levels or even increased. We found that this deregulation was due to different mechanisms. While p62 accumulated in the nuclear aggregates, mainly in the striatum and hippocampus, NBR1 remained cytoplasmic. We found that, besides mutant huntingtin, an age-dependent decrease of Xpo-1 levels could also contribute to this nuclear p62 accumulation in the mutant huntingtin aggregates. Finally, we observed that *in vitro* inhibition of Xpo-1 could impair autophagy.

Since our results suggested alterations in the autophagic flux, we wondered whether these could result from changes in mTOR activity. Thus, the second aim of this Thesis consisted in dissecting out the mTOR pathway along the progression of the disease to understand its contribution to the possible autophagic deregulation, and to the previously reported over-activation of the pro-survival kinase AKT in the striatum, but not in the cortex and hippocampus, of Huntington's disease mouse models (Saavedra *et al.,* 2010). Results obtained during the development of the second aim of this Thesis pointed to a deregulation in the AKT-mTOR pathway, but not in the mTOR regulation of the autophagic pathway in the striatum of R6/1 mice. We found that while

mTOR protein levels were not altered at any of the ages analyzed, protein levels of mTOR phosphorylated at two different sites, pSer2448 mTOR and pSer2481 mTOR, increased from 12 weeks of age onwards. Moreover, we found that Rictor, but not Raptor, protein levels were increased in the striatum of R6/1 mice at all ages analyzed, and in human Huntington's disease putamen. Thus, we hypothesized that mTORC2 complex would be over-activated in Huntington's disease pathology. However, we failed to find further alterations in the levels of mTORC2 downstream-phosphorylated substrates, other than pSer473 AKT. Protein levels of two, out of three, mTORC1 downstream-phophorylated substrates analyzed were not changed. However we found an increase in 4EBP1 levels from early 8 weeks onwards. Finally, since pSer757 ULK1 levels were not altered at any of the time-points analyzed, we suggest that the alterations in autophagy that we have found in this model of the disease seem unrelated to mTOR signalling.

Finally, analyzing several mTORC2 substrates, we found PKCα protein levels decreased in the striatum of R6/1 brains. Interestingly, different evidences suggest a deregulation of the PKC protein family. PKCβII protein levels are decreased in the striatum of human Huntington's disease brains (Hashimoto *et al.,* 1992). Moreover, PKCβII mRNA levels are decreased in brains of R6/2 mouse model of the disease (Harris *et al.,* 2001). On the other hand, PHLPP1, whose protein levels are diminished in brains of R6/1 mice (Saavedra *et al.,* 2010), is known to mediate dephosphorylation and degradation of some PKC isoforms (Gao *et al.,* 2008), suggesting an accumulation of those in the same animal models of Huntington's disease. Thus, we have also examined the protein levels of distinct PKC isoforms along the progression of the disease in the striatum, cortex and hippocampus of R6/1 mice. We have shown that all isoforms analyzed present a reduction in their protein levels in the R6/1 mouse model. Protein levels of PKCδ were the ones most importantly affected, starting to decrease already at 8 weeks of age, and could represent a compensatory pro-survival mechanism induced by mutant huntingtin expression.

1 - DEREGULATION OF p62 AND NBR1 IN R6/1 MICE BRAINS

We have shown that p62 and NBR1 protein levels are deregulated in R6/1 mice in brain-region- and age-dependent manner. Interestingly, the dynamics at a protein level of both cargo receptors were similar at all ages and regions analyzed in R6/1 mice brains when compared to their wild-type littermates. At early stages of the disease (12

weeks of age) p62 and NBR1 protein levels were lower than in the wild-type mice in all regions examined. However, this was not the case for later stages of the disease (30 weeks of age) when p62 and NBR1 protein levels followed different dynamics in the cerebral regions examined. Their protein levels returned to control levels in the striatum and accumulated in the hippocampus, while in the cortex they kept decreased when compared to wild-type animals.

Alterations at protein level might not be only caused by autophagy. Other factors could have a role in inducing these alterations, such as gene expression or degradation by other means distinct than autophagy. Thus, we analyzed by Q-PCR the expression of p62 and NBR1 within the striatum and the cortex, since in these two areas we observed totally distinct p62 and NBR1 protein levels dynamics. Since NBR1 expression was not altered in any of the regions and ages analyzed, and p62 mRNA levels were increased in the striatum and cortex at ages at which p62 protein levels were decreased, the deregulation of p62 and NBR1 protein levels in the R6/1 mouse brain cannot be explained by changes in gene expression. Anyhow, the increase in both striatum and cortex of p62 mRNA levels is in accordance with a previous study, showing that p62 gene is up-regulated upon mutant huntingtin expression in an *in vitro* system (Nagaoka *et al.,* 2004). Previous studies have established p62 as a stress response protein induced by oxidative stress, via the transcription factor Nrf2, and also by proteasome inhibitors (Ishii *et al.,* 2000; Ishii *et al.,* 1997; Jain *et al.,* 2010). Interestingly, elevated lipid peroxidation appears to affect specifically vulnerable brain regions in R6/1 mice (Perez-Severiano *et al.,* 2000). Moreover, p62 is among the targets that are up-regulated by the transcription factor TFEB (Settembre *et al.,* 2011), and we have shown that protein levels of this transcription factor are increased in the two brain areas examined at 30 weeks of age. Our results are in good agreement with others that have observed an increase in the TFEB mRNA levels in induced pluripotent stem cells carrying the Huntington's disease mutation (Castiglioni *et al.,* 2012). The transcription factor TFEB regulates autophagy by inducing the expression of a large number of lysosomal and autophagic genes. TFEB plays a role in transcription, at least in response to starvation, and controls autophagy by positively regulating autophagosome formation and autophagosome-lysosome fusion (Settembre *et al.,* 2011). Moreover, an over-expression of this transcription factor induces lysosomal biogenesis and increases mutant huntingtin degradation (Sardiello *et al.,* 2009). Although it is still unclear how exactly the cell regulates TFEB activity according to its needs, TFEB translocation to the nucleus seems to be finely regulated by phosphorylation at different residues (Pena-Llopis *et al.,* 2011; Settembre *et al.,* 2011,

2012). We finally show that the increase in TFEB protein levels is also observed in nuclear-enriched fractions. An increase in the TFEB levels could account, or at least participate in up-regulating the p62 gene. ERK and mTORC1 have been proposed to phosphorylate TFEB upon starvation, and regulate in an opposite way its nuclear translocation. While ERK phosphorylation on TFEB interferes with its nuclear localization, mTORC1 phosphorylation is needed for TFEB nuclear translocation (Settembre *et al.,* 2011, 2012). In the R6/1 model of Huntington's disease phosphorylated ERK protein levels increase at 20 and 30 weeks of age (Saavedra *et al.,* 2011), not correlating with the nuclear increase in TFEB protein levels. mTORC1 could participate in this TFEB nuclear increase, since another mTORC1 substrate, 4EBP1, is increased from 8 weeks onwards. However, TFEB is phosphorylated in more than ten different sites, and other kinases and phosphatases could contribute to the nuclear accumulation of TFEB (Dephoure *et al.,* 2008; Mayya *et al.,* 2009; Yu *et al.,* 2011).

Another mechanism that could explain the deregulation of p62 and NBR1 in the R6/1 mouse brain is an alteration in their degradation. On one hand, proteasomal inhibitors induce a prominent increase in the amount of p62 protein in cells (Kuusisto *et al.,* 2001; Nagaoka *et al.,* 2004; Thompson *et al.,* 2003), thus indicating that p62 can be degraded by the proteasome. However, the same works also show a significant increase in p62 mRNA levels under the same conditions, which the authors attribute to a cell response to an increase in the level of ubiquitinated proteins, consistent with previous reports (Ishii *et al.,* 2000). Moreover, autophagy could play a more important role than the proteasome in p62 degradation, since there is a marked elevation of p62 in mouse embryonic fibroblasts and primary neurons, treated with agents that inhibit autophagy, such as chloroquine, NH_4Cl and Baf A1, and in $Atg5^{-/-}$ mouse embryonic fibroblasts (Myeku & Figueiredo-Pereira, 2011). Pulse and chase experiments, in which ^{35}S-labeled methionine was added in cells and p62 was specifically immunoprecipitated, indicate a half-life of p62 of 6 h, and almost all of the radiolabeled protein is lost after 24h of chase. Rapamycin treatment causes a decrease of endogenous p62, whereas treatment with BafA1 for 18h results in an accumulation of endogenous p62 (Bjorkoy *et al.,* 2005). On the other hand, the role of the proteasome in NBR1 protein stability has been addressed by Kirkin *et. al.*, who used the proteasomal inhibitors epoxomycin and proteasome inhibitor I. They found that proteasome inhibition did not significantly alter NBR1 protein levels (Kirkin *et al.,* 2009). In fact, time course experiments using lysosomal inhibition by BafA1 showed that NBR1 is turned over by lysosomes considerably faster than p62 (Kirkin *et al.,* 2009).

Moreover, Baf1 treatment leads to an accumulation of NBR1 in vesicle-like structures (Kirkin *et al.,* 2009). Finally, and as already explained in the introduction, global proteasome system activity is not altered in the R6/1 mouse brain (Maynard *et al.,* 2009), thus, suggesting a relation between p62 and NBR1 protein levels and selective autophagic activity (Johansen & Lamark, 2011).

The deregulation of these cargo receptors, and the fact that their protein levels followed similar dynamics along the progression of the disease and in different brain regions, suggested that autophagic activity was deregulated in the brains of R6/1 mice. At early stages of the disease, both p62 and NBR1 were reduced in all regions examined, suggesting that the expression of mutant huntingtin increases basal levels of selective autophagy by an unknown mechanism. Accordingly, autophagy is induced in striatal cells over-expressing full-length or truncated mutant huntingtin (Kegel *et al.,* 2000; Ravikumar *et al.,* 2004), and in mouse models of Huntington's disease (Heng *et al.,* 2010; Ravikumar *et al.,* 2004). Moreover, autophagy is activated as a compensatory response in other neurodegenerative conditions characterized by the presence of abnormal protein aggregates (Butler *et al.,* 2006). However, at late stages of the disease, the deregulation observed for p62 and NBR1 was brain region-dependent, which hinted that mutant huntingtin-induced changes in the selective autophagic flux were cell-type specific. Autophagy in cortical neurons seemed to keep over-activated at all the ages analyzed, whereas in the striatum and hippocampus this over-stimulation did not occur at late stages of the disease, in which autophagy seemed to function at normal levels in the striatum or even collapse in the hippocampus. Peroxisomes are degraded by selective autophagy (Kim *et al.,* 2008). In good correlation with NBR1 protein levels, we detected decreased and unchanged levels of the peroxisome marker catalase in 30 week-old R6/1 mice cortex and striatum, respectively. These results reinforce the hypothesis that at late stages of the disease autophagy in R6/1 cortex is over-activated, while in the striatum is similar to that in control animals.

2 - ACCUMULATION OF p62, BUT NOT NBR1, WITHIN MUTANT HUNTINGTIN NUCLEAR AGGREGATES

It is well known that mutant huntingtin is not only prone to aggregate with itself but also has the tendency to aggregate with other selected proteins such as CBP, HSP70 or p53, and disrupt their function (Busch *et al.,* 2003; Steffan *et al.,* 2000; Suhr *et al.,*

2001). Moreover, p62 and NBR1 selectively bind ubiquitinated proteins that must be degraded by autophagy. Thus, we also explored the possibility that p62 and NBR1 could get hampered into the mutant huntingtin aggregates and be retained in the stacking gels of the western blots, inducing alterations in their protein levels. Although p62 interaction with mutant huntingtin is an event that has been already shown by other groups (Martinez-Vicente *et al.,* 2010; Nagaoka *et al.,* 2004), NBR1 is only known to interact with ubiquitin and LC3, but any group has shown yet whether it is able to co-immunoprecipitate together with mutant huntingtin. We show by immunoprecipitation for the first time that not only p62, but also NBR1, interact with mutant, but not with wild-type, huntingtin in the R6/1 mouse striatum and cortex. However, previous results from another group also showed an interaction of p62 with wild-type huntingtin in autophagic vacuoles (Martinez-Vicente *et al.,* 2010). This difference could be due to the use of a different methodological approach, since here we used total protein extracts from the striatum of wild-type and R6/1 mice, and the authors of this study analyzed the interaction of p62 with huntingtin specifically in autophagic vacuoles obtained from a cell culture fractionation. Interestingly, the authors of the study show that the amount of p62 associated with mutant huntingtin was markedly higher than that associated with wild-type huntingtin. The fact that both p62 and NBR1 co-immunoprecipitate with mutant huntingtin, means that both proteins recognize ubiquitinated mutant huntingtin and are both potentially able to direct it to the autophagy machinery. By analyzing the stacking gel of the western blots, we failed to detect p62 and NBR1, while we indeed found aggregated mutant huntingtin at 30 weeks of age in the striatum of R6/1 mice. This is in agreement with the fact that p62 is recruited to polyQ protein aggregates as a result of its ability to bind polyubiquitin chains via its UBA domain (Donaldson *et al.,* 2003), and that this binding is non-covalent (Vadlamudi *et al.,* 1996). Thus, the differences in p62 and NBR1 protein levels were more likely related to their turnover.

Mutant huntingtin aggregates have been found progressively accumulated in brains from mice, and also human individuals, affected by Huntington's disease, in both cytoplasmic and nuclear cell compartments (Davies *et al.,* 1997; DiFiglia *et al.,* 1997; Gutekunst *et al.,* 1999). Further analyzis of p62 intracellular distribution in nuclear and cytoplasmic enriched fractions, and of its intracellular localization by immunohistochemistry, revealed an accumulation of p62 in the nucleus of R6/1 mice neurons, mainly in the striatum and hippocampus, only at late stages of the disease. At 30 weeks of age, p62 colocalized with mutant huntingtin inclusions in neuronal nuclei, similarly to what has been previously observed in R6/2 mice brain (Nagaoka *et al.,*

2004). Interestingly, we observed that most cytoplasmic mutant huntingtin aggregates were not positive for p62 labelling, according to decreased levels of p62 detected in cytoplasmic enriched fractions. Finally, we confirmed our results in human Huntington's disease brains, where p62 is localized aggregated in the nucleus, but not in the cytoplasm, of striatal and cortical neurons. This finding adds Huntington's disease to a list of human brain, liver and muscle pathologies characterized by protein aggregates also containing p62 (Kuusisto *et al.,* 2008; Nogalska *et al.,* 2009; Strnad *et al.,* 2008; Zatloukal *et al.,* 2002).

Autophagy is a process that is restricted to the cytoplasm, and thus, it efficiently degrades polyQ aggregates that are located in the cytoplasm, but not the ones that are located within the nucleus (Iwata *et al.,* 2005). p62 is able to shuttle between the nucleus and the cytoplasm, thanks to NLS and NES tags in their sequence. However, very little attention has been paid to this fact, although some functions have been already attributed to the p62 shuttling. On one hand, in the nucleus, p62 has been shown to recruit ubiquitinated proteins to promyelocytic leukemia bodies (Pankiv *et al.,* 2010) that function as one of the nuclear sites for proteasomal degradation of misfolded proteins (Rockel *et al.,* 2005). Interestingly, nuclear mutant hutntingtin is localized in these structures (Kegel *et al.,* 2002). On the other hand, p62 can also export ubiquitinated substrates from the nucleus into the more degradation-potent cytoplasmic compartment (Pankiv *et al.,* 2010). Thus, it is tempting to speculate that nuclear p62 binds ubiquitinated mutant huntingtin in order to promote the degradation of the toxic protein by any of these two mechanisms. However, p62 could end up being trapped in the mutant huntingtin nuclear aggregates.

p62 has been suggested to work also as a scaffold protein to generate protein aggregates. Some groups support the idea that p62 is actually indispensable for protein aggregation (Komatsu *et al.,* 2007), since in autophagy deficient mice, protein aggregates accumulate in cells from liver and brain, which are present in a much lesser amount in autophagy and p62 deficient mice. However, we have found that p62 colocalizes with nuclear mutant huntingtin aggregates, and with almost no cytoplasmic aggregates. Thus, we think that p62 is not needed for protein aggregation, or at least for mutant huntingtin aggregation, and p62 may be recruited into aggregates later on, in an attempt to get them degraded by autophagy. Our results are similar to previous studies showing that that aggregate formation also occurs in the absence of p62 (Bjorkoy *et al.,* 2005; Nagaoka *et al.,* 2004).

In 30-week-old R6/1 mice, we found not only p62 nuclear accumulation, but also a decrease in the p62 cytoplasmic levels in all the brain areas examined. p62 is not only a cargo receptor for autophagy but also a scaffold protein that seems to play a pleiotropic role in the regulation of cellular signalling and homeostasis of multiple proteins. Thus, less levels of cytoplasmic p62 could impair its function in both autophagy and signalling, and induce pathology. p62 regulates cell survival and death signalling pathways (Moscat & Diaz-Meco, 2009), and has been recently shown to regulate the mTORC1 pathway (Duran *et al.,* 2011). In fact, deficiency of p62 leads to an accumulation of hyperphosphorylated Tau, neurofibrillary tangles, and neurodegeneration, followed by increased anxiety, depression, loss of working memory, and reduced serum BDNF levels (Wooten *et al.,* 2008). Increasing evidences link alterations in p62 to the pathology of Alzheimer's disease, such as p62 retention in neurofibrillary tangles (Salminen *et al.,* 2012), and a marked reduction of the cytoplasmic p62 levels that has been found in the frontal cortex of Alzheimer's disease patients compared to that of control subjects (Du *et al.,* 2009). This reduction might be the result of both impaired p62 transcription (Du *et al.,* 2009), and also of the binding of p62 to neurofibrillary tangles (Babu *et al.,* 2005). Reduced p62 protein levels have been linked to less p62 signalling in Alzheimer's disease. It would be noteworthy to analyze whether similar alterations could be found in Huntington's disease, since we show a reduction in the levels of cytoplasmic p62 in R6/1 mice that could lead to a reduction in the p62 signalling.

In contrast to p62, any nuclear tag has been described in the sequence of NBR1, nor any role of this protein within the nucleus. Consistent with this are the results that we obtained by immunohistochemistry in human Huntington's disease brains, showing that NBR1 displayed a granular cytoplasmic staining pattern, whereas no staining was observed within the nucleus. Thus, accumulation of NBR1 in whole cell lysates obtained from R6/1 mice brain at late stages of the disease seems to take place by a different mechanism than p62. Accumulation of NBR1 in 30-week-old R6/1 mice hippocampus should be the result of decreased selective autophagic flux, whereas nuclear accumulation of p62 could be due to its sequestration into intranuclear mutant huntingtin inclusions. In addition, and in contrast to that observed in human liver, muscle and neurological pathologies (D'Agostino *et al.,* 2011; Kirkin *et al.,* 2009; Odagiri *et al.,* 2012), our results show that in Huntington's disease p62 and NBR1 do not colocalize in the same aggregates, since p62 is sequestered in intranuclear mutant huntingtin inclusions whereas NBR1 remains cytoplasmic. This suggests that p62 and NBR1 may act independently and have redundant functions as previously shown

(Itakura & Mizushima, 2011; Waters *et al.,* 2009). Therefore, in the absence of p62, cytoplasmic NBR1 may still exert its function as a cargo receptor in cells expressing mutant huntingtin, as long as autophagy works efficiently. In the p62 knockout mice, neuronal disturbances appear later in their lifespan, which indicates that other mechanisms, such as NBR1, could compensate for the deficiency of p62 protein (Kirkin *et al.,* 2009).

Our findings showed a significant nuclear redistribution of p62 from early to late stages of the disease, being more evident in the striatum and hippocampus than in the cortex. This progressive accumulation resembles that of mutant huntingtin, which is dependent upon Xpo-1 that guides NES-containing proteins nuclear export. p62, as well as mutant huntingtin, needs Xpo-1 to travel from the nucleus to the cytoplasm (Chan *et al.,* 2011; Pankiv *et al.,* 2010). In agreement with previous results showing reduced Xpo-1 levels in both aged wild-type and R6/2 mice whole brain (Chan *et al.,* 2011), we detected a decline of Xpo-1 protein levels in the striatum and hippocampus, but not in the cortex, of R6/1 mice at late stages of the disease. Thus, we conclude that lower Xpo-1 levels could trigger longer permanence of p62 within the nucleus, leading to higher probability of interaction with mutant huntingtin inclusions only in R6/1 brains, and could explain the differential accumulation of this protein in striatal, hippocampal and cortical neuronal nuclei.

3 - INHIBITION OF XPO-1 AND AUTOPHAGIC ACTIVITY

Here, we show a region-dependent deregulation of Xpo-1 along the progression of the disease. While in the striatum and hippocampus Xpo-1 levels decrease from an early to a late stage of the disease, correlating with a reduction in the selective autophagy, in the cortex, where autophagy is over-activated at all stages of the disease, Xpo-1 levels are unaltered. Thus, we analyzed if a decrease in the Xpo-1 levels could impact in autophagy function, by impairing the functional localization of proteins essential for the process, such as p62.

Treatment of striatal cells *in vitro* with an Xpo-1 inhibitor led to an accumulation of autophagosomes, due to a reduction in their clearance. However, we failed to detect alterations in long-lived protein degradation. Although acute treatment with an Xpo-1 inhibitor *in vitro* did not alter long-lived proteins degradation, we cannot discard that a more chronic and progressive reduction in Xpo-1 levels, as observed *in vivo*, would

induce an impaired autophagy-mediated elimination of aggregates or damaged cellular organelles. Similarly, in autophagy-deficient neurons, accumulation of aggregates occurs gradually over time (Chan *et al.,* 2011; Hara *et al.,* 2006; Komatsu *et al.,* 2006).

The mechanism by which Xpo-1 could impair autophagy remains unknown, since over-expression or knock-down of p62 does not affect autophagosome numbers (Korolchuk *et al.,* 2009). Moreover, p62 knock-down does not affect autophagic flux (Komatsu *et al.,* 2007; Korolchuk *et al.,* 2009; Shvets *et al.,* 2008), although there is an accumulation of hyperphosphorylated Tau and neurofibrillary tangles in aged mice (Babu *et al.,* 2005). Therefore, our results suggest that in addition to p62, other important proteins for autophagosome degradation should be retained in the nucleus after Xpo-1 inhibition. Besides p62, the autophagic proteins found to undergo nucleocytoplasmic shuttling so far are Beclin 1, diabetes- and obesity-regulated gene and Alfy (Knaevelsrud & Simonsen, 2010). While the first two are required for the induction of autophagy upon stress and starvation, the latter one, together with p62, is more implicated in selective autophagy. None of these proteins seem to participate in the autophagic disruption observed upon LMB treatment, since none of them participate in the fusion of autophagosomes to lysosomes (Knaevelsrud & Simonsen, 2010). We speculate that some proteins important for autophagosome maturation might get impaired after LMB treatment, thus leading to autophagic dysfunction. The histone deacetylase (HDAC) 6 was first related to the formation of aggregates, by transporting miss-folded proteins along the microtubules through interaction with dynein (Kawaguchi *et al.,* 2003). More recently, HDAC6 has also been implicated in the creation of an actin network that facilitates the autophagosome-lysosome fusion and clearance of autophagic substrates (Lee *et al.,* 2010). This effect was specific to basal quality-control autophagy, and dispensable for starvation-induced autophagy. Moreover, a fraction of the cytosolic protein relocalizes into the nucleus upon LMB treatment (Verdel *et al.,* 2000). Since HDAC6 is sensitive to Xpo-1 inhibition, nuclear relocalization of this histone deacetylase might impair the fusion of autophagosomes and lysosomes. Thus, it should be further explored whether autophagy inhibition upon LMB treatment could be in part HDAC6-dependent.

4 - NBR1 AND p62 AS MARKERS TO TRACK SELECTIVE AUTOPHAGY IN HUNTINGTON'S DISEASE

We show that, although p62 and NBR1 have similar functions, in cells expressing mutant huntingtin they are differentially deregulated due to their distinct intracellular distribution. Since we observe that p62 is being sequestered into the nuclear mutant huntingtin aggregates as the disease progresses, we suggest that cytoplasmic NBR1 may be important to maintain the flux of selective autophagy. In addition, our results put forward the analyzis of NBR1, but not p62, protein levels as an indicator of the basal selective autophagic activity in Huntington's disease brain regions (Fig. 44).

NBR1 protein levels alterations indicate that autophagy is induced at an early stage of the disease in the striatum, cortex and hippocampus. At later stages of the disease autophagy is still induced in the cortex but not in the other regions, where the autophagic flux recovers to normal levels, as seen in the striatum, or even collapses, as observed in the hippocampus. Interestingly, autophagy is also activated in other neuropathological situations characterized by the presence of protein aggregates, and this activation has been suggested to represent a compensatory response (Butler *et al.,* 2006). Since mutant huntingtin is degraded by autophagy (Sarkar *et al.,* 2008), activation of selective autophagy could hamper the formation of cytoplasmic mutant huntingtin aggregates, whereas a reduction as the disease progresses could result in more mutant huntingtin aggregation. Accordingly, cytoplasmic aggregates containing mutant huntingtin increase with age in R6/1 mice brain (Bayram-Weston *et al.,* 2012; Cummings *et al.,* 2007) and, at late stages of the disease, R6/2 mice display larger number of aggregates in the striatum and hippocampus than in the cortex (Weiss, *et al.,* 2008).

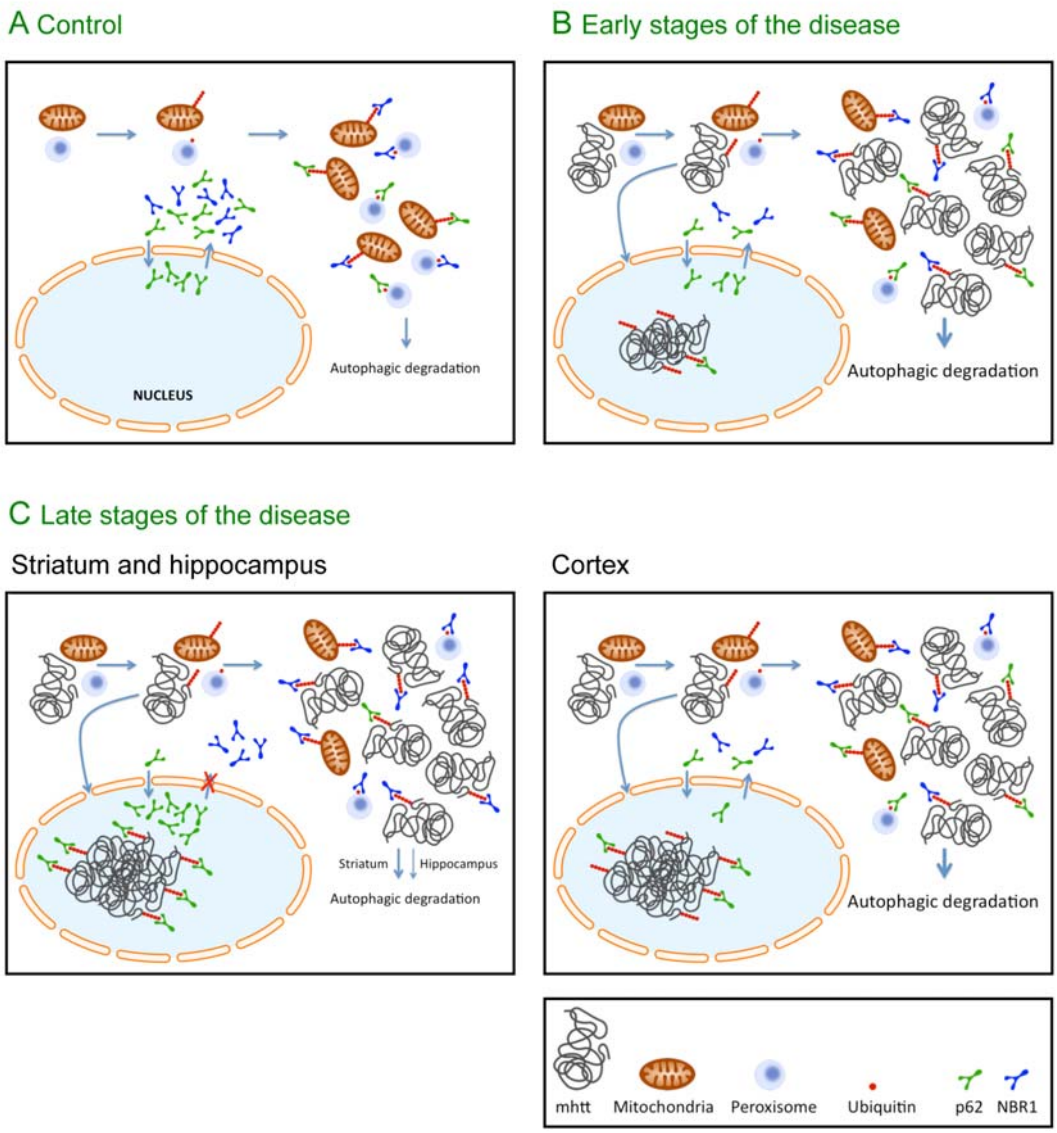

Figure 44. Scheme showing changes in p62 and NBR1 during the disease progression in R6/1 mouse brain and the proposed consequences on selective autophagy. p62 and NBR1 recognize unfolded proteins (such as mutant huntingtin) and damaged organelles (peroxisomes and mitochondria) tagged with ubiquitin. (A) In control conditions, selective autophagy is functioning normally and p62 and NBR1 are degraded together with the cargo. (B) At early stages of the disease, p62 and NBR1 protein levels are decreased in all the brain areas examined compared to control condition where mutant huntingtin is lacking. These decreased levels of autophagic cargo receptors could be the result of increased selective autophagy in response to the presence of mutant huntingtin by an unknown mechanism. (C) At late stages of the disease, regulation of p62 and NBR1 differs depending on the brain region. p62 starts getting accumulated within the nucleus, probably by interacting with mutant huntingtin. (c1) In the striatum and hippocampus, Xpo-1 levels are decreased compared to levels detected at earlier stages, which might contribute to a longer permanence of p62 within the nucleus. Since p62 is retained in the nucleus, we detect higher levels of this protein compared to those in wild-type mice, in the striatum and hippocampus. Cytoplasmic NBR1 would be the hope for selective autophagy in these cerebral areas in front of a pathological redistribution of p62. However, we detect higher NBR1 protein levels in 30 week-old R6/1 mice hippocampus suggesting an impairment of selective autophagy with age, whereas in the striatum selective autophagy seems to function at similar levels as in wild-type animals. (c2) In contrast, in cortical tissue, p62 also interacts with nuclear mutant huntingtin, although it is less retained in the nucleus. In the

cytoplasm its function seems not to be impaired and, together with NBR1, is still degraded by autophagy at an enhanced rate, like peroxisomes.

5 - PHOSPHORYLATION OF mTOR IN THE STRIATUM OF R6/1 MICE

Since our previous results analyzing p62 and NBR1 indicated changes in autophagic activity in Huntington's disease, we analyzed whether deregulation of mTOR activity could account for these changes.

Four phosphorylation sites have been described for mTOR up to date. The ones that have been most deeply studied are the rapamycin-sensitive pSer2448 mTOR and the autophosphorylated pSer2481 mTOR. While the first one is regulated by both AKT and S6K1, and is sensitive to rapamycin treatment (Holz & Blenis, 2005; Nave *et al.,* 1999), the latter is the result of an autophosphorylation and, thus, it serves as a biomarker to analyze intrinsic mTOR catalytic activity (Soliman *et al.,* 2010). We have found that protein levels of these two phosphorylation forms of mTOR are already increased from early stages of the disease (12 weeks of age) in the striatum of R6/1 mice. In addition, mTOR protein levels trend to increase at 8 weeks of age, however, no changes were observed at any other time-point of the disease analyzed. These results, together with the fact that phosphorylated AKT at Ser473 increases at the same time-points as phosphorylated mTOR (Saavedra *et al.,* 2010), suggested a deregulation of the whole pathway in the striatum of R6/1 mice. Consistent with this are the findings showing that short exposure to mutant huntingtin in a cell culture inhibits mTOR activity and AKT phosphorylation, which are reversed by longer exposure-times to mutant huntingtin in a time-dependent manner (Hyrskyluoto *et al.,* 2012). Contrary to this, however, is the fact that mTOR gets sequestered into both nuclear and cytoplasmic mutant huntingtin aggregates in cells transfected with the exon 1 of mutant huntingtin, in brains of N171-82Q mice models of Huntington's disease and in Huntington's disease human brain (Ravikumar *et al.,* 2004). These authors suggested that this interaction with mutant huntingtin inhibits mTOR function. Our immunohistofluorescence results show that increases in the phosphorylation of mTOR in the R6/1 mouse brain is not due to an accumulation and inactivation of this protein into the mutant huntingtin aggregates, as we fail to detect mTOR colocalizing with mutant huntingtin aggregates. Interestingly, we show that although both pSer2448 mTOR and pSer2481 mTOR are mainly localized in the cytoplasm, they have a different distribution pattern that is conserved from wild-type to R6/1 mice, so this

intracellular distribution is mutant huntingtin-independent. While pSer2448 mTOR localization is more diffuse and cytoplasmic, pSer2481 is accumulated in denser puncta that are distributed in the cytoplasm, but also within the nucleus. mTOR was first considered a cytoplasmic kinase and very few data exist about its intracellular localization and also about of the complexes that it builds. Some evidences have revealed the capability of mTOR to shuttle to the nucleus. mTOR has been found predominantly in the nucleus of many types of cancer cells (Zhang *et al.,* 2002), but also in the cytoplasm in HeK293 cells (Kim & Chen, 2000; Zhang *et al.,* 2002). It is still not well known what functions it may have within the nucleus. Interestingly, it has been found that mTOR and also some of its partners, mLST8, Rictor and sin1, are predominantly cytoplasmic in non-immortalized human primary fibroblasts (Rosner & Hengstschlager, 2008). Although Raptor is also abundant in the nucleus, the mTORC1 complex is predominantly cytoplasmic, whereas the mTORC2 complex is abundant in both compartments (Rosner & Hengstschlager, 2008). This might be consistent with our results showing a cytoplasmic localization of pSer2448 mTOR, which is enriched in the mTORC1 complex (Copp *et al.,* 2009), whereas pSer2481 mTOR is part of both complexes (Soliman *et al.,* 2010), and thus, shows a cytoplasmic and nuclear localization.

6 - REGULATION OF mTORC1 AND mTORC2 COMPLEXES IN THE STRIATUM OF R6/1 MICE

Increased levels of mTOR phosphorylation could indicate an increase in its activity. Thus, we dissected out whether mTOR partners and substrates could be altered at any time-point of the disease.

Raptor, PRAS40, Deptor and mLST8/GβL are the four mTOR-partners that compose the complex mTORC1, to promote protein synthesis and cell growth, and inhibit autophagy (Zoncu *et al.,* 2011). Here, we have shown that protein levels of Raptor are not altered along the disease progression in the striatum of R6/1 mice, and neither in human Huntington's disease putamen. Raptor functions as a scaffolding protein that facilitates the recruitment of substrates containing a TOR signal motif to the mTOR kinase. Accordingly, rapamycin treatment results in a conformational change in mTORC1 that is believed to alter and weaken the interaction with Raptor, impeding the phosphorylation of its substrates. Moreover, reducing the endogenous levels of Raptor by siRNA methods significantly impairs the mTOR kinase activity towards 4EBP1 (Hara

et al., 2002). Thus, Raptor protein levels might be important to build up functional mTORC1 complexes. Unaltered levels of Raptor in the striatum of R6/1 mice might indicate that mTORC1 substrates are recruited to the mTOR kinase with the same efficiency as in control mice, thus leading to unaltered mTORC1 kinase activity.

Similarly to mTORC1, mTOR and some accessory proteins compose the complex mTORC2 (Sarbassov *et al.,* 2004). Deptor and mLST8/GβL are also present in this complex, and bind mTOR together with three mTORC2-specific accessory proteins, Rictor, Protor and mSIN1, to induce cell survival and actin cytoskeleton organization (Zoncu *et al.,* 2011). In contrast to Raptor, Rictor levels increase from en early age (8 weeks) onwards in the striatum of R6/1 mice when compared to their control littermates, and also in the putamen of Huntington's disease patients. Rictor, similarly to Raptor, has been found to be essential for the mTORC2 complex signalling. Loss of Rictor in worm, fly, mouse and human cells results in complete loss of AKT phosphorylation at Ser473 (Sarbassov *et al.,* 2005). Furthermore, Rictor knockout mice, which result in embryonic lethality, are, at the time of embryonic arrest (E10.5), slightly smaller and developmentally delayed compared to control littermates (Guertin *et al.,* 2006). In contrast, over-expression of Rictor has been detected in gliomas, which correlates with mTORC2 elevated activity and AKT phosphorylation (Masri *et al.,* 2007). Interestingly, disruption of the Rictor-mTOR complex also leads to a decrease in pSer473 AKT (Chen *et al.,* 2010), suggesting that not only protein levels of Rictor and mTOR are essential for AKT phosphorylation, but also their interaction in the mTORC2 complex is very important. Finally, over-expression of Rictor increases its association with mTOR and decreases the association of Raptor with mTOR, thus affecting mTORC1 activity (Chen *et al.,* 2010).

Downstream substrates of mTORC1 include S6K1, 4EBP1, and ULK1 (Beretta *et al.,* 1996; Fox *et al.,* 1998; Hosokawa *et al.,* 2009). While total levels of these three proteins were not altered in the brains of R6/1 mice, phosphorylated forms were differentially regulated. We have analyzed specific-mTOR phosphorylation levels of these substrates, and we show that while pSer371 S6K1 and pSer757 ULK1 were not altered at any time-point analyzed, phosphorylation at the Thr37/46 4EBP1 increases from early 8 weeks, and is maintained until the last age examined. Finally, we further show that phosphorylated levels of the S6K1-specific downstream substrate ribosomal protein S6 are neither altered, according to the pSer371 S6K1 levels. Thus, in the striatum of R6/1 mice, alterations in mTOR phosphorylation may not impact on protein translation through S6K1. Since the increase in phosphorylated 4EBP1 protein levels occurs at earlier time-points than pSer2481 mTOR, it is more likely that other

mechanisms would account for the regulation of 4EBP1 phosphorylation. We have only examined here mTOR and Raptor protein levels. However, it is believed that not only total protein levels of Raptor can modulate mTORC1 activity, but also phosphorylations on Raptor mediated by Rheb, RSK, AMPK and ERK, could finely tune mTORC1 activity (Carriere *et al.,* 2008; Carriere *et al.,* 2011; Gwinn *et al.,* 2008). mTORC1 containing phosphorylation site-defective Raptor exhibits reduced *in vitro* kinase activity toward the substrate 4EBP1 (Carriere *et al.,* 2008; Foster & Fingar, 2010). Interestingly RSK protein levels are increased at 8 weeks of age in the striatum of R6/1 mice (Xifro *et al.,* 2011), which could result in an increase in phosphorylated Raptor and have a role in the increase of pThr37/46 4EBP1. Another possibility that could lead to an increase in pThr37/46 4EBP1 levels earlier than the increase in the mTOR autophosphorylation, is that 4EBP1 could be phosphorylated in an mTOR-independent manner (Choo & Blenis, 2009). Interestingly, mTORC1 inhibition with rapamycin or the rapamycin derivative RAD001 affects S6K1 but not 4EBP1 phosphorylation status (Choo *et al.,* 2008; Nawroth *et al.,* 2011). It has been suggested that 4EBP1 phosphorylation is also regulated directly or indirectly by PI3K (Nawroth *et al.,* 2011). This could lead to miss-interpretation of mTORC1 activity *in vivo*. One last explanation for this differential substrate regulation could be due to the fact that S6K1 interacts more weakly with Raptor than 4EBP1 does (Schalm & Blenis, 2002; Schalm *et al.,* 2003). It would be interesting to know whether ULK1 binds mTORC1 as weakly as S6K1 does, since that would explain the differences that exist when analyzing the phosphorylation levels of the three substrates. It should be interesting to know whether protein levels and phosphorylation levels of the other accessory proteins present in the mTORC1 complex could also have some influence in directing the mTOR kinase towards a specific substrate. Finally, alterations in mTORC1 activity seem not to influence autophagy, since pSer757 ULK1 levels are not modified along the disease progression. Thus, the ULK1-Atg13-FIP200 complex activity might not result impaired, at least in an mTOR-dependent manner, and this complex would exert its function in autophagosome nucleation in R6/1 at the same rate as it does in control brains. In the striatum of R6/1 mice we have detected an over-activation of autophagy at an early stage of Huntington's disease (12 weeks), which returned to control levels at late stages of the pathology. At 12 weeks of age, autophagy could be induced in an mTOR-independent manner. Thus, it would be interesting to examine the levels of the proteins that compose the class III PI3K complex (Vps34, Beclin-1, Vps15 and Atg14) and the activity of the complex, since an increase in its activity could result in an induction of autophagy (Fimia *et al.,* 2007; Takahashi *et al.,* 2007).

An increase in Rictor protein levels, together with enhanced pSer2481 mTOR, could lead to an increased activity of the mTORC2 complex in the striatum of R6/1 mice brains. mTORC2 triggers phosphorylation on the hydrophobic motif of the AGC kinases AKT, SGK and PKCα (Gao *et al.,* 2005; Garcia-Martinez & Alessi, 2008; Ikenoue *et al.,* 2008; Sarbassov *et al.,* 2005). Consistent with our results showing increased Rictor protein levels from early stages of the disease, and of enhanced pSer2481 mTOR from 12 weeks of age onwards in the striatum of R6/1 mice, pSer473 AKT protein levels have been found increased also from 12 weeks of age onwards in the striatum of the same mouse model (Saavedra *et al.,* 2010). However, we failed to find further increase in the phosphorylation levels of SGK and PKCα. Total amounts of SGK and PKCα protein levels were down-regulated upon the disease progression, and so did their phosphorylated forms. Similarly to mTORC1, differences in the phosphorylation levels of mTORC2 substrates could be due to other factors altered in the pathology that would govern substrate-specificity, however very few data do exist about this issue. In contrast to mTORC1, where TOR signal motif-containing substrate is recognized by Raptor, no such a motif has been found in mTORC2 substrates. Since AKT and SGK interact with mTORC2, but not mTORC1, the existence of a parallel mechanism that might be operating for mTORC2 substrates has been suggested (Lu *et al.,* 2011). Interestingly, it has been shown that mSIN1, but not Rictor, recruits SGK, but not AKT, into the mTORC2 complex to undergo phosphorylation (Lu *et al.,* 2011). Although mTORC2 mediates AKT phosphorylation, it may use a distinct strategy independent of mSIN1, such as binding to Rictor, thus suggesting a mechanism for differential regulation of these two signalling kinases. Furthermore, as well as Raptor, Rictor can also undergo phosphorylation events at multiple sites, having a role in the mTORC2-mediated AKT phosphorylation (Chen *et al.,* 2011a). Interestingly, Rictor can also interact with Cullin1 in an mTOR-independent manner, acquiring the ability to ubiquitinate and degrade SGK (Gao *et al.,* 2010). Furthermore, in accordance with the decreased SGK protein levels detected in the striatum of R6/1 mice, the expression of SGK is elevated in Rictor null cells (Gao *et al.,* 2010), suggesting that a sustained induction of Rictor in the R6/1 could account for the reduction in the SGK total protein levels.

Summarizing, the AKT-mTOR pathway seems to be deregulated in the striatum of R6/1 mouse models of Huntington's disease. An increase in Rictor and phosphorylated mTOR levels could trigger enhanced activity of the complex mTORC2 to phosphorylate, specifically, AKT. Importantly AKT is a key protein in the regulation of cell survival. An increase in AKT phosphorylation and activity is known to induce cell

survival upon mutant huntingtin expression (Humbert *et al.,* 2002), with the implication of a broad branch of substrates. An early increase in pAKT, described in distinct Huntington's disease models, has been suggested to counteract the toxic effect triggered by mutant huntingtin, by phosphorylating and inactivating different pro-apoptotic substrates, such as FoxO and GSK3β (Gines *et al.,* 2003; Saavedra *et al.,* 2010). Furthermore, AKT is also known to phosphorylate mutant huntingtin at Ser421, reducing the caspase-mediated mutant huntingtin cleavage and its toxicity (Humbert *et al.,* 2002; Warby *et al.,* 2009). However, further experiments are needed in order to demonstrate that over-expression of Rictor together with increased pSer2481 mTOR, account for the already published increase in pSer473 AKT protein levels. One such experiment would be to examine by co-immunoprecipitation whether the increase in Rictor protein levels, observed in the striatum of R6/1 mice, entails in an increase in Rictor-mTOR interaction, and to analyze whether this results in enhanced AKT phosphorylation, using an *in vitro* activity kinase assay.

7 - REGULATION OF PKC ISOZYMES IN HUNTINGTON'S DISEASE PATHOLOGY

Decreased PHLPP1 protein levels in brains of R6/1 mice (Saavedra *et al.,* 2010) could also result in changes in the protein levels of PKCs (Gao *et al.,* 2008). Therefore, we have analyzed the protein levels of two cPKCs, PKCα and PKCβII, and one nPKC, PKCδ. Although different isozymes can trigger overlapping functions or even opposite effects depending on the cell type analyzed (Mischak *et al.,* 1993), PKCα and PKCβII are generally considered to induce cell survival, whereas PKCδ is classically involved in apoptosis (Reyland, 2007). We have shown a decrease of the three isoforms protein levels in three different R6/1 mouse brain areas. PKCδ is the isoform whose protein levels start to get reduced earlier in the pathology, and whose protein levels present a greater reduction when compared to control littermates. Moreover, PKCδ protein levels are also reduced in the putamen of Huntington's disease patients. Interestingly, this decrease gets accentuated along the progression of the disease and is not dependent on the brain region, since striatum, cortex and hippocampus present a similar PKCδ protein reduction. Many mechanisms, such as gene expression or PKC degradation could lead to the protein down-regulation that occurs for the three PKC isoforms along the progression of the disease in R6/1 mice brains. On one hand, mutant huntingtin has been related to affect several transcription activities by sequestering and rendering inactive many important transcriptional factors (Steffan *et al.,* 2000; Nucifora *et al.,* 2001). Consistent with our protein results, decreased PKCβII gene expression levels

have been described in R6/2 mouse brains (Harris *et al.,* 2001), which could result in less PKCβII protein levels. In the same direction, we analyzed PKCα and PKCδ mRNA levels, but failed to find alterations at any time-point of the disease, suggesting that the down-regulation of these isoforms could take place by another mechanism than gene expression. On the other hand, PKC stability is dependent on factors, such as phosphorylation and binding to scaffold proteins (Newton, 2010). Phosphorylation represents a key event that is required for PKC maturation, which primes PKC for second messenger-activation and protects it from degradation, since most unphosphorylated PKC isoforms are rapidly degraded (Newton, 2010). Interestingly, PKCs that have been activated by second messengers have an increased sensitivity to phosphatases (Dutil *et al.,* 1994), and prolonged PKC activation, as occurs with phorbol esters (functional analogues of diacylglycerol), results in the dephosphorylation and degradation of PKCs (Hansra *et al.,* 1999). PHLPP is known to dephosphorylate PKC specifically at the hydrophobic motif and shunt it for further dephosphorylation, ubiquitination and proteasomal degradation (Gao *et al.,* 2008; Newton, 2010). However, since PHLPP1 is already down-regulated in the R6/1 mouse model of Huntington's disease (Saavedra *et al.,* 2010), other mechanisms should account for the down-regulation of PKCs. Lack of enzyme activation could also lead to PKC degradation (Battaini & Pascale, 2005), and thus, it would be interesting to measure the levels of membrane-bound PKCα, PKCβII and PKCδ. Moreover, PKC binding to scaffold proteins, such as RACKs, AKAPs and 14-3-3 proteins helps in stabilizing the enzyme (Klauck *et al.,* 1996; Ron *et al.,* 1994; Van Der Hoeven *et al.,* 2000), so that alterations in those anchoring proteins could plausible lead to less sustained activation of PKCs and faster dephosphorylation and degradation.

8 - PUTATIVE MECHANISMS LEADING TO INCREASED PKCδ DEGRADATION IN HUNTINGTON'S DISEASE

It is noteworthy to mention that some PKC isoform-specific mechanism for signalling termination may exist (Melnikov & Sagi-Eisenberg, 2009; Srivastava *et al.,* 2002). Since the greatest reduction at a protein level was found for PKCδ, and this reduction could represent an early pro-survival compensatory mechanism activated in the R6/1 model of Huntington's disease, further experiments were performed in order to find the mechanism that would lead to PKCδ down-regulation. Interestingly, while dephosphorylation commonly triggers PKC degradation, experiments in which phosphatases were specifically inhibited revealed an increase in PKCδ degradation

(Srivastava *et al.,* 2002). A specific phosphorylation of PKCδ at Thr505 was found to prime PKCδ for degradation (Srivastava *et al.,* 2002). This phosphorylation site is located at the activation loop of PKCδ but, differing from other PKC isozymes, is not necessary for protein activation (Stempka *et al.,* 1997). PKCε has been suggested to trigger this phosphorylation on PKCδ (Garczarczyk *et al.,* 2009). We further analyzed this phosphorylation event in brains of R6/1 mice at two different ages, at 8 weeks of age, when PKCδ protein levels started to decrease, and at 30 weeks of age. We observed that the pThr505 PKCδ/PKCδ ratio was increased only at later stages of the disease in the striatum, cortex and hippocampus, correlating with decreased PKCδ protein levels. The absence of alterations in pThr505 PKCδ at 8 weeks of age, suggests that in the presence of mutant huntingtin other mechanisms would contribute to PKCδ degradation. These findings suggest that phosphorylation at Thr505 is enhanced at later stages of the disease and that this is an event that could participate in PKCδ down-regulation. However, there must be another mechanism that accounts for PKCδ down-regulation earlier in the pathology, such as ubiquitination. Thus, it would be interesting to also analyze the levels of ubiquitinated PKCδ.

9 - EFFECTS OF PKC DOWN-REGULATION UPON MUTANT HUNTINGTIN EXPRESSION

Down-regulation of PKCα, PKCβII and PKCδ could lead to different effects due to their functional disparity.

Loss of PKCα and PKCβII could trigger dysfunction and also account for the synaptic loss that has been observed in R6/1 mice (Spires *et al.,* 2004), as they have been implicated in the regulation of survival and also in changes of pre- and post-synaptic ultra-structure (Hongpaisan & Alkon, 2007). Furthermore, they could account for memory deficits in Huntington's disease, as their function has been tightly linked to memory storage (Nelson *et al.,* 2008), and its deficiency has been widely associated with memory-loss diseases such as Alzheimer's disease (Alkon *et al.,* 2007; Cole *et al.,* 1988; Masliah *et al.,* 1990). In particular, PKCα has a neuroprotective effect in an Alzheimer's disease mouse model upon its forced activation with bryostatin-1, which has been shown to restore synaptic loss, cognitive deficits and also reduce soluble β-amyloid levels (Hongpaisan *et al.,* 2011). Mice deficient in PKCβ showed normal brain anatomy and normal hippocampal synaptic transmission, normal long-term potentiation and normal sensory and motor responses, but exhibited a loss of learning (Weeber *et*

al., 2000). Interestingly, no motor alterations have been observed in PKCα and PKCβ-deficient mice, since they exhibit indistinguishable motor performance on the rota-rod when compared to control mice (Weeber *et al.,* 2000; Zhao *et al.,* 2011), thus suggesting that loss of PKCα and PKCβII could account for the cognitive deficits in Huntington's disease, but probably not for motor disturbances.

In contrast, PKCδ has emerged as a general pro-apoptotic intermediate in a broad number of cells, mainly in non-proliferating cells, including neurons (Fujii *et al.,* 2000; Maher 2001). Thus, PKCδ down-regulation could be beneficial for cells to avoid apoptosis upon mutant huntingtin expression. Many different cell types from PKCδ-null mice have a suppressed response to apoptotic agents *in vitro* (Humphries *et al.,* 2006; Leitges *et al.,* 2001; Allen-Petersen *et al.,* 2010). Most notably, parotid salivary glands from PKCδ-null mice lack efficient *in vivo* apoptotic response (Humphries *et al.,* 2006). Furthermore, PKCδ is up-regulated after transient global brain ischemia, and its induction precedes neuronal death (Koponen *et al.,* 2000). Upon apoptotic stimuli, PKCδ is phosphorylated at tyrosines 64 and 155 and translocates to the nucleus, where it is cleaved by caspase-3 at the hinge region generating the constitutive catalytic fragment δCF that is able to induce apoptosis (Humphries *et al.,* 2008). However, although this δCF is sufficient to induce apoptosis, it seems not to be that essential, and PKCδ-mediated apoptosis can still occur in the absence of proteolytic cleavage by caspase-3 (Fujii *et al.,* 2000; Maher 2001). Our results show a similar decrease of PKCδ in both cytoplasmic and nuclear compartments in R6/1 brains when compared to control littermates. In addition, we do not detect any difference in the protein levels of δCF, suggesting that PKCδ is not mediating cell death in response to mutant huntingtin expression. In contrast to our results, others have shown an association of three PKC isozymes, including PKCδ, with mutant huntingtin and ubiquitinated aggregates in cerebellar sections of R6/2 mice (Zemskov *et al.,* 2003). We failed to observe colocalization of ubiquitin-positive inclusions with PKCδ in R6/1 mice striatum. Since the mouse model that we have used for our study is very similar to the R6/2, this difference could be mainly due to the fact that in this study the authors analyzed cerebellar sections, whereas we have analyzed the striatum. We cannot discard that PKCδ protein dynamics behave distinctly in the cerebellum than in the striatum. In any case, association of PKCδ with ubiquitinated aggregates could also result in PKCδ inhibition. Finally, PKCδ not only regulates different apoptotic events, including caspase activation and DNA fragmentation, as well as loss of mitochondrial membrane potential (Matassa *et al.,* 2001), but also it has been suggested to play an important role in inducing mitochondrial fission in neurons upon oxidative stress (Qi *et*

al., 2011). Thus, early reduction of PKCδ protein levels in striatal, cortical and hippocampal cells could represent a pro-survival mechanism to avoid, or at least delay, neurodegeneration induced by mutant huntingtin. In accordance to this hypothesis, PKCδ-deficient mice do not present motor abnormalities, as examined with the rota-rod test, but loss PKCδ is protective and partially restores the motor deficits triggered by methamphetamine-induced dopaminergic toxicity (Shin *et al.,* 2011). This is consistent with our results showing that over-expression of PKCδ together with mutant huntingtin, but not with wild-type huntingtin, increases cell death in an immortalized striatal cell line.

10 - SELECTIVE AUTOPHAGY AND mTOR AND PKCδ SIGNALLING PATHWAYS, AS COMPENSATORY PRO-SURVIVAL MECHANISMS ACTIVATED IN HUNTINGTON'S DISEASE

Compensatory pro-survival mechanisms are essential to counteract mutant huntingtin-induced toxicity and delay the progression of Huntington's disease. We have studied here three different mechanisms along the progression of the disease in R6/1 mice brains that could enhance cell survival and delay cell dysfunction (Fig. 45).

We found an early activation in selective autophagy that was maintained in the cortex at late stages of the disease (Fig. 45), but returned to normal or even decreased levels in the striatum and hippocampus of R6/1 mice, respectively. Moreover, we have detected increased pmTOR and Rictor protein levels that could account for the over-activation of AKT and consequently for AKT-mediated cell survival. We failed to find differences in pSer757 ULK1 protein levels in the striatum of R6/1 mice, suggesting that early selective autophagy over-activation could be induced through an mTOR-independent pathway. Pharmacological induction of autophagy could be a good therapeutic approach to reduce the amount of the toxic protein. In fact, activation of mTOR-dependent autophagy with rapamycin attenuated the toxic effects of mutant huntingtin in fly and mouse models of Huntington's disease (Ravikumar *et al.,* 2004). However, rapamycin treatment has side-effects, and it is noteworthy to mention that prolonged inhibition of mTOR with rapamycin can also impair mTORC2 activity, by sequestering the cellular pool of mTOR in a complex with rapamycin linked to FKBP12, making it unavailable for assembly into mTORC2 (Sarbassov *et al.,* 2006). Inhibition of mTORC2 could impair the over-activation of the pro-survival kinase AKT in Huntington's disease. Moreover, mTOR inhibition could also lead to deficits in learning

and memory processes, since mTOR, at least at basal levels, regulates those processes (Garelick & Kennedy, 2011; Swiech *et al.,* 2008). Furthermore, rapamycin might modulate the levels of misfolded mutant huntingtin via other pathways than autophagy, since it decreases mutant huntingtin also in autophagy-deficient cells (King *et al.,* 2008). For all these reasons, we suggest that rather than inducing autophagy by inhibiting mTOR, a better approach might be the use of mTOR-independent autophagy enhancers, which, in fact, have already been suggested for treatment in Huntington's disease (Sarkar & Rubinsztein, 2008). Furthermore, from our point of view, the best time-point of the disease to administer and autophagy enhancer, would be at early stages of the disease, when both autophagy cargo receptors, p62 and NBR1, are available. We have shown that at later stages of the disease p62 gets sequestered into the nuclear mutant huntingtin aggregates, which could impair the efficiency of autophagy inducers in degrading cytoplasmic mutant huntingtin.

Finally, we have detected very low levels of the pro-apoptotic PKCδ in R6/1 brains when compared to their control littermates (Fig. 45). We have also found reduced levels of two other PKC isoforms, PKCα and PKCβII (Fig. 45). These results do not correlate with the decrease in PHLPP1 levels. Thus, other mechanisms must account for the reduction in PKC protein levels, such as low gene expression, low protein levels of anchoring proteins or low PKC activation. Since we have observed that mutant huntingtin expressing cells present enhanced susceptibility to PKCδ over-expression, decreased PKCδ protein levels could be added to the list of kinases that are deregulated in Huntington's disease to induce pro-survival signalling (Anglada-Huguet *et al.,* 2012; Gines *et al.,* 2003; Humbert *et al.,* 2002; Lievens *et al.,* 2002; Saavedra *et al.,* 2010, 2011; Xifro *et al.,* 2011).

Huntington's disease is a progressive neurodegenerative disease with the most common onset in adulthood, although mutant huntingtin is expressed from early in development. Interestingly, while cell death does not occur in almost any mouse model of the disease, mice develop motor and cognitive deficits (Table 1). The lack of cell death in these models lets hypothesize that these deficits are more related to cell dysfunction. Interestingly, in the conditional HD94 model of the disease, mutant huntingtin gene silencing in mice with already 20% cell death, protected not only from additional loss but also recovered mice from their motor deficits (Diaz-Hernandez *et al.,* 2005).

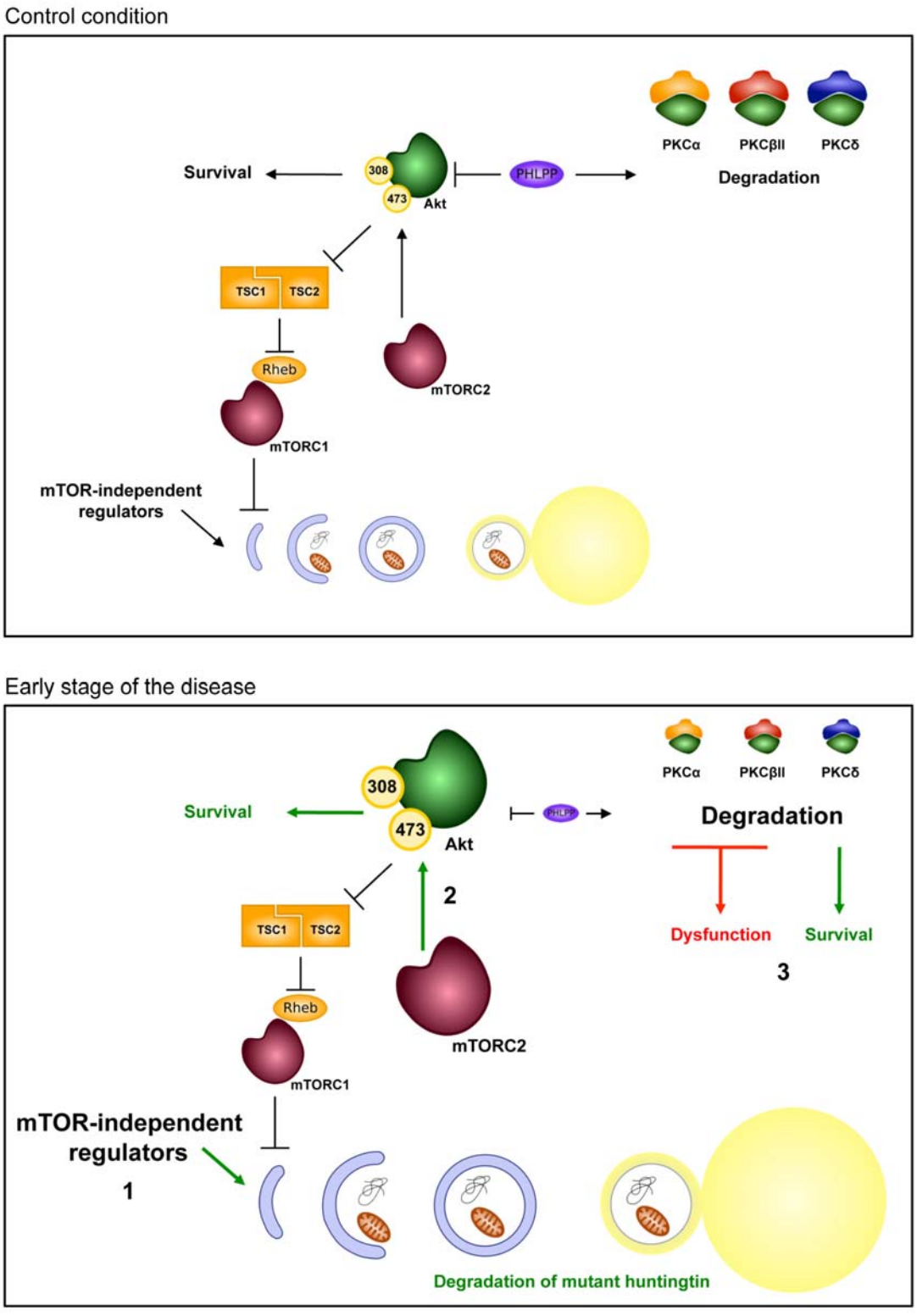

Figure 45. Scheme summarizing results obtained in this Thesis. (1) In an early stage of the disease, selective autophagy is increased by an mTOR-independent mechanism, suggesting an enhanced degradation of mutant huntingtin that could compensate for its toxicity. (2) Rictor levels could contribute to enhance mTORC2 activity, which, in turn, could lead to increased cell survival through AKT signalling. (3) Distinct PKCs protein levels are down-regulated in cells expressing mutant huntingtin. While decreases in PKCα and PKCβII could lead to cell dysfunction, down-regulation of PKCδ could trigger cell survival and counteract mutant huntingtin toxicity.

Thus, pro-survival mechanisms are highly important in Huntington's disease, and if in a therapeutical approach pro-survival mechanisms were potentiated while neurons are still alive, neuronal function might be improved and Huntington's disease progression might be delayed.

VI. CONCLUSIONS

1. Selective autophagic activity is deregulated in R6/1 mice brains in a region- and age-dependent manner, as monitored by the analyzis of NBR1 protein levels.

2. NBR1 might be a better marker than p62 to study selective autophagy flux at late stages of Huntington's disease, since p62 gets sequestered into the mutant huntingtin nuclear aggregates but NBR1 remains in the cytoplasm.

3. Age-dependent Xpo-1 protein levels decrease could contribute for the observed macroautophagy block at late stages of the disease.

4. Macroautophagy deregulation in the R6/1 mouse model of Huntington's disease does not seem related to mTOR activity, at least not in an ULK1-dependent manner in the striatum of R6/1 mice.

5. Striatal increased levels of Rictor and phosphorylated mTOR could contribute to enhance mTORC2 activity in striatal cells expressing mutant huntingtin.

6. Increased levels of striatal AKT phosphorylation, and the consequent pro-survival signalling, might be triggered not only by a decrease in PHLPP1 levels, but also by an increase in mTORC2 activity.

7. The pro-apoptotic protein PKCδ is down-regulated in Huntington's disease probably by an increase in its targeting for degradation.

8. Decreased levels of PKCδ could represent a common pro-survival compensatory mechanism in different cell types to delay neurodegeneration induced by mutant huntingtin.

VII. REFERENCES

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VIII. ANNEX

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Neurobiology of Disease

Striatal-Enriched Protein Tyrosine Phosphatase Expression and Activity in Huntington's Disease: A STEP in the Resistance to Excitotoxicity

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Striatal-enriched protein tyrosine phosphatase (STEP) is highly expressed in striatal projection neurons, the neuronal population most affected in Huntington's disease. Here, we examined STEP expression and phosphorylation, which regulates its activity, in N-terminal exon-1 and full-length mutant huntingtin mouse models. R6/1 mice displayed reduced STEP protein levels in the striatum and cortex, whereas its phosphorylation was increased in the striatum, cortex, and hippocampus. The early increase in striatal STEP phosphorylation levels correlated with a deregulation of the protein kinase A pathway, and decreased calcineurin activity at later stages further contributes to an enhancement of STEP phosphorylation and inactivation. Accordingly, we detected an accumulation of phosphorylated ERK2 and p38, two targets of STEP, in R6/1 mice striatum at advanced stages of the disease. Activation of STEP participates in excitotoxic-induced cell death. Because Huntington's disease mouse models develop resistance to excitotoxicity, we analyzed whether decreased STEP activity was involved in this process. After intrastriatal quinolinic acid (QUIN) injection, we detected higher phosphorylated STEP levels in R6/1 than in wild-type mice, suggesting that STEP inactivation could mediate neuroprotection in R6/1 striatum. In agreement, intrastriatal injection of TAT–STEP increased QUIN-induced cell death. R6/2, Tet/HD94, and Hdh$^{Q7/Q111}$ mice striatum also displayed decreased STEP protein and increased phosphorylation levels. In Tet/HD94 mice striatum, mutant huntingtin transgene shutdown reestablished STEP expression. In conclusion, the STEP pathway is severely downregulated in the presence of mutant huntingtin and may participate in compensatory mechanisms activated by striatal neurons that lead to resistance to excitotoxicity.

Introduction

Striatal-enriched protein tyrosine phosphatase (STEP), encoded by the *Ptpn5* gene, is a brain-specific phosphatase involved in neuronal signal transduction. STEP is enriched in the striatum (Lombroso et al., 1991) and expressed at lower levels in the cortex, hippocampus, and amygdala (Boulanger et al., 1995). STEP mRNA is alternatively spliced into the membrane-associated $STEP_{61}$ and the cytosolic $STEP_{46}$ (Bult et al., 1997). Both isoforms are expressed in the striatum, whereas the hippocampus and cortex only express $STEP_{61}$ (Boulanger et al., 1995).

STEP activity is regulated through phosphorylation/dephosphorylation of a serine residue within its kinase interacting motif domain. Stimulation of dopamine D_1 receptors (D_1Rs) activates the cAMP-dependent protein kinase A (PKA) (Stoof and Kebabian, 1981), which phosphorylates $STEP_{46}$ and $STEP_{61}$, thereby inactivating them (Paul et al., 2000). In contrast, glutamate stimulation of NMDA receptors (NMDARs) results in the dephosphorylation and activation of STEP through a calcineurin/PP1 pathway (Paul et al., 2003; Valjent et al., 2005). Once activated, STEP dephosphorylates the glutamate receptor subunits NR2B (Pelkey et al., 2002; Snyder et al., 2005; Braithwaite et al., 2006) and GluR2 (Zhang et al., 2008), leading to their endocytosis, and the kinases ERK1/2 (extracellular signal-regulated kinase 1/2), p38, and Fyn, thereby controlling the duration of their signal (Pulido et al., 1998; Nguyen et al., 2002; Muñoz et al., 2003; Paul et al., 2003).

Striatal projection neurons are specially affected in Huntington's disease (HD) (Reiner et al., 1988), a dominantly inherited

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MOLECULAR NEURODEGENERATION

RESEARCH ARTICLE Open Access

Increased 90-kDa ribosomal S6 kinase (Rsk) activity is protective against mutant huntingtin toxicity

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Abstract

Background: The 90-kDa ribosomal S6 kinase (Rsk) family is involved in cell survival. Rsk activation is regulated by sequential phosphorylations controlled by extracellular signal-regulated kinase (ERK) 1/2 and 3-phosphoinositide-dependent protein kinase 1 (PDK1). Altered ERK1/2 and PDK1 phosphorylation have been described in Huntington's disease (HD), characterized by the expression of mutant huntingtin (mhtt) and striatal degeneration. However, the role of Rsk in this neurodegenerative disease remains unknown. Here, we analyzed the protein levels, activity and role of Rsk in *in vivo* and *in vitro* HD models.

Results: We observed increased protein levels of Rsk1 and Rsk2 in the striatum of $Hdh^{Q111/Q111}$ and R6/1 mice, $STHdh^{Q111/Q111}$ cells and striatal cells transfected with full-length mhtt. Analysis of the phosphorylation of Rsk in Hdh mice and STHdh cells showed reduced levels of phospho Ser-380 (dependent on ERK1/2), whereas phosphorylation at Ser-221 (dependent on PDK1) was increased. Moreover, we found that elevated Rsk activity in $STHdh^{Q111/Q111}$ cells was mainly due to PDK1 activity, as assessed by transfection with Rsk mutant constructs. The increase of Rsk in $STHdh^{Q111/Q111}$ cells occurred in the cytosol and in the nucleus, which results in enhanced phosphorylation of both cytosolic and nuclear Rsk targets. Finally, pharmacological inhibition of Rsk, knock-down and overexpression experiments indicated that Rsk activity exerts a protective effect against mhtt-induced cell death in $STHdh^{Q7/Q7}$ cells transfected with mhtt.

Conclusion: The increase of Rsk levels and activity would act as a compensatory mechanism with capacity to prevent mhtt-mediated cell death. We propose Rsk as a good target for neuroprotective therapies in HD.

Keywords: cell death, ERK, Huntington's disease, knock-in mouse, neuroprotection, PDK1, R6/1 mouse, striatum

Background

The 90-kDa ribosomal S6 kinase (Rsk) is a family constituted by four isoforms (Rsk1-4) of serine/threonine kinases broadly expressed in the brain that regulate important cellular functions, including cell survival [1]. Rsk is activated by extracellular signal-regulated protein kinase (ERK) 1/2 [2] and 3-phosphoinositide-dependent protein kinase 1 (PDK1) [3] by sequential phosphorylations in the C-terminal kinase domain (CTKD) and N-terminal kinase domain (NTKD) [1,4], respectively. Briefly, sequential phosphorylations are initiated by ERK1/2 at Thr-573/574 of CTKD leading to the autophosphorylation of Rsk at Ser-380. This phosphorylation allows the dockage of PDK1 to the hydrophobic motif and enables PDK1-dependent phosphorylation in the NTKD of Rsk at Ser-221, resulting in its maximal activation [1,4]. When activated, Rsk promotes the phosphorylation of many cytosolic and nuclear targets. In the cytosol, Rsk induces the inactivation of certain pro-apoptotic proteins, such as Bad [5], glycogen synthase kinase 3β (GSK-3β) [6] or death-associated protein kinase (DAPK) [7], whereas in the nucleus it activates transcription factors involved in the synthesis of anti-apoptotic proteins, namely cAMP response element

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Submitted

Brain region- and age-dependent deregulation of p62 and NBR1 in Huntington's disease

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Running Title: p62 and NBR1 in Huntington's disease

Keywords: Aggregates, autophagy, R6/1 mouse

Abstract

Huntington's disease (HD) is characterized by the formation of protein aggregates, which can be degraded by macroautophagy. Here, we studied protein levels and intracellular distribution of p62 and NBR1, two macroautophagy cargo receptors, during disease progression. In R6/1 mice, p62 and NBR1 protein levels were decreased in all brain regions analyzed early in the disease, whereas at late stages they accumulated in the striatum and hippocampus, but not in the cortex. The accumulation of p62, but not NBR1, occurred in neuronal nuclei, where it co-localized with mutant huntingtin inclusions, both in R6/1 and HD patients. Moreover, exportin-1 was selectively decreased in old R6/1 mice brain, and could worsen p62 nuclear accumulation. In conclusion, p62 interacts with mutant huntingtin and is retained in the nucleus along the progression of the disease, mostly in striatal and hippocampal neurons. Thus, cytoplasmic NBR1 might be important to maintain basal levels of selective macroautophagy in these neurons. Finally, the analysis of NBR1 protein levels could be an indicator of selective macroautophagic activity in HD brain.

Reciprocal negative crosstalk between Liver X receptors and STAT1: effects on IFN-γ-induced inflammatory responses and LXR-dependent gene expression

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Running title: Negative crosstalk between LXRs and STAT1

Keywords: Monocytes/Macrophages, Microglia, Interferon-gamma, Liver X Receptors; Neuroinflammation; STAT1.

Abstract

Liver X receptors (LXRs) exert key functions in lipid homeostasis and in the negative control of inflammation. In this work we have explored the impact of LXR activation on the macrophage response to the endogenous inflammatory cytokine IFN-γ. Transcriptional profiling studies demonstrate that approximately 38% of the IFN-γ-induced transcriptional responses are repressed by LXR activation in macrophages. LXRs also mediated inhibitory effects on selected IFN-γ-induced genes in primary microglia and in a model of IFN-γ-induced neuroinflammation *in vivo*. LXR activation resulted in reduced STAT1 recruitment to the promoters tested in this study without affecting STAT1 phosphorylation. We have also analyzed whether IFN-γ signaling exerted reciprocal effects on LXR target genes. Treatment with IFN-γ interfered negatively, in a STAT1-dependent manner, with the capability of LXRs to upregulate selective targets, including ABCA1 and SREBP1c. Downregulation of ABCA1 expression correlated with decreased cholesterol efflux to apolipoprotein A1 in macrophages stimulated with IFN-γ. The inhibitory effects of IFN-γ on LXR signaling did not involve reduced binding of the LXR/RXR heterodimer to target gene promoters. The results shown in this study suggest an important level of bidirectional negative crosstalk between IFN-γ/STAT1 and LXRs with implications both in the control of IFN-γ-mediated immune responses and in the regulation of lipid metabolism.