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The role of 4E-BP1 in Huntington's disease: modulation of translation initiation as a therapeutic strategy

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RESUM

La malaltia de Huntington (MH) és un trastorn neurodegeneratiu hereditari dominant causat per una expansió d'un codó CAG en l'exó 1 del gen de la huntingtina (HTT). Els primers símptomes inclouen alteracions psiquiàtriques com la depressió o la irritabilitat a les quals progressivament s'uneixen alteracions cognitives i motores, ambdues relacionades amb la disfunció hipocampal i de la via corticoestriatal (Vonsattel et al., 1985). Tot i que durant els últims anys s'ha avançat de forma notable en el desenvolupament de tractaments dirigits a reduir l'expressió del producte del gen defectuós, no existeix encara cap tractament efectiu capaç d'aturar la progressió de la malatia. Per aquest motiu, la investigació dels mecanismes implicats en la neurodegeneració segueix sent clau per al disseny de noves teràpies pel control dels símptomes motors i conductuals relacionats amb la MH.

La desregulació de la traducció d'ARNm altera la viabilitat cel·lular i representa la causa molecular d'algunes malalties. Les neurones són especialment sensibles a la desregulació traduccional ja que aquesta és essencial per a la plasticitat sinàptica i la supervivència neuronal. Per tant, la regulació precisa de la iniciació de la traducció té un paper crític en els processos d'aprenentatge i memòria, i defectes en aquest procés s'han relacionat amb nombrosos trastorns cognitius incloent tant transtorns neurològics com l'addicció, la síndrome de l'X fràgil o l'autisme, com també diversos trastorns neurodegeneratius com la malaltia de Parkinson (Gkogkas et al., 2013; Martin et al., 2014; Santini et al., 2013, 2017).

La interacció entre els factors d'iniciació de la traducció eIF4E i eIF4G, per tant l'ensamblatge del complex eiF4F, és un punt de regulació clau en la iniciació de la traducció cap-depenent. Aquest mecanisme s'executa mitjançant una família de proteïnes anomenades proteïnes d'unió a eIF4E (4E-BPs; 4E-*Binding Protein*), sent 4E-BP1 la més estudiada. Funcionalment, 4E-BP1 actua com a factor repressor de la iniciació de la traducció cap-depenent i la seva activitat és regulada per fosforilació. Quan està actiu, i per tant, no fosforilat, s'uneix amb gran afinitat a eiF4E bloquejant la formació del complex de preiniciació 48S. Alternativament, el 4E-BP1 inactivat principalment per la quinasa mTOR, concretament per el complex 1 de mTOR (mTORC1), perd la seva afinitat per eIF4E i permet l'ensamblatge dels factors d'iniciació.

Resultats anteriors del nostre grup van descriure l'augment de la formació del complex proteic eIF4F i de la inactivació de 4E-BP1 acompanyat de l'augment aberrant de la taxa de traducció *de novo* a les neurones estriatals del ratolí R6/1, model de la MH. Tanmateix, els nivells de fosforilació de S6K1, un altre substrat de mTORC1 important, roman inalterat en aquest model, cosa que suggereix la implicació d'un mecanisme independent de mTOR. Curiosament, la infusió intracerebroventricular de 4EGI-1, un potent inhibidor de la interacció eIF4E i eIF4G, va ser capaç de normalitzar la traducció estriatal i millorar significativament els símptomes motors en ratolins R6/1. Aquests resultats donen suport a la hipòtesi de que un augment aberrant en la traducció cap-depenent contribueix en la disfunció neuronal i en la simptomatologia de la MH. Malgrat aquests resultats prometedors, la contribució precisa de la traducció cap-depenent a la progressió de la MH requereix més investigació per poder oferir opcions terapèutiques més precises. Per aquesta raó, en aquesta tesi hem avaluat el potencial de la modulació de l'activitat 4E-BP1, com a pas limitant clau en la iniciació de la síntesi de proteïnes cap-depenent, en la reducció de la patologia estriatal i hipocampal que es produeix en la MH. A més, hem explorat el potencial d'alguns fàrmacs genèrics ja aprovats per la FDA i àmpliament distribuïts per normalitzar l'augment de la traducció cap-depenent i millorar la simptomatologia replicant l'efecte observat amb la injecció intraventricular del 4EGI-1, que encara és un fàrmac experimental, en ratolins R6/1.

Malgrat l'existència d'estudis descrivint resultats contradictoris en l'activitat de la via de mTOR en la MH, el tractament amb 4EGI-1 en ratolins R6/1 suggereix que l'augment aberrant de síntesi proteica i la formació del complex eIF4F contribueixen als dèficits motors, la desregulació de l'energètica cel·lular i la disfunció sinàptica en els ratolins R6/1. Per tant, tot i que la HTT mutada (mHTT) podria estimular la traducció a través de vies alternatives, la inhibició de mTOR és capaç d'activar el repressor de traducció 4E-BP1 en el context molecular de la MH que podria ser suficient per exercir un efecte beneficiós sobre el fenotip. No obstant això, atès que s'ha demostrat que 4EGI-1 pot tenir altres dianes moleculars, un millor coneixement de la contribució de la inactivació de 4E-BP1 a la patologia de la MH és essencial per a oferir millors opcions de tractament. Per aquest motiu, en aquesta tesi, ens vàrem preguntar si la recuperació de l'activitat de 4E-BP1 era suficient per millorar la patologia estriatal en el model de ratolí R6/1. Per fer-ho, es va sobreexpressar una forma catalíticament activa de 4E-BP1 (4E-BP1^{F113A}) al nucli estriat de ratolins control (WT) i R6/1. Malauradament, la sobreexpressió de 4E-BP1^{F113A} no va normalitzar la síntesi de proteïnes al nucli estriat

de ratolins R6/1. En canvi, vam detectar un augment compensatori de la síntesi *de novo* de proteïnes y una hiperfosforilació del factor 4E-BP1 als ratolins WT que els va conduir al desenvolupament d'un fenotip motor i una degeneració estriatal semblant al dels ratolins R6/1. Malgrat inesperats, els nostres resultats donen suport a la hipòtesis que l'augment de la síntesi de proteïnes és un putatiu nou mecanisme molecular subjacent a la patogènesi de la MH.

Tot i que la inhibició de mTORC1 es pot aconseguir mitjançant inhibidors al·lostèrics clàssics com la rapamicina o els seus anàlegs, aquests afecten principalment la fosforilació del seu altre substrat S6K i només inhibeixen dèbilment la fosforilació de 4E-BP1. Atès que la generació de compostos amb capacitat inhibitòria selectiva de l'eix mTORC1/4E-BP1 encara és un repte per a la investigació, el desenvolupament i l'aplicació de models experimentals *in vitro* que repliquin la desregulació de la traducció cap-depenent és essencial pel cribratge inicial de nous compostos amb potencial terapèutic. Tanmateix, la replicació dels trets fenotípics de la MH depenen del model cel·lular. Com que els models transgènics de ratolí requereixen un número de repeticions CAG molt elevat per generar un fenotip, els cultius primaris de fibroblasts de pacients de la MH es van presentar com un model prometedor a causa de les seves longituds d'expansió de poliglutamina clínicament significatives. A més, el fet que derivin de teixit perifèric humà podria ser molt útil per definir biomarcadors moleculars precisos de la progressió de la MH. Tanmateix, els nostres resultats indiquen que els cultius primaris de fibroblasts no són útils per a aquest objectiu, ja que no es van trobar diferències entre genotips o estadis de la malaltia en els nivells de traducció. Aquests resultats acompanyats de les evidències que demostren que hi ha una correlació entre els nivells de traducció i la taxa de proliferació cel·lular, ens van suggerir la hipòtesi que les línies cel·lulars proliferants no eren aptes com a model *in vitro* per estudiar alteracions traduccionals en la MH. Per aquest motiu, el descobriment de l'augment de la inactivació de 4E-BP1 i la taxa de síntesi proteica en cultius neuronals estriatals dels ratolins R6/1 ens va conduir a l'ús d'aquests cultius com a model cel·lular per a la identificació de compostos amb la capacitat de normalitzar les alteracions traduccionals en la MH.

Aprofitant aquest model *in vitro*, en el següent apartat de la Tesi, hem identificat la sertralina, un inhibidor selectiu de la recaptació de serotonina (ISRS) ja aprovat per la FDA i àmpliament distribuït, amb fàcil translació a la clínica, com a possible opció

terapèutica per a la MH dirigida a la normalització de l'activitat de 4E-BP1 i de la traducció cap-depenent. Estudis previs duts a terme per grups independents assenyalen que el tractament crònic amb sertralina és capaç d'augmentar la supervivència, reduir l'atròfia estriatal i millorar el rendiment motor en la prova de rotarod dels models de ratolí transgènics R6/2 i N171-82Q. En ambdós casos, els efectes beneficiosos del tractament amb sertralina es van correlacionar amb una normalització dels nivells de BDNF i un augment en la taxa de neurogènesi adulta que atribueixen a la modulació del sistema serotoninèrgic ja que s'ha descrit que l'administració d'ISRS augmenta els nivells de BDNF. En la mateixa línia, el nostre treball amplia l'estudi de l'efecte del tractament crònic amb sertralina sobre la coordinació i l'aprenentatge motor i descriu la capacitat d'aquest fàrmac per normalitzar la síntesi de proteïnes i l'activitat de 4E-BP1 en el nucli estriat dels ratolins R6/1. Tenint en compte els efectes beneficiosos sobre la funció motora induïts per la normalització de la síntesi de proteïnes mitjançant el tractament amb 4EGI-1, aquests resultats suggereixen que aquest mecanisme podria estar involucrat, almenys en part, en els efectes beneficiosos del tractament amb sertralina sobre el fenotip descrits en diversos models animals de la MH.

Per confirmar els efectes beneficiosos del tractament amb sertralina en pacients de la MH, hem investigat l'impacte d'aquest tractament, prescrit a una cohort de pacients de MH com a teràpia per als símptomes depressius, en la progressió longitudinal de la malaltia a nivell motor i funcional durant un període de set anys utilitzant la base de dades Enroll-HD. Notablement, el tractament amb sertralina exhibeix un efecte beneficiós en retardar la disminució prevista del rendiment funcional d'aquesta cohort de pacients de la MH, però malauradament, no hem pogut identificar clarament els efectes positius o negatius de la sertralina sobre la funció motora a causa de la petita mida de la mostra.

Tot i que els símptomes cognitius i psiquiàtrics són els més devastadors per als pacients i les seves famílies, els assajos clínics es concentren principalment en els símptomes motors i la capacitat funcional general. En aquesta Tesi ampliem la hipòtesi de la desregulació de la traducció cap-depenent com a mecanisme molecular que contribueix a la patogènesi de la MH fora del nucli estriat. Els nostres resultats mostren que la inactivació aberrant de 4E-BP1 també afecta a l'hipocamp i a l'escorça motora dels ratolins R6/1. Aquests resultats suggereixen que les cèl·lules de l'hipocamp

podrien patir un canvi dràstic en el translatoma que podria resultar neurotòxic i generar estrès cel·lular. Com que la inactivació del factor 4E-BP1 augmenta la traducció d'un subconjunt específic d'ARNm's, ens hem preguntat si la desregulació de la traducció afecta a un conjunt de proteïnes concret. Per resoldre aquesta qüestió, hem aplicat el mètode SUnSET en talls de cervell que contenen l'hipocamp combinat amb l'anàlisi per espectrometria de masses dels pèptids naixents marcats amb puromicina per tal d'identificar les proteïnes sintetitzades *de novo* a l'hipocamp dels ratolins R6/1. L'anàlisi de vies KEGG ha revelat que la majoria de les proteïnes alterades estan relacionades amb vies metabòliques per a la producció d'energia i la utilització de nutrients com la glucòlisi i el metabolisme del piruvat. Curiosament, varis estudis independents han relacionat la traducció de gens implicats amb aquestes vies amb la inactivació del factor 4E-BP1. A més, hem detectat una alteració d'un nombre notable de proteïnes sinàptiques, majoritàriament regulades a la baixa, a l'hipocamp dels ratolins R6/1. Aquests resultats reforcen la hipòtesis de la importància de la desregulació del factor 4E-BP1 com a mecanisme patogènic en la MH.

A continuació, vam plantejar la hipòtesi que l'augment de la traducció a l'hipocamp podria estar contribuint al deteriorament cognitiu dels ratolins R6/1. La nostra investigació dona suport als resultats d'estudis anteriors que mostraven que els ratolins R6/1 exhibeixen dèficits de memòria i flexibilitat cognitiva, de manera similar als pacients de la MH i proporciona evidència que la desregulació de la traducció cap-depenent contribueix a la simptomatologia depenent de l'hipocamp en un model de ratolí de la MH. La normalització de la síntesi *de novo* de proteïnes en les cèl·lules hipocampals s'ha validat mitjançant l'aplicació del mètode SUnSET en talls hipocampals combinat amb l'anàlisi per espectrometria de masses utilitzat anteriorment en aquesta Tesi per avaluar els canvis de translatoma en els ratolins R6/1 en comparació amb els WT. Curiosament, hem detectat la recuperació de diverses proteïnes associades a la resposta a l'estrès metabòlic i oxidatiu. Notablement, hem detectat una augment de la traducció de la proteïna RBM3, una proteïna d'unió a ARNm de resposta a l'estrès coneguda per estimular la traducció global de proteïnes mitjançant la fosforilació de factors d'iniciació de la traducció i la formació activa de polisomes. Sorprenentment, s'ha descrit que la sobreexpressió de RBM3 augmenta la fosforilació d'eIF4E i 4E-BPs, per la qual cosa es presenta com possible causa molecular de la desregulació de l'activitat de 4E-BP1 en la MH. Finalment, com que els canvis en la traducció s'han relacionat amb una falta de plasticitat sinàptica, hem

avaluat l'LTP (*Long Term Potentiation*) a les regions CA3-CA1 de l'hipocamp per determinar si la millora cognitiva mostrada en els ratolins R6/1 tractats amb 4EGI-1 anava acompanyada d'una millora en la plasticitat sinàptica en aquesta via. Els nostres resultats mostren que l'administració de 4EGI-1 en els ratolins R6/1, tot i no assolir valors significatius, augmenta lleugerament l'efecte LTP en comparació amb els ratolins tractats amb vehicle.

Per últim, creixent evidència suggereix que la metformina, un fàrmac de primera línia per a la diabetis mellitus tipus 2, pot tenir efectes neuroprotectors en una varietat de trastorns cerebrals, inclosa la MH. Per exemple, s'ha descrit que l'administració de metformina al model de ratolí zQ175 millora els símptomes neuropsiquiàtrics i motors, i prevé la disminució del BDNF i l'agregació de mHTT. Addicionalment, l'ús de metformina també es va relacionar amb millors resultats en les proves cognitives i es va identificar una tendència favorable cap a la millora de la funció motora en un anàlisi estadístic d'una mostra de població de pacients de la base de dades Enroll-HD. D'altra banda, s'ha demostrat que la metformina actua com a inhibidor de mTORC1 mitjançant l'activació de la Ser/Thr quinasa AMPK que donaria lloc a una activació de 4E-BP1 i una disminució global de la síntesi de proteïnes. Com que prèviament hem vinculat la normalització de la síntesi de proteïnes i els nivells de fosforilació de 4E-BP1 amb una millora del fenotip motor en els ratolins R6/1, el nostre objectiu en aquesta part de la tesi va ser explorar la correlació entre l'efecte de la metformina sobre la síntesi de proteïnes i la millora del fenotip descrita anteriorment. Per fer-ho, hem investigat la capacitat de la metformina per normalitzar la síntesi de proteïnes cap-depenent en el context molecular de la MH. Els nostres resultats mostren que la metformina és capaç de disminuir els nivells de síntesi *de novo* de proteïnes, així com els nivells de fosforilació de 4E-BP1 en cultius de neurones estriatals primàries i en talls d'hipocamp dels ratolins R6/1. Malauradament, no hem pogut reproduir els resultats als talls corticoestriatals, tot i que es va detectar una tendència cap a la disminució. En conjunt, la nostra investigació mostra per primera vegada que la metformina és capaç d'inhibir la hiperfosforilació de 4E-BP1 en dos models de la MH i obren una nova línia d'investigació cap a la recerca de la via molecular exacta a través de la qual la metformina és capaç de reduir la traducció.

ABBREVIATIONS

43S PIC	43S Pre-Initiation Complex
4E-BP1	Eukaryotic Translation Initiation Factor 4E-Binding Protein 1
4E-BPs	4E-Binding Proteins
5-HT	Serotonin
5′ UTR	5' Untranslated Regions
AD	Alzheimer’s Disease
AMPK	AMP-Activated Protein Kinase
ASOs	Antisense Oligonucleotides
ATF4	Activating Transcription Factor 4
BAC	Bacterial Artificial Chromosome
BDNF	Brain Derived Neurotrophic Factor
CA	Cornu Ammonis
CDK1	Cyclin-Dependent Kinase 1
CDK12	C-Terminal Domain Kinase
CNS	Central Nervous System
DG	Dentate Gyrus
eEF2K	Eukaryotic Elongation Factor-2 Kinase
eIF2B	Eukaryotic Translation Initiation Factor 2B
eIF2α	Eukaryotic Initiation Factor 2α
eIF4A	Eukaryotic Initiation Factor 4A
eIF4E	Eukaryotic Initiation Factor 4E
eIF4F	Eukaryotic Initiation Factor 4F
eIF4G	Eukaryotic Initiation Factor 4G
eIFs	Eukaryotic Initiation Factors
ER	Endoplasmic Reticulum
FAS	Functional Assessment Scale
FMPR	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
GABA	Gamma-Aminobutyric Acid
GCN2	General Control Non-Depressible 2 Kinase
GEF	Guanine Nucleotide Exchange Factor
GP	Globus Pallidus
GPe	Globus Pallidus Externus
GPi	Globus Pallidus Internus

GSK3β	Glycogen Synthase Kinase 3 Beta
HD	Huntington's Disease
HRI	Heme-Regulated Inhibitor
HTT	Huntingtin (Human Gene)
HTT	Huntingtin (Human Protein)
Htt	Huntingtin (Mouse Gene)
Htt	Huntingtin (Mouse Protein)
iPSC	Induced Pluripotent Stem Cells
IRES	Internal Ribosome Entry Site
IS	Independence Score
LFS	Low Frequency Stimulation
LRRK2	Leucine-Rich Repeat Kinase 2
LTD	Long-Term Depression
LTP	Long-Term Potentiation
m*HTT*	Mutant HTT (Gene)
mHTT	Mutant HTT (Protein)
MSCs	Mesenchymal Stromal Cells
MSN	Medium-Sized Spiny Neuron
mTOR	Mechanistic Target Of Rapamycin
mTORC1	Mtor Complex 1
mTORC2	Mtor Complex 2
NII	Intranuclear Inclusions
NMDA	N-Methyl-D-Aspartate
OCD	Obsessive Compulsive Disorder
PABP	Poly(A)-Binding Protein
PD	Parkinson’s Disease
PERK	PKR-Like Endoplasmic Reticulum (ER) Kinase
PIM2	Proviral Integrations Of Moloney Virus
PKR	Protein Kinase RNA-Activated
PSD95	Postsynaptic Density Protein 95
RFs	Release Factors
Ribo-seq	Genome-Wide Ribosome Profiling
RNAi	RNA Interference
RNAPII	RNA Polymerase II

RSK	Ribosomal S6 Kinase
S6k	S6 Kinase
SN	Substantia Nigra
SNc	SN Pars Compacta
SNr	SN Pars Reticulata
SSRIs	Selective Serotonin Reuptake Inhibitors
STN	Subthalamic Nucleus
TFC	Total Functional Capacity
TMS	Total Motor Score
TOS	C-Terminal TOR Signalling Motif
TSC2	Tuberous Sclerosis Complex 2
UHDRS	Unified Huntington's Disease Rating Scale
UPS	Ubiquitin-Proteasome System
VMAT2	Vesicular Monoamine Transporter 2
WT	Wild-Type
YAC	Yeast Artificial Chromosome

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INTRODUCTION

2. HUNTINGTON'S DISEASE (HD)

2.1 History and etiology

HD is an inherited autosomal dominant neurodegenerative disorder described by George Huntington in 1982. Although other physicians described similar conditions in earlier times, Huntington's exhaustive characterization described in 1872 in a paper entitled 'On chorea' published in the Medical and Surgical Reporter earned him the eponym "Huntington's disease". He first referred to this disorder as Huntington's Chorea (from the Greek "choreia" meaning dance) given that the most distinguishing feature is a clonic spasm affecting voluntary muscles and voluntary movement impairment that progresses into a gradually increasing rigidity and stiffness. Cognitive and psychiatric symptoms also appear as the disease progresses (Huntington, 2003). After this publication, decades went by with little progress in the knowledge of this disorder until the 1970s decade when many affected families' associations were raised with the purpose of drawing the scientific community's attention. A crucial moment was the Centennial Symposium on Huntington's disease, in Columbus, Ohio, in 1972, where researchers and affected families gathered to commemorate Huntington's contribution and define future directions on the research of this disorder. In this line, this meeting put the spotlight on an interesting small fishing community from the shores of Lake Maracaibo in Venezuela where a local physician, Americo Negrette, had encountered an unusually high prevalence of HD due to a strong founder's effect. The rare features of this community allowed Negrette to accurately trace back the families' history and identify the dominant autosomal inheritance pattern of the disease (Okun & Thommi, 2004). After attending the meeting, Nancy Wexler, a psychoanalyst whose mother and grandfather were diagnosed with HD, flew to Venezuela and started an ambitious research project culminating with the identification of the genetic marker for HD. Together with James Gusella, they identified the HD causative gene and located it on chromosome 4p16.3 (Gusella et al., 1983).

2.2 Genetics

Ten years later, the causative mutation for HD was identified as an expansion of a polymorphic CAG trinucleotide repeat in the exon 1 of a new gene, *IT15*, which codes for a 350 kDa protein called huntingtin (HTT) (MacDonald et al., 1993). The $(CAG)_n$ repeat region codes for a polyglutamine chain (polyQ) that when exceeding more than 39

repeats leads invariantly to the expression of the HD phenotype (McNeil et al., 1997). Interestingly, *HTT* alleles containing 36 to 39 repeats present a form of incomplete penetrance of the disease, with a very late age of onset of the symptoms (Walker, 2007). Despite the dominant pattern of inheritance, individuals expressing between 27 and 35 CAG repeats, although they do not manifest the HD symptomatology, are at high risk of transmitting the disorder to their offspring (Killoran et al., 2013; Ranen et al., 1995). Interestingly, these individuals with an intermediate allele, first thought to be asymptomatic, were later observed to present a greater cognitive decline than healthy individuals (Kenney et al., 2007; Squitieri et al., 2011). Even though other factors are associated with the age of onset, CAG repeat length accounts for more than 60% of the total variance, with longer CAG repeats being associated with earlier onset and faster disease progression (Andrew et al., 1993; Djoussé et al., 2003; Duyao et al., 1993; Gusella et al., 2014). In fact, the most severe and rapidly progressing form of HD is the ultrarare juvenile form which starts presenting symptoms before the age of 20 and usually appears in patients carrying more than 60 CAG repeats (Quarrell et al., 2013). These cases are a result of genomic anticipation due to CAG instability. Although CAG repeat length changes occur when the transmission comes from both male and female parents, it seems to occur more frequently and result in larger CAG expansions with paternal transmission since CAG instability seems higher in spermatogenesis than oogenesis (Duyao et al., 1993; Kremer et al., 1995; Telenius et al., 1994).

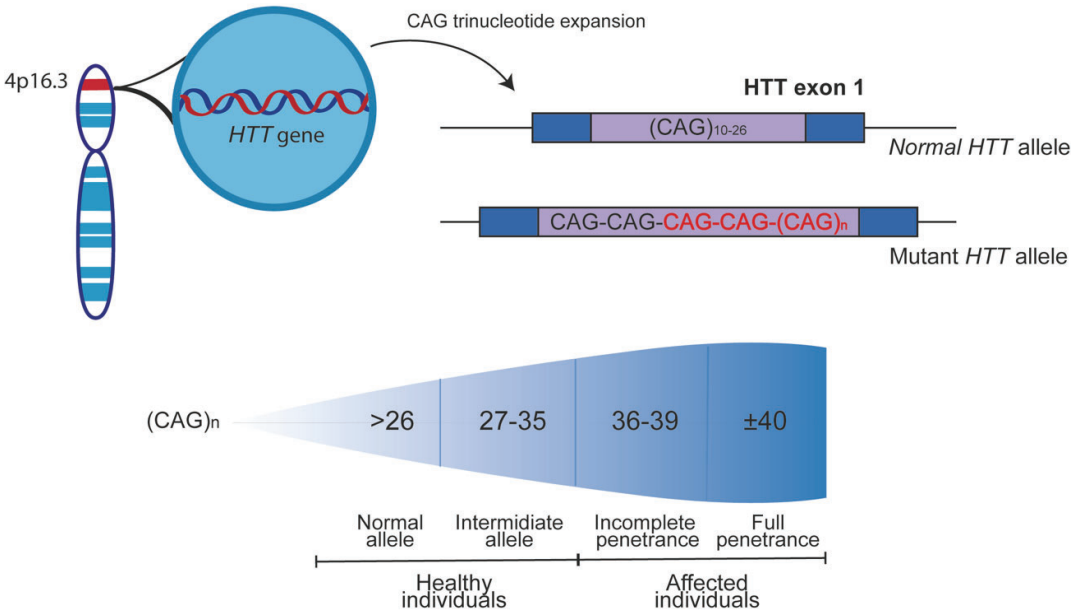


Figure 1.- HD causative mutation Representation of the chromosome localization and HD mutation in the HTT gene (top). Expected phenotypes corresponding to the CAG repeat expansion are depicted (bottom).

2.3 Epidemiology

In the matter of epidemiology, although HD cases occur in all human populations, the prevalence of this disease is highly dependent on geographical factors. As reviewed by Pringsheim et al. (2012), HD is much more common in Caucasians and hence, populations of European descent, being European migration a determining historic factor of its prevalence. In broad terms, the prevalence is higher in North America, Europe, and Australia reaching 5.70 per 100,000 people and it is notably rarer in Asia with an incidence of 0.40 per 100,000 people.

2.4 Clinical aspects

HD symptomatology is defined by a triad of motor, psychiatric and cognitive deficits that appear at an average age of onset of 45 years and causes the life expectancy to drop to 15-20 years after diagnosis due to disease-related complications such as pneumonia and cardiovascular diseases (Bates et al., 2015; Sørensen & Fenger, 1992). Before HD diagnosis, asymptomatic patients who carry the HD mutation are termed “premanifest”. Generally, the diagnosis is usually based on the appearance of motor symptoms that designate the start of the “manifest” disease stage. However, in the “premanifest” stage, patients usually start experiencing subtle symptoms, mainly cognitive and psychiatric, labelled by clinicians as “prodromal” symptoms (Stout et al., 2011). In this “prodromal” phase, patients still do not fulfil all diagnostic criteria for an HD diagnosis but are expected to develop concrete motor symptoms soon.

As mentioned earlier, motor symptoms in early and moderate stages appear as involuntary movements and then progress to voluntary movement impairment in later stages. The most striking symptom is the chorea, the term used to designate the hyperkinetic stage with involuntary and excessive movements that start with the twitching of facial muscles and distal extremities and extend to the entire body. Choreatic movements, often accompanied by dystonia, myoclonic jerks and tics, although at first may not compromise patients’ quality of life to a great extent, they gradually lead to dysarthria, dysphagia and constant loss of balance. Bradykinesia and rigidity overcome the hyperkinetic state as the disease progresses exacerbating gait problems and falls (Ghosh & Tabrizi, 2018). Nonetheless, the balance between hyper- and hypokinesia varies from patient to patient. Although clinical diagnosis depends

mainly on motor symptoms onset, subtle cognitive deficits and behavioural signs appear as far as 15 years before such as deficits in visual attention, psychomotor speed and visuospatial abilities (Duff et al., 2007; Paulsen et al., 2001; Tabrizi et al., 2013). HD is classified as a subcortical dementia and in later stages, it presents with alterations in executive function, information processing, verbal fluency and procedural and explicit motor learning (reviewed in Giralt et al., 2012). Prevalent and invalidating psychiatric symptoms are also present at different points of the disease progression including major depression, apathy, anxiety, irritability, mania and psychosis (Paoli et al., 2017). HD population also presents an elevated suicidality rate, with major depression as the main risk factor (Hubers et al., 2012). Finally, HD patients also suffer from alterations in peripheral tissues such as weight loss, skeletal muscle wasting and osteoporosis and metabolic and immune disturbances (van der Burg et al., 2009).

2.5 Neuropathology

The most remarkable and first described neuropathological feature in the HD brain is the bilateral neurodegeneration of the neostriatum which shrinks in correlation with disease severity and progression (Vonsattel & DiFiglia, 1998). This striatal atrophy and astrogliosis appear in a very distinctive and consistent spatial and temporal pattern which allowed the establishment of the Vonsattel grading system based on the striatal macroscopic appearance, the visible microscopic extent of the striatal neuronal loss and degree of reactive astrogliosis. This grading system classifies HD brain into five grades, from 0 to 4. Brains classified as grade 0 present normal macroscopic features and no astrogliosis, but histological examination shows 30-40% neuronal loss in the caudate. Astrogliosis and striatal atrophy progressively increase in correlation with Vonsattel grades reaching grade 4 when the striatal neuronal loss extends to 95%. Neurodegeneration is cell-type specific and affects the GABAergic neuronal population of medium-sized spiny neurons (MSNs) that represents 90-95% of total striatal neurons (Kassubek et al., 2004; Vonsattel & DiFiglia, 1998).

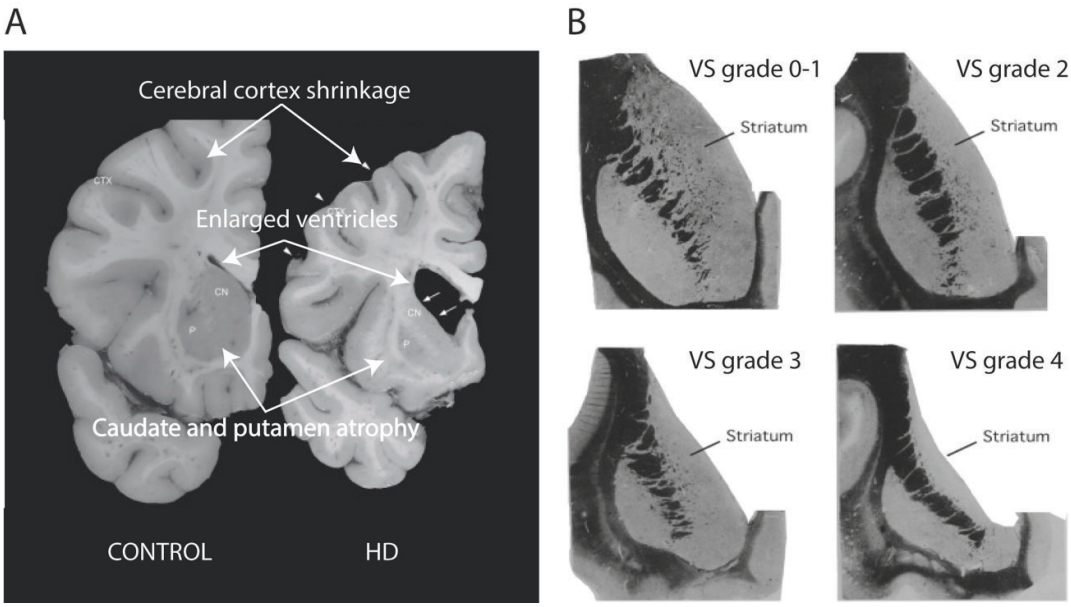


Figure 2.- Neuropathology of HD. (A) Coronal sections from the left hemisphere of human brain at the level of the striatum from a control case of a 35-year-old male and a VS grade 3/4 HD patient. CN: caudate nucleus; CTX: cerebral cortex; P: putamen. (B) Schematic illustrations of caudate at HD Grades 0-4 according to the Vonsattel scale. This illustration is adapted from Reiner et al. (2011) and Waldvogel et al. (2014).

However, the reductionistic view of HD as a neurodegenerative disorder exclusively confined to the caudate and putamen (striatum) has been gradually replaced by a multisystemic view with a broader distribution pattern of neurodegeneration also affecting other areas. For instance, the complex interconnection between the subcortical nuclei forming the basal ganglia leads to major effects on the areas receiving projections from the striatum, mainly the globus pallidus (GP) and substantia nigra pars reticulata (SNr). Additionally, in advanced cases, the cerebral cortex is itself severely affected, especially layers III, V and VI. In fact, cortical neuropathology in HD is very variable between patients and disease stages, which leads to a broad spectrum of symptoms (Halliday et al., 1998; Hedreen et al., 1991; Rosas et al., 2002; Thu et al., 2010; Tippett et al., 2007). Furthermore, although at first the hippocampus was believed to be spared from degeneration, significant volume reductions, neuronal loss and reactive gliosis have been later observed in this region. Specifically, the selective vulnerability of the CA1 hippocampal region has been observed (Spargo et al., 1993). In general, although in Grades 0, 1 and 2 non-striatal structures of the brain are apparently normal, or show only mild atrophy, structures such as the neocortex, the GP, the

substantia nigra (SN), the hippocampus, the thalamus, the subthalamic nucleus, the white matter, and the cerebellum appear smaller than normally expected in more advanced stages of HD (Vonsattel & DiFiglia, 1998).

2.6 Pathophysiology

The functional implications of HD neuropathology can be explained by the circuitry and connectivity alterations derived from it. In this regard, motor symptomatology in HD can be attributed to a very specific pattern of cortico-striatal dysfunction. Basal ganglia, including the striatum, the GP, the subthalamic nucleus (STN) and the SN are a group of interconnected cortical nuclei that play an important role in motor function and learning. The main afferent basal ganglia nucleus is the striatum, which mainly receives excitatory glutamatergic input from the cortex but also from the thalamus as well as dopaminergic projections from the SN pars compacta (SNc) that modulate cortico-striatal synapses (Fonnum et al., 1981). The fore-mentioned MSNs, GABAergic neurons that represent 90-95% of the striatal neuronal population and present selective neurodegeneration in HD, process and integrate these excitatory inputs and project to other nuclei through two main inhibitory pathways: direct and indirect (Kemp & Powell, 1971; Vonsattel et al., 1985). Both pathways are considered to control movement in opposing ways, initiating and inhibiting movement, respectively. Consequently, a fine balance between both circuits is essential for smooth movement control (Alexander & Crutcher, 1990). On the one hand, the direct pathway starts with the cortical and thalamic glutamatergic excitation of a population of striatal MSNs expressing substance P, dynorphin, and D1-like dopamine receptor which in turn inhibit the GP internus (GPi) and SNr. The inhibition of the GABAergic neurons in these nuclei ultimately results in a decrease in the inhibition of the thalamus, and therefore, the excitation of the motor cortex which translates into the initiation of voluntary movement (Albin et al., 1989; DeLong, 1990). On the other hand, the striatal MSNs from the indirect pathway, expressing enkephalin (enk) and D2-like dopamine receptors, inhibit the GABAergic neurons from the GP externus (GPe) which results in less inhibition in the STN and culminates in the excitation of the GABAergic neurons in GPi and SNr resulting in greater inhibition of the thalamus and signalling to the motor cortex (Alexander & Crutcher, 1990).

Interestingly, the temporal pattern of these pathways' dysfunction correlates with the observed symptomatology in HD patients, being the MSNs from the indirect pathway

the first ones to degenerate resulting in a hyperkinetic or choreic phenotype in the early stages and progressing to rigidity and bradykinesia with the later dysfunction of the direct pathway (Deng et al., 2004).

Although cognitive deficits were first attributed to cortico-striatal dysfunction, it is now well-accepted that non-striatal dysfunction also contributes to HD non-motor symptoms (Ruocco et al., 2006). Hippocampal HD pathology is of particular interest in this thesis as growing evidence suggests its role as an important contributor to cognitive symptoms including not only the appearance of mild cognitive impairment in pre-motor stages of the disease but also the presence of deficits in classical hippocampal-dependent cognitive functions such as episodic memory, visuospatial perception and executive function in the clinical stages (Duff et al., 2010). Anatomically, the hippocampus is part of a larger structure called the hippocampal formation composed of the dentate gyrus (DG), the hippocampus proper and the subiculum. In like manner, the hippocampus proper is formed by the cornu ammonis (CA) fields: the CA1 and CA3 fields and the smaller, much less studied, CA2 field. The simplicity of the hippocampal circuitry compared to other brain structures due to the unidirectionality of its main projections has been a great appeal for researchers in search of a simpler model for electrophysiology studies. Consequently, synaptic plasticity models such as long-term potentiation (LTP) and depression (LTD) are well-established in the hippocampus and have been deeply studied in the HD context through animal models (Bliss & Lømo, 1973). Although basal neurotransmission appears normal at early stages, alterations in hippocampal synaptic plasticity, both short- and long-term, have been described in different HD models. For instance, deficient LTP induction and maintenance are described in the R6/1 and R6/2 mouse models of HD before the appearance of any symptomatology, suggesting that cognitive decline in HD might be attributed to dysfunctional plasticity. In addition, both models show an aberrant N-methyl-D-aspartate (NMDA) receptor-dependent form of LTD induced by low-frequency stimulation (LFS) suggesting the existence of a tendency towards synaptic depression in these models (Milnerwood et al., 2006; K. P. S. J. Murphy et al., 2000). Notably, impaired hippocampal LTP has also been shown in pre-symptomatic knock-in mouse models of HD (HdhQ92 and HdhQ111) and CAG140 HD mice and shown to be restored by Brain Derived Neurotrophic Factor (BDNF) administration (Brito et al., 2014; Lynch et al., 2007; Simmons et al., 2009). Intrinsic membrane properties are also altered in R6/1 mouse hippocampal neurons at later stages suggesting that alterations in synaptic

plasticity have a synaptic origin (Milnerwood et al., 2006). Specifically, spared paired-pulse facilitation indicates that dysfunction mainly affects post-synaptic level (K. P. S. J. Murphy et al., 2000). Although it is well-known that NMDA receptor function is essential for hippocampal LTP, they seem to be unaltered at protein level in early stages, supporting the idea that their signalling pathways are altered at an intracellular level (Cepeda et al., 2001; Jarabek et al., 2004; Torres-Peraza et al., 2008).

2.7 Mechanisms of neurodegeneration

HTT is a large protein containing over 3100 amino acids and a molecular mass of 350 kDa depending on the length of the polyglutamine chain (MacDonald et al., 1993). Although ubiquitously expressed throughout the body and regardless of the cell type, it is highly expressed in the testis and brain, concretely in the neocortex, striatum, hippocampus and cerebellum (Borrell-Pagès et al., 2006; Trottier et al., 1995). Cellular localization studies have reported a close association of the wild-type (WT) HTT with organelles such as the Golgi apparatus, the endoplasmic reticulum, mitochondria, synaptic vesicles and the cytoskeleton (Landles & Bates, 2004). At a lower degree, it is also localized inside the nucleus (Kegel et al., 2002). While its function has not been entirely elucidated, WT HTT binds to numerous proteins exerting its role in a variety of intracellular functions including protein trafficking, vesicle transport and anchoring to the cytoskeleton, clathrin-mediated endocytosis, postsynaptic signalling, transcriptional regulation and anti-apoptotic function. For instance, WT HTT interacts with postsynaptic density 95 (PSD95) regulating post-synaptic NMDA receptor dynamics (Y. Sun et al., 2001). WT HTT is also relevant for the regulation of BDNF levels by preventing the nuclear translocation of the transcription factor complex REST-NRSF and promoting BDNF vesicular transport (Gauthier et al., 2004; Zuccato et al., 2003). Importantly, WT HTT has been demonstrated to have an anti-apoptotic role by preventing the formation of a functional apoptosome complex and the consequent activation of caspase-9 and caspase-3 or physically interacting with active caspase-3 and inhibiting its proteolytic activity (Rigamonti et al., 2000; Y. Zhang et al., 2006). Thus, loss-of-function effects due to the decrease in WT HTT expression or its sequestration into mutant HTT (mHTT) aggregates are suggested to contribute to HD pathology. However, it is believed to be the sum of both, the toxic gain of function of the mutant form and the loss of function of the WT protein that lead to the dysregulation of many canonical intracellular pathways culminating in the observed neurodegeneration.

The pathogenic mechanisms triggered by mHTT contribute at different levels to HD pathology. These, which have been explored through different *in vitro*, *in vivo* and post-mortem studies, include abnormal protein aggregation, NMDA receptor-mediated excitotoxicity, mitochondrial dysfunction, dysregulated autophagy, and disrupted gene transcription, as well as loss of trophic support. As described in other polyglutamine diseases, mHTT forms perinuclear accumulations of mHTT, named intranuclear inclusions (NII), as well as neurital **protein aggregates** that are localized mainly in neurons from the cortex and striatum, the most affected regions in HD (DiFiglia et al., 1997; Sapp et al., 1999). However, their role in the disease pathogeny is not clear as the higher frequency of aggregates does not correspond to the most vulnerable cell types in HD. In fact, interneurons that are spared are the ones showing the highest aggregate frequency. Hence, this fact raises the question of whether aggregates could play a neuroprotective role instead of contributing to mHTT toxicity (Kuemmerle et al., 1999). On the other hand, the presence of mHTT is hypothesized to exert **NMDA receptor-mediated excitotoxicity** to the GABAergic MSN population due to an aberrant increase in NMDA receptor function. This hyperactivation due to, among other mechanisms, an increase in glutamate release from cortical afferents and a compromised glutamate uptake from glial cells culminates in a disruption of calcium homeostasis and mitochondrial function leading to cellular death (Arzberger et al., 1997; Landwehrmeyer et al., 1995). **Decreased neurotrophic support** is another main pathological mechanism in HD. As described above, in addition to the suggested deleterious effects of the loss of the WT HTT function, mHTT also reduces BDNF transcription levels, which suggests that the lower cortical BDNF levels in HD are due to both the loss of WT HTT function and increased toxicity of the mutant form (Zuccato et al., 2001, 2005). Along with the processes described above, **autophagy dysfunction** has also been associated with HD. On the one hand, as the disease progresses, normal clearance pathways are unable to degrade mHTT aggregates (Pircs et al., 2018; Weihl, 2013). Moreover, autophagosomes impaired ability to recognize their cytosolic target cargo leads to a net imbalance of protein degradation and energy expenditure (Martinez-Vicente et al., 2010). In support, autophagic system failure is thought to contribute to HD neuronal death and is also associated with the **mitochondrial dysfunction** observed in HD patients and mouse models through the accumulation of defective mitochondria (Cortes & la Spada, 2014; Kamat et al., 2014; Koga et al., 2011). Furthermore, mHTT directly interacts with mitochondrial proteins affecting processes such as mitochondrial fragmentation which affects energy production and increases

oxidative stress resulting in increased cell death (Knott et al., 2008; Knott & Bossy-Wetzel, 2008). Lastly, **transcription dysregulation** has been described in transcriptional profiling studies in HD human brain samples and several disease models. These progressive changes in gene expression, usually attributed to aberrant interactions of mHTT with transcription factors, include many neuronal genes namely neurotransmitters, neurotrophins and their receptors, as well as genes involved in stress-response pathways (Cha, 2007; Helmlinger et al., 2006; Ross & Tabrizi, 2011). Relevant dysregulated genes include *BDNF*, dopamine receptors 2 and 1a (DRD2, DRD1A) and protein phosphatase 1 regulatory subunit 1B (*PPP1R1B*) (Hodges et al., 2006; Kuhn et al., 2007; Seredenina & Luthi-Carter, 2012; Vashishtha et al., 2013; Yildirim et al., 2019).

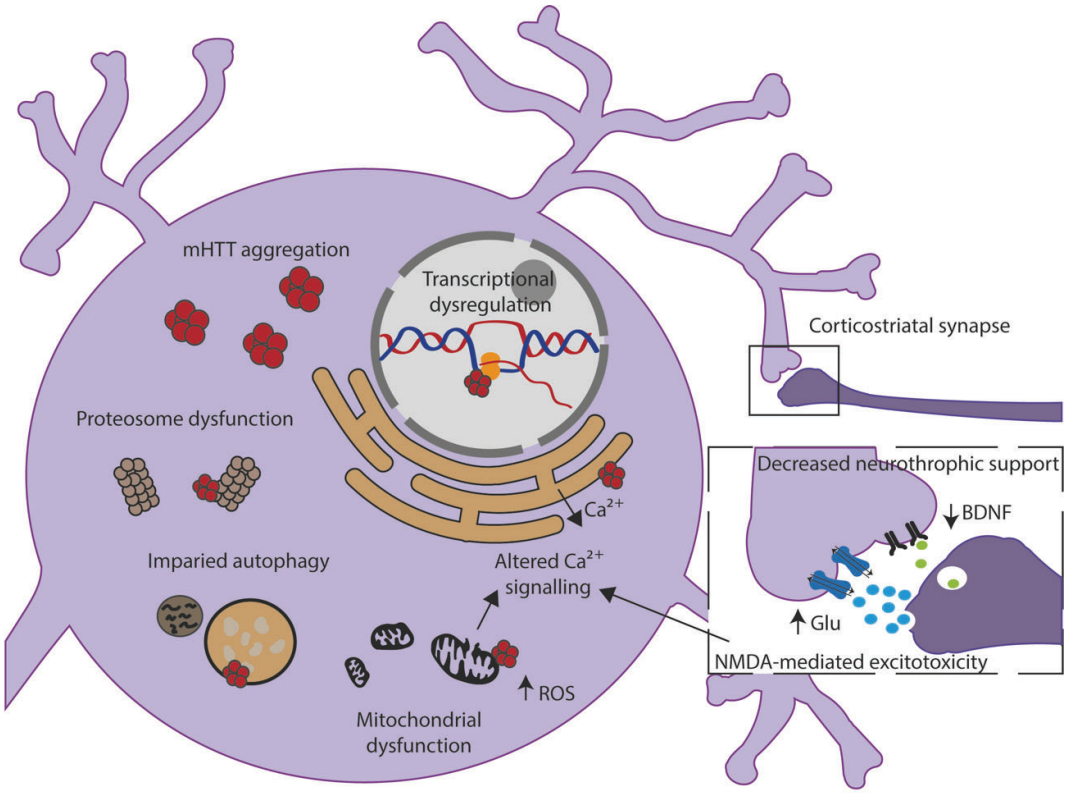


Figure 3.- Molecular mechanisms involved in the pathogenesis of HD. Schematic representation of the disrupted molecular pathways by mHTT products involved in striatal neurons vulnerability in HD.

3. HD MODELS

3.1 HD animal models

The discovery of the causative HD mutation in 1993 (MacDonald et al., 1993) enabled the generation of genetically engineered animal models from simple model organisms such as the fly fruit, C. elegans and zebrafish to mammalian species (Brignull et al., 2006; Karlovich et al., 1998; Marsh et al., 2003). Although non-mammalian organisms have been very useful in the identification of novel therapeutic targets in early research due to their easy genetic manipulation, the findings still needed further validation in higher complexity organisms, being the mice, the most used (Farshim & Bates, 2018). However, in the last decade, while murine models still dominate the current HD research field, big steps have been taken into the generation and characterization of larger animal models that could more accurately reproduce the human HD phenotype and disease progression such as rats, minipigs, sheep and non-human primates (Howland et al., 2020). In fact, preclinical studies on the TgHD (N548) minipig model have recently enabled, for the first time, the FDA approval of an HTT-lowering therapeutic approach based on a study performed on a large HD animal model resulting in the start of a Phase I/II clinical trial (Evers et al., 2018).

3.2 HD mouse models

Until the generation of the first genetic mouse model of HD in 1996, only toxin-induced models based on the administration of quinolinic and 3-nitropropionic acids were accessible (Beal et al., 1991; Ouary et al., 2000). These models are still used as acute models to study neuroprotective and cell replacement therapies because they reproduce the characteristic striatal cell loss. However, they do not replicate HD neuropathological features and distinctive slow progression.

With the identification of the HD mutation (MacDonald et al., 1993), as mentioned above, came the generation of the first genetic mouse model in the laboratory of Gillian Bates which provided a valuable tool for the study of the disease progression (Mangiarini et al., 1996). Since then, diverse HD mouse models have been created to address specific mechanistic questions and can be categorized into two main groups regarding the expression of mHTT: transgenic (n-terminal or full-length) or knock-in models. The main difference between them is that the gene insertion to generate knock-in models was

targeted specifically to the mouse *HTT* gene locus while in transgenic models it was randomly inserted in the genome with its own promoter leading to higher levels of expression.

3.3 Transgenic n-terminal fragment mouse models

As previously mentioned, the first HD transgenic mice were the R6/1 and R6/2 mice. They both express the N-terminal fragment of the human *HTT* gene containing promoter sequences, the exon 1 and approximately 262 bp of intron 1. The inserted fragment is translated to an exon 1 HTT protein which shows that the expression of this mutant exon 1 fragment is sufficient to produce HD-related phenotypes. When first developed, R6/1 and R6/2 mice expressed 116 and 144 CAG repeats respectively, however, CAG repeats are unstable through generations. Both mouse models are the first chosen for the evaluation of therapeutic approaches as they show the fastest progression. The R6/1 mice, which is the animal model used in this thesis and will be explained in more detail, show decreased lifespan (32-40 weeks) and decreased weight loss starting at 22 weeks of age, although it appears very variable between individuals (J. Y. Li et al., 2005). It develops progressive neuropathological changes that advance in correlation with evident symptomatology including enlargement of the ventricles, the presence of mHTT aggregates and neuronal degeneration. Appealingly, the R6/1 mouse develops cognitive deficits before motor symptoms appear, mimicking the pattern of the disease progression in HD patients and confirming the presence of cortical and hippocampal dysfunction in this model (Puigdellívol et al., 2016). The main downside of this model is the lack of cell death, which is an important neuropathological hallmark of HD (Francelle et al., 2014). On balance, the R6/1 model appears to be a good model for preclinical studies for new drug development as it shows early onset molecular biomarkers, and the slow progression of the disease makes it suitable for both pre- and symptomatic studies as well as chronic treatments with slow-acting drugs. Moreover, its strong symptomatology permits a better quantification of behavioural changes that can be unnoticeable in other models with milder affection (Naver et al., 2003). R6/2 mice recapitulate R6/1 features but show a much faster progression with a lifespan critically decreased to 10-13 weeks of age difficulting the evaluation of the changes in the symptomatology. Finally, other N-terminal mouse models were generated later, such as the N171-82Q (Schilling et al., 1999). In this model, the *HTT* human gene fragment is expressed under the mouse prion protein restricting the expression mainly to neurons.

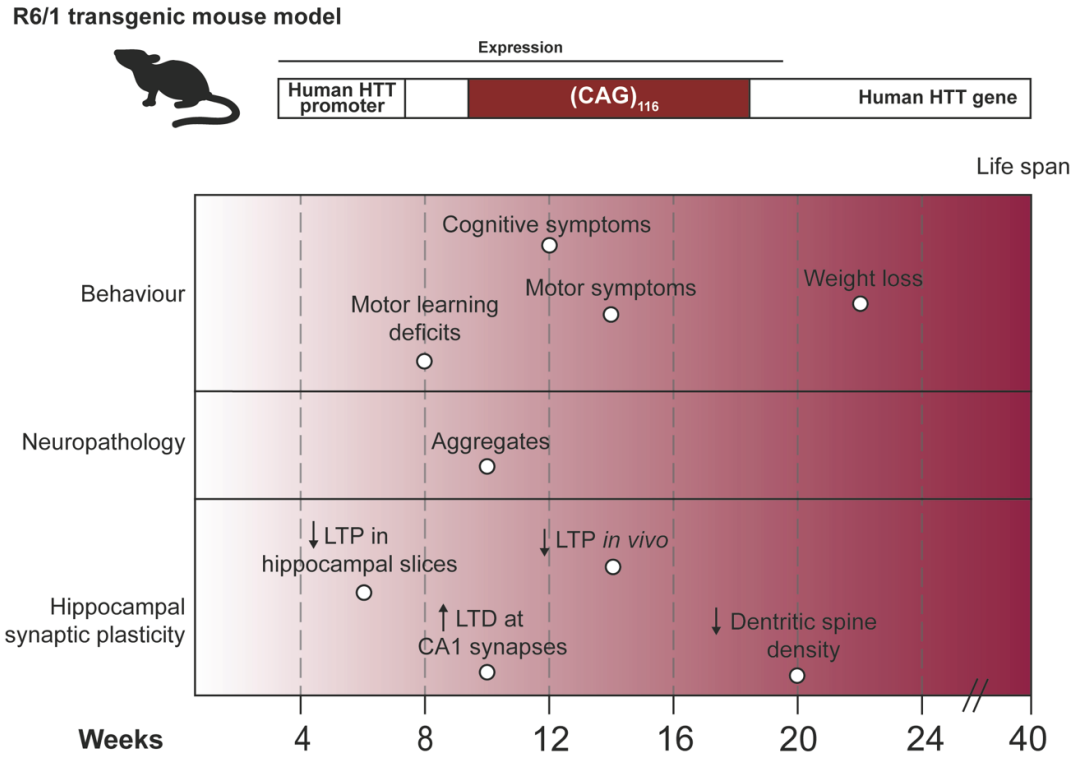


Figure 4.- R6/1 transgenic mouse model of HD. The construct used for the generation of the R6/1 mouse model is depicted on top. Timeline of the R6/1 model's behavioural and neuropathological characteristics. The chart shows the age at which motor and cognitive abnormalities, weight loss, synaptic plasticity dysfunction and mHtt aggregates (measured by EM48 immunostaining) first appear (in weeks).

3.4 Transgenic full-length mouse models

Transgenic mouse models expressing mutant versions of the full-length HTT protein with all the introns, exons and regulatory sequences have been generated using a yeast artificial chromosome (YAC) or a bacterial artificial chromosome (BAC) as vector. Interestingly, the human origin of the transgene makes it very valuable for the evaluation of preclinical genetic therapies targeting human m*HTT* such as the use of the CRISPR/Cas9 gene-editing system. The most used ones are the YAC128 and the BACHD mouse model which express 128 and 97 CAG repeats respectively (Gray et al., 2008; Hodgson et al., 1999; Slow et al., 2003). They both show a slow progression of the disease with robust motor and cognitive phenotype and a relatively normal life span. The main asset in this animal models is the presence of selective striatal neuronal loss which allows the study of molecular mechanisms involved in cell death. However, these

mice develop very few mHTT aggregates and tend to present increased body weight compared to WT littermates which makes them unsuitable for metabolism-related studies (Menalled et al., 2014; van Raamsdonk et al., 2006). Very recently, a novel BAC-generated model, called BAC226Q has been reported to accurately recapitulate the full spectrum of HD phenotypes including decreased life span, weight loss, motor and cognitive phenotypes, selective brain atrophy with the presence of striatal neuronal death, mHTT aggregation and even gliosis (Shenoy et al., 2022).

3.5 Knock-in mouse models

The knock-in mouse models are generated through the modification of the endogenous *Htt* mouse gene locus. Two approaches have been followed for genetic manipulation. On the one hand, the *Hdh*Q150 mice are generated by the replacement of the polyQ endogenous tract by an expanded CAG sequence of 150 CAGs resulting in the only difference between the HD and the normal allele (C. H. Lin et al., 2001). On the other hand, a different approach was to completely replace the murine exon 1 with the mutated human exon 1 resulting in the expression of a hybrid protein. The most popular model generated using this method is the *Hdh*Q111 mouse which contains 111 glutamines (Wheeler et al., 2000). Knock-in models are considered the most accurate when recapitulating the natural slow progression and neuropathology of HD as they are the most precise in terms of genetic context, which leads to a more natural temporal and spatial pattern of expression (Heng et al., 2008). However, symptomatology in these models is mild and, like R6 models, they do no present neuronal loss. In general, the late appearance of the motor symptoms makes them suitable for the study of preventive therapies and early disease stages.

3.6 In vitro and ex-vivo HD models

In vitro models, although not valuable to evaluate the outcome of a new therapeutic strategy on HD symptomatology, are a useful tool for drug screening used to evaluate novel compounds for the treatment of HD. They are also convenient for the study of molecular mechanisms and cellular events triggered by mHTT toxicity on an equal genetic background (Sipione & Cattaneo, 2001). The most used cell lines include immortalized neuronal cell lines, primary cultures and induced pluripotent stem cells. PC-12 and the striatal cell line STHdh$^{Q7/Q111}$ are the most used transformed cell lines.

The last, used in this Thesis, were generated from neuronal progenitors isolated from striatal primordia dissected out from E14 *Hdh*Q111 mouse embryos and immortalized through the expression of tsA58/U19 large T antigen through a retroviral vector. As transformed cells, this cell line provides a uniform population with an easy expansion that become great assets in drug screening processes. Moreover, unlike primary neuronal cultures, they undergo cell division and can be used to evaluate effects on cell cycle, which is a likely neurotoxic target (Trettel et al., 2000). However, as dividing cells, they significantly differ from neurons in terms of cell cycle regulation pathways which can be misleading.

In contrast, neuronal primary cultures have also been suggested to have the potential to overcome many of the disadvantages inherent to cell lines. However, isolating and culturing primary neurons from brains of adult patients is technically complex and is limited to the availability of tissue extracted from neurosurgical brain specimens (T. I. H. Park et al., 2020). Therefore, primary neurons are usually obtained and cultured from embryonic murine brain tissue. Skin fibroblast cultures from HD patients are also contemplated as an attractive model for in vitro studies, specifically for the research of peripheral biomarkers. The obtention, although from a human origin, is uncomplicated and minimally invasive and the effect of the mutation can be studied in the correct genetic background. Studies in primary skin fibroblast cultures obtained from patients have generated a broad impact on research due to their ability to mimic HD pathophysiological characteristics such as early mitochondrial bioenergetics defects or increased lamin B1 levels and disrupted nuclear structure (del Hoyo et al., 2006; Garcia-Forn et al., 2023; Jędrak et al., 2018; Squitieri et al., 2010). Future investigations are needed to reveal the pathways involved and to show if they relate to changes in the central nervous system (CNS).

Additionally, the generation of models based on disease-specific induced pluripotent stem cells (iPSC) from human skin fibroblast or mesenchymal stromal cells (MSCs) have recently emerged as one of the most rapidly evolving topics in this research field (Mattis et al., 2012; Takahashi & Yamanaka, 2006). In this line, many groups have pointed at HD-specific iPSC as a potential cell-based therapy but also several studies have reported the usefulness of HD *in vitro* models derived from HD–iPSCs suggesting their value in the research of pathological mechanisms and screening of possible therapeutics (Carter et al., 2014; Y. Liu et al., 2017; Szlachcic et al., 2015). However,

recent studies have highlighted the challenges in reproducing published data from human iPSC-derived neurons as large differences were observed between differentiation protocols (le Cann et al., 2021).

Although primary dissociated cultures, cell lines and IPSC cultures have provided important knowledge about the mechanisms of HD and potential therapeutic targets, they do not mimic the proper cellular and anatomical environment. Hence, brain slice cultures and organotypic slices demonstrated to be useful tools as they provide remarkable advantages over *in vivo* and *in vitro* models (Daviaud et al., 2013; Gähwiler et al., 1997). For instance, tissue structures and therefore, synapse circuitry is preserved thus replicating many aspects of the *in vivo* context (S. Cho et al., 2007). Further advantages of brain slice cultures include a reduced cost, as well as low technical complexity and simplicity of analysis. Recently, different HD organotypic models have been developed. They can be obtained from transgenic mice but they can also be prepared from WT rodents and posteriorly achieve neuron loss by other methods such as neurotoxin injection of the m*HTT* gene delivery (Arsenault & O'Brien, 2013; Johnson et al., 2006; R. C. Murphy & Messer, 2001, 2004; Ortiz et al., 2011; Reinhart et al., 2011).

3.7 Current treatment options

Current HD treatment focuses on symptom management because of a lack of disease progression-modifying therapies (Sturrock & Leavitt, 2010). The diverse symptomatology and individual response to different treatments prompt the need for a personalized treatment plan. In the last 20 years, HD treatment has not significantly advanced (Walker, 2007). Briefly, chorea symptoms are classically addressed through selective non-competitive inhibitors of vesicular monoamine transporter 2 (VMAT2) that produce depletion of releasable neuronal monoamine neurotransmitters such as tetrabenazine and deutetrabenazine. Psychiatric symptoms are commonly handled through antipsychotic drugs, being Olanzapine the second most frequently prescribed for HD treatment (Coppen & Roos, 2017). Its mechanism of action includes inhibition of dopamine receptors (D1, D2 and D4), serotonin (5-HT) receptors (5-HT2A, 5-HT2C), histamine receptors (H1) as well as a1-adrenergic and muscarinic receptors (Fulton & Goa, 1997). HD often presents with depressive symptoms which are commonly treated with selective serotonin reuptake inhibitors (SSRIs) such as Fluoxetine, Citalopram and Sertraline described to inhibit the reuptake of 5-HT into the presynaptic nerve terminal.

Anticonvulsants like sodium valproate, lamotrigine and carbamazepine are also prescribed to treat HD-associated behavioural symptoms like obsessive compulsive disorder (OCD), bipolar disorder and aggression (Naarding et al., 2001; Paulsen et al., 2005; Walker, 2007). The development of therapies that directly target mHTT has been the main research direction in the last few years (Wild & Tabrizi, 2017). RNA targeting therapies, mainly antisense oligonucleotides (ASOs) and RNA interference (RNAi) are currently the most promising and are enrolled in more advanced clinical trials (Evers et al., 2018; McColgan & Tabrizi, 2018). However, even if these therapeutic strategies are proven to be beneficial and stop HD progression, there are still many challenges they must overcome to reach the general population like proving the safety of lowering HTT, a high target specificity, selective for the mutant allele, and a less invasive route of administration (Wild & Tabrizi, 2017). DNA therapies, in particular CRISPR therapies, are also emerging as promising therapeutical strategies, as well as antibody-based and stem cell transplants, although they are still in preclinical trials (Denis et al., 2019; Ekman et al., 2019; Maucksch et al., 2013; Wild & Tabrizi, 2017). Overall, although a substantial part of the research in HD therapeutical strategies is aimed at blocking the disease-triggering mutation the identification of the affected downstream targets might allow the development of drugs that can restore their activity and modify the disease progression.

4. PROTEIN TRANSLATION DYSREGULATION AS A PATHOGENIC MECHANISM IN NEURODEGENERATIVE DISORDERS

Translational dysfunction can critically impact cell homeostasis and emerging evidence suggests translational dysregulation as a potential pathogenic mechanism for the development of neurological diseases (Baleriola & Hengst, 2015; Chen et al., 2019; Kwiatkowski et al., 2009). Neurons are thought to present a slow overall protein turn-over compared to other tissues with higher metabolic rates, reaching an average of 3 times longer half-time lives than the average (Price et al., 2010). Because of the extension of protein half-lives in neural tissue, translational alterations have longer-term effects, thus, making neuronal cells especially vulnerable to defects in every stage of mRNA translation (Dörrbaum et al., 2018). In fact, pathogenesis in a subset of neurodegenerative illnesses may be influenced by global decreases in translation initiation driven by mutations in the translational machinery or incorrect activation of the integrated stress response. Translation infidelity and ribosome stalling, which lead to neurodegeneration, can also be a consequence of the dysfunction of key elements of the elongation machinery, including tRNAs and associated enzymes. When taken as a whole, disruption of mRNA translation is emerging as a common pathophysiologic mechanism for numerous neurodegenerative disorders (reviewed in Kapur et al., 2017). Translation of proteins involves three steps: initiation, elongation and termination, with the first one being the most tightly regulated. This tight regulation allows cells to change their proteome within short periods of time, having an impact on cell processes in a much faster way than mRNA transcription changes (Anderson & Kedersha, 2006). Phosphorylation of multiple proteins involved in the initiation of protein translation is the main mechanism by which this important process is modulated.

4.1 Protein synthesis overview

Translation initiation is defined as either cap-dependent when the ribosome binds initially at the 5' cap mRNA structure, or as cap-independent, when the ribosome directly binds into a position upstream, or directly at the initiation codon via a specific cis-acting RNA element in the 5' untranslated region (5’ UTR).

Cap-dependent translation initiation’s first step, the assembly of the eukaryotic translation initiation factor 4F (eIF4F) complex, is the most critically regulated step. This

protein complex consists of the 5′ mRNA cap-binding protein eIF4E, the scaffolding protein eIF4G and the ATP-dependent RNA helicase eIF4A, which is thought to unwind RNA secondary structures in the 5'-UTR during scanning and to prevent reannealing in the generated single-stranded regions (Marintchev et al., 2009; Svitkin et al., 2001). Moreover, eIF4G also interacts with the poly(A)-binding protein (PABP) which is thought to circularize mRNA and hence, bring the 3' UTR where most regulatory sequences are located close to the translation initiation complex(Wells et al., 1998). After the eIF4F complex assembly, it participates in the recruitment of the 43S pre-initiation complex (PIC) which consists of the assembly of several initiation factors (eIFs) including eIF1, eIF1A, eIF3, eIF5, the 40S small ribosomal subunit and the ternary complex (composed of the methionine loaded tRNA (Met-tRNAi) and the GTP-loaded eIF2 (Sonenberg & Hinnebusch, 2009). At the next step, the resulting 48S PIC complex scans the 5′ UTR of the mRNAs in a 5′ to 3′ direction for the identification of the start codon (mostly AUG). Upon the start codon identification, GTP bound to eIF2 is hydrolyzed to GDP, which allows the recruitment of the 60S large ribosomal subunit to finally form the 80S ribosome (40S and 60S subunits) to initiate the elongation step (Jackson et al., 2010; Sonenberg & Hinnebusch, 2009).

Under specific conditions like cellular stress or viral infection, an alternative mechanism for translation initiation through an internal ribosome entry site (IRES) triggered by the mammalian target of rapamycin (mTOR) signalling inhibition replaces cap-dependent translation. The endogenous proteins translated through cap-independent translation play a crucial role in the stress response to such cellular conditions (Y. Yang & Wang, 2019). In this scenario, eIF4E remains sequestered by an activated eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; dephosphorylated) inhibiting cap-dependent translation and eIF4G expression increases and mediates IRES-dependent translation.

Elongation is a sequential process. Like in the initiation step, non-ribosomal proteins, in this case known as elongation factors, are required for the elongation stage. tRNAs charged with the proper amino acid that enters the A site of the large ribosomal subunit are later shifted to the P site and finally to the E (exit) site where they exit the ribosome uncharged. Energy for the conformational changes required in each step comes from the hydrolyzation of GTP as several elongation factors behave as GTPases. When compatible codon-anticodon interaction occurs between the aminoacyl-tRNA entering

the A site and the mRNA codon there, the peptidyl transferase, an enzyme integrated into the large ribosome subunit, catalyzes the formation of the peptide bond between the free amino group of the aminoacyl-tRNA in the A site and the ester bond attaching the developing polypeptide to the tRNA in the ribosomal P site. Next, the uncharged tRNA in the P site moves to the E (exit) site and leaves the ribosome. The peptidyl-tRNA in the A site is then translocated back to the P site leaving the A site free for a new aminoacyl-tRNA to enter the ribosome as mRNA advances in the 3′ direction.

Termination occurs when a stop codon enters the A site and it's not recognized by any aminoacyl-tRNA but by a set of proteins called release factors that bind to the A-site and trigger hydrolysis of the polypeptide-tRNA, hence releasing the completed protein and leading to translation termination.

4.2 Regulation of cap-dependent translation

Gene expression can be modulated at different levels and its regulation is key for cell homeostasis maintenance. Although part of the regulation is done at a transcriptional level, translational control has more rapid and greater effects on the proteome that allow cells to meet the changing requirements and adapt to new cellular conditions. Translation can be regulated at a global or mRNA-specific level. Global translational control is commonly exerted by changing the activity or availability of eIFs through phosphorylation. The two most well-established mechanisms included in this category are the regulation of eIF4E availability by 4E-binding proteins (4E-BPs) and the modulation of active ternary complex levels by eIF2α phosphorylation (Gebauer & Hentze, 2004).

4.3 4E-BPs regulation of cap-dependent translation

As mentioned above, the interaction between eIF4E and eIF4G, thus the assembly of the eiF4F complex, is an important regulation point of cap-dependent protein synthesis. This mechanism is executed through a family of related proteins named eIF4E-binding proteins (4E-BPs), being 4E-BP1 the most thoroughly studied. Functionally, 4E-BP1 acts as a repressor of cap-dependent translation initiation. When active, and thus, not phosphorylated, it strongly binds to eiF4E blocking the formation of the 48S pre-initiation complex (Marcotrigiano et al., 1999). Alternatively, inactivated 4E-BP1, mainly

by mTOR phosphorylation, loses its affinity for eIF4E and allows the assembly of the eIFs. As explained above, initiation factors eIF4E, eIF4A and eIF4G constitute the eIF4F protein complex which recruits mRNA to the ribosome and helps to scan the 5′ UTR in pursuit of an initiation codon (Jackson et al., 2010). Interestingly, 4E-BP1 and eIF4G share a common eIF4E-binding motif explaining the competitive binding that allows translational activity control (Mader et al., 1995).

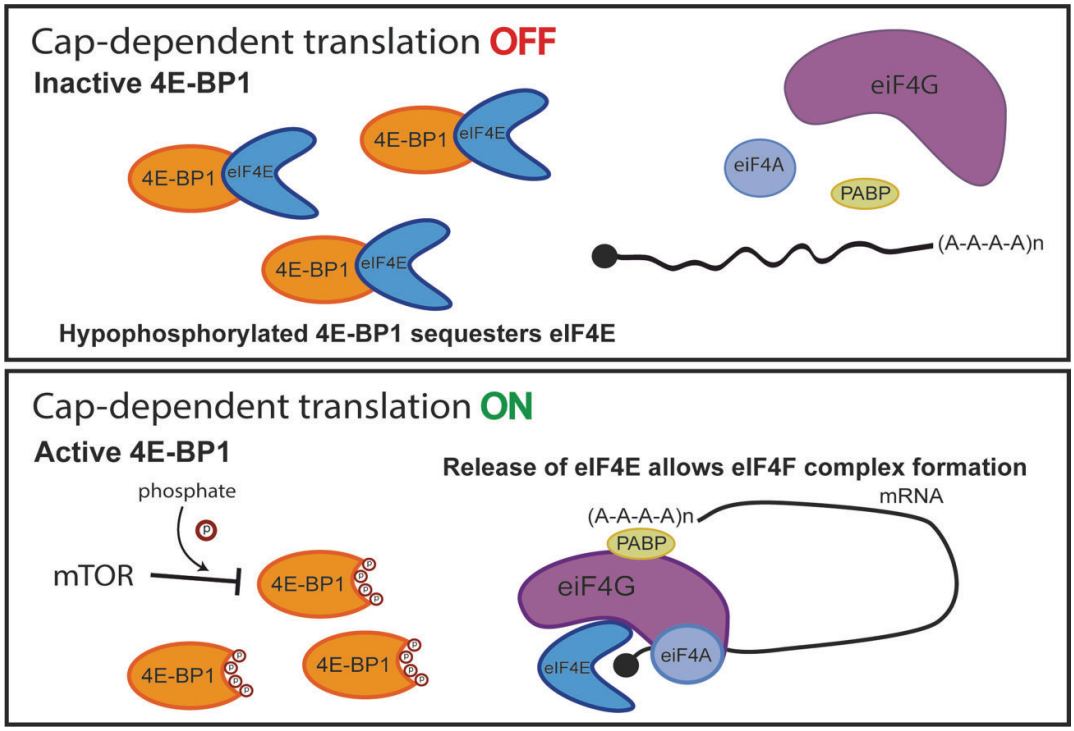


Figure 5.- Schematic illustration of eIF4F assembly regulation by 4E-BP1 and its impact on cap-dependent translation rate. (A) Non-phosphorylated 4E-BP1 presents high affinity for eIF4E thus preventing eIF4F formation and leading to a decrease in overall translation rate. (B) Phosphorylated 4E-BP1 is not able to bind eIF4E and as a result allows the eIF4F formation, resulting in an increase of overall translation rate.

4.4 Translational control by eIF2 phosphorylation

Another important translation initiation regulation mechanism involves the phosphorylation of the GTPase eIF2. This phosphorylation at Ser 52 of the eIF2α subunit decreases the availability of active ternary complex by preventing the recycling of eiF2. In short, eIF2 is only able to form the ternary complex by interacting with Met-tRNAi when bound to GTP (Jennings et al., 2017; Kapp & Lorsch, 2004). The posterior

interaction with the GTPase activating protein eIF5 promotes GTP hydrolysis and its release from the ribosome, still bound to eIF5. eIF2 recycling, in order to be able to be engaged in the following round of translation initiation, requires the initiation factor eIF2B which removes eIF5 and acts as a guanine nucleotide exchange factor (GEF) to reactivate eIF2 and allows its interaction with Met-tRNAi once-again(Jennings et al., 2017). However, under cellular stress, eIF2α is phosphorylated by several kinases that respond to different cellular stimuli dramatically increasing its affinity with eIF2B hence reducing the availability of eIF2-GTP and inhibiting global translation (Bogorad et al., 2018; Krishnamoorthy et al., 2001). Different stress stimuli induce eIF2α phosphorylation by four related protein kinases: the heme-regulated inhibitor (HRI) only in red blood cells, the protein kinase RNA-activated (PKR) that responds to viral infection activated by binding to double-stranded RNA, the PKR-like endoplasmic reticulum (ER) kinase (PERK) that senses endoplasmic reticulum (ER) stress caused by the accumulation of misfolded proteins, and the general control non-depressible 2 kinase (GCN2) that responds to low levels of certain amino acids (Taniuchi et al., 2016).

Although phosphorylation of eIF2α is thought to be a mechanism for global translation initiation regulation, some specific transcripts are actively translated during the cellular stress response. For instance, the mammalian activating transcription factor 4 (ATF4) is translated during ER stress conductions when it protects the cell against oxidative stress and counteracts the lack of amino acid availability (Harding et al., 2003).

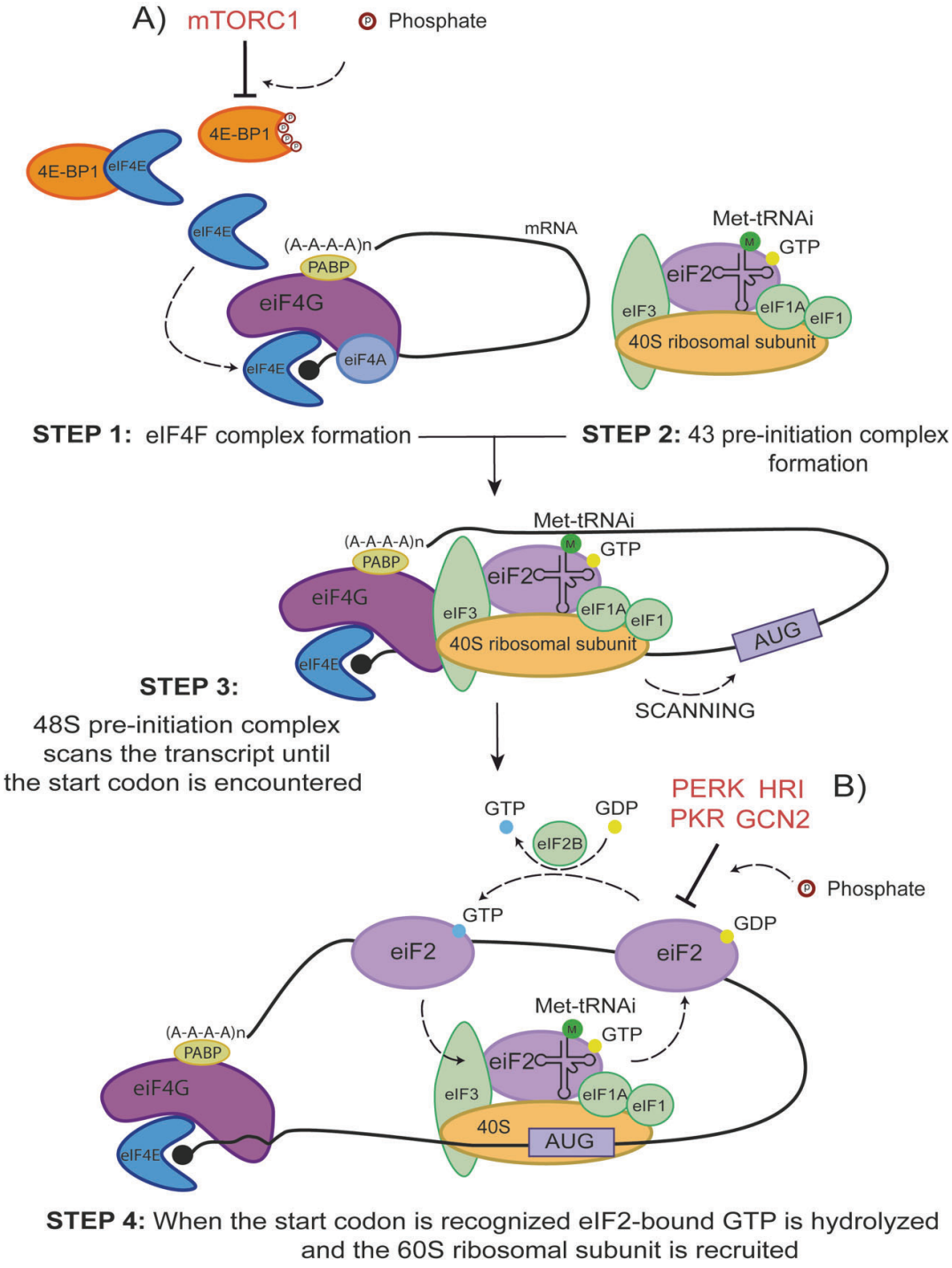


Figure 6.- Overview of the cap-dependent translation initiation process. Main rate-limiting steps in the regulation of the initiation process include: (A) eIF4E is retained in an inactive form by 4E-BP1 but it is released after phosphorylation of the 4E-BP1 mainly by mTORC1. eIF4E release allows binding to the mRNA 5' cap structure (black circle) and the recruitment of the other eIFs to stabilize the small ribosomal subunit on the mRNA. (B) Upon start codon recognition eIF2-bound GTP is hydrolyzed and the 60S ribosomal subunit is recruited. After a round of

translation, eIF2 must be recharged with GTP by the GEF eIF2B for the ternary complex to be reformed for subsequent cycles of translation initiation. Under stress conditions, eIF2 is phosphorylated by stress-induced kinases preventing eIF2B from exchanging eIF2-bound GDP for GTP.

4.5 Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1)

As this thesis is focused on the role of 4E-BP1 activity alterations in the pathophysiology of HD, it will be further explained. As mentioned before, eukaryotic initiation factor 4E-binding protein 1 (EIF4EBP1) better known as 4E-BP1 or phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I), is one of mTOR complex 1 (mTORC1) main downstream effectors (R. Sun et al., 2019). 4E-BP1 belongs to a family of eIF4E-binding proteins, which includes 4E-BP2 and -3, each of which is encoded by a different gene. Despite that, they show a high degree of homology with one another (Dowling et al., 2010). The 4E-BP1 protein structure is depicted in Fig. 7. In short, 4E-BP1 protein structure carries 3 relevant functional domains: The N-terminal RAIP motif, the C-terminal TOR signalling motif (TOS) and the eIF4E binding domain, which gives the protein its name. The first two are both required for the binding with the mTOR scaffold protein Raptor which is necessary for the mTOR phosphorylation and thus, inactivation, of 4E-BP1 (V. H. Y. Lee et al., 2008; Pause et al., 1994). Within 4E-BP1 structure, seven phosphorylation sites have been identified: Thr 37, Thr 46, Ser 65, Thr70, Ser 83, Ser 101, and Ser 112 (numbering of human 4E-BP1; numbers are lower by one in rodents; Martineau et al., 2012). Phosphorylation events are sequential and phosphorylation of Thr 37 and Thr 46 are thought to be the priming event followed by Thr 70 and Ser 65 (Gingras et al., 2001).

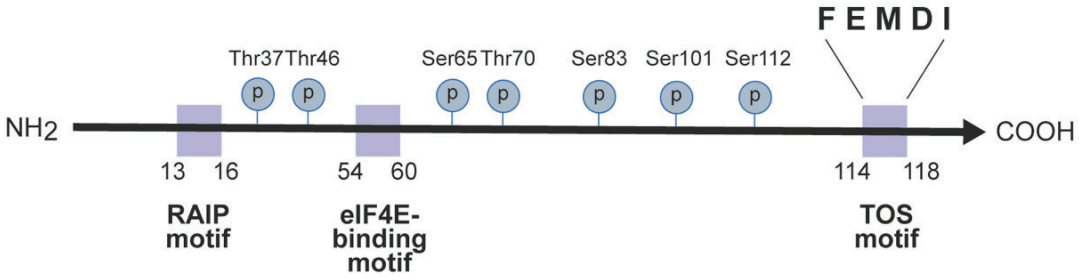


Figure 7.- Protein structure and phosphorylation sites of 4E-BP1. 4E-BP1 is a 15 kDa protein (118 amino acids in humans and 117 in rodents) presenting three several highly conserved

functional domains: the ^{54}Y*XXXX*LΦ^{60} eIF4E- binding domain (X: any AA; Φ: hydrophobic residue) (Marcotrigiano et al., 1999; Matsuo et al., 1997); a C-terminal TOR signalling motif (TOS) and an N-terminal RAIP, which have a role in the association with raptor and mTOR-mediated phosphorylation of 4E-BP1 (Beugnet et al., 2003b; Schalm et al., 2003; Wang et al., 2005).

4E-BP1 activity regulation

mTORC1/4E-BP1 axis

4E-BP1 activity is mainly regulated by the mTOR kinase. In fact, 4E-BP1 is considered the most important downstream effector when considering mTOR's role in cap-dependent protein translation regulation. mTOR is a Ser/Thr kinase forming two multiprotein complexes, mTORC1 and mTORC2, with distinct protein binding partners. These complexes respond to distinct stimuli and regulate different important cell functions. Importantly, the main function of mTORC1 is to regulate protein synthesis and cell growth through the phosphorylation of its substrates 4E-BP1 and S6K in response to different extra- and intracellular stimuli such as amino acid availability, and oxygen and energy status of the cell (Saxton & Sabatini, 2017). Specifically, mTORC1 promotes 4E-BP1 dissociation from eIF4E allowing the formation of the eIF4F complex and the initiation of cap-dependent translation by directly phosphorylating 4E-BP1 at several sites (Thr 37/46, Thr 70, Ser 65) (Gingras et al., 2001). In more detail, cellular energy status is sensed by the AMP-activated protein kinase (AMPK), a protein that responds to the AMP/ATP increased ratio activating tuberous sclerosis complex 2 (TSC2) an upstream inhibitor of mTOR by phosphorylation and consequently inhibiting mTORC1. On the other hand, a family of GTPases termed tag proteins activate mTORC1 in a cellular state of amino acid sufficiency (Nguyen & Olzmann, 2017). Another important pathway positively regulating protein translation through the mTOR/4E-BP1 axis is the Ras-ERK pathway. On the one hand, ERK also phosphorylates TSC2 directly or through the ribosomal S6 kinase (RSK) promoting its dissociation and leading to mTORC1 activation (Ma et al., 2005). Moreover, RSK also directly affects mTORC1 activity through the phosphorylation of Raptor (Carrière et al., 2008). Interestingly, the Ras/ERK pathway also influences the activity of the eIF4F complex through the activation of the ERK-activated protein kinases MNK1 and MNK2 which, when activated, directly phosphorylate eiF4E at Ser 209 (Waskiewicz et al., 1999). eIF4E phosphorylation enhances the formation of the eIF4F complex by

increasing its affinity with the mRNA and eIF4G, thus increasing cap-dependent translation (Minich et al., 1994). Lastly, mTORC1, also regulates protein translation initiation through the phosphorylation of other substrates in a 4E-BP1-independent manner. Predominantly, S6 kinase (S6K), another well-established substrate of mTORC1, phosphorylates several substrates involved in the translation initiation machinery such as eIF4B, the eukaryotic elongation factor-2 kinase (eEF2K) and PDCD4, which suppresses eIF4A activity among others (Holz et al., 2005; Wang et al., 2001; H.-S. Yang et al., 2003).

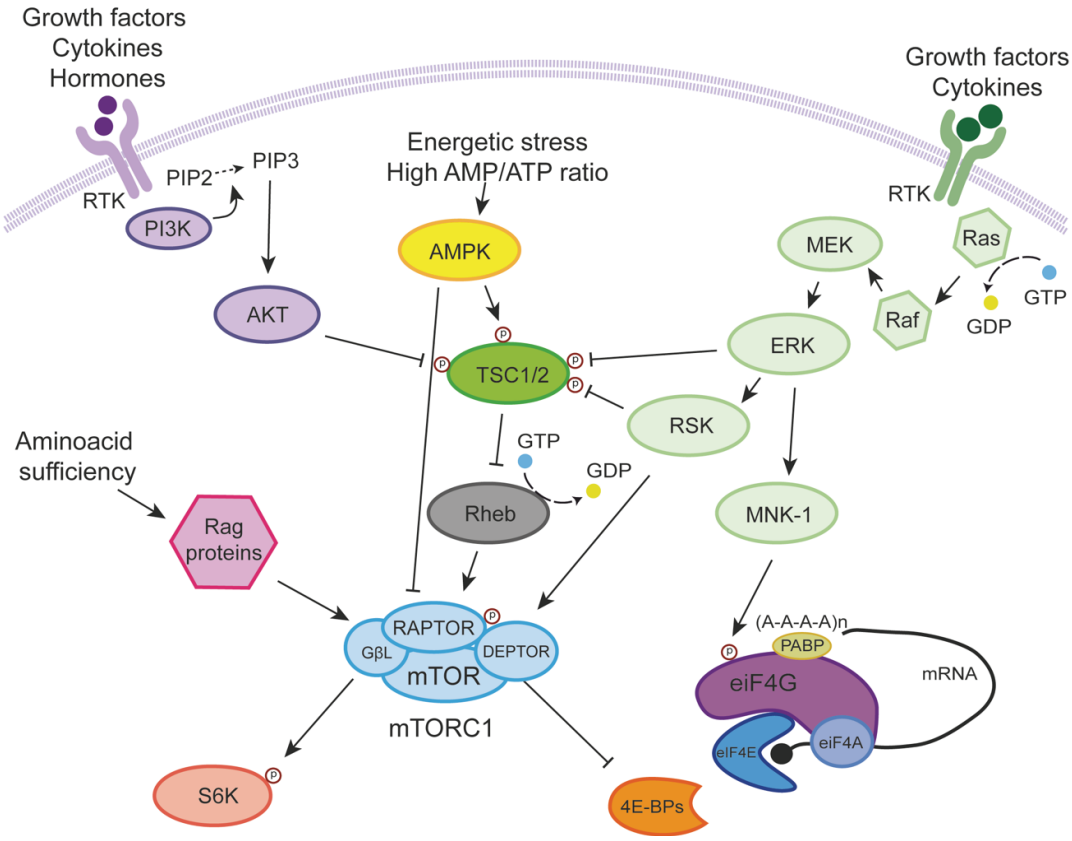


Figure 8.- Signaling pathways regulating cap-dependent translation through eIF4F complex activity. Arrows represent activation and T bars represent inhibition within a pathway. See the text for details.

4.6 Other kinases regulating 4E-BP1 activity

The observation of high levels of total and phosphorylated 4E-BP1 in most human cancer cellular models has put the spotlight on the mTOR/4E-BP1 cascade inhibition as a promising therapeutic target. Interestingly, some of these studies working on mTOR inhibitors reported that the inhibition of mTOR was, in some conditions, not sufficient to block 4E-BP1 phosphorylation (Choo et al., 2008; Ducker et al., 2013; Mi et al., 2015; Y. Zhang & Zheng, 2012). These findings indicate that mTOR may not be the only kinase capable of 4E-BP1 phosphorylation in contrast to the classical mTOR/4E-BP1 cascade which accentuates the already high complexity of the phosphorylation of mTORC1 substrates due to multiple regulatory mechanisms and feedback loops that extend its regulation far beyond the simple sequence motif of each phosphorylation site (Kang et al., 2013). In this line, it has been reported that glycogen synthase kinase 3 beta (GSK3β) phosphorylates 4E-BP1 at Thr 37 and Thr 46 and thereby decreases its association with eIF4E and could be able to regulate the phosphorylation of other sites (Ser 65 and/or Thr 70) in some specific cell cellular context (Shin et al., 2013). As mentioned before, it has been proposed that following ionizing radiation ERK stimulates protein synthesis both by direct phosphorylation of 4E-BP1 at Ser 65 and by blocking TSC2 activity, leading to greater mTOR activation and increased phosphorylation of 4E-BP1 (Braunstein et al., 2009). Throughout the cell cycle, 4E-BP1 phosphorylation exhibits different patterns. Interestingly, during mitosis, cyclin-dependent kinase 1 (CDK1)/cyclin B complex can phosphorylate 4E-BP1 at Ser 83 which can be used as a specific mitotic marker although it seems to not affect eIF4E:4E-BP1 interaction (Velásquez et al., 2016). Moreover, CDK1/cyclin B can also phosphorylate other sites, including the priming Thr 37/46 in an mTOR-independent manner (Shuda et al., 2015). Another important CDK, The RNA polymerase II (RNAPII) C-terminal domain kinase (CDK12) known for its role in mRNA biosynthesis of DNA repair genes and genomic stability regulation has been recently reported to promote translation of mTORC1-dependent mRNAs through Ser 65 and Thr 70 phosphorylation after mTORC1 phosphorylation of the priming sites. Genome-wide ribosome profiling (Ribo-seq) further identified that CDK12 regulated the translation of a specific set of target mRNAs involved in mitotic chromosome stability (S. H. Choi et al., 2019). The leucine-rich repeat kinase 2 (LRRK2), has been also described as one of the physiological kinases for 4E-BP1 being at least involved in the phosphorylation of the priming Thr 37/46 sites. Intriguingly, pathogenic mutations in LRRK2 found in a form of

heritable Parkinson's disease (PD) seem to inactivate 4E-BP1 and deregulate protein translation, eventually resulting in the loss of dopaminergic neurons (Imai et al., 2008). In response to several cellular stresses such as UVB irradiation, MAP kinase 38 inactivates 4E-BP1 through its downstream effector kinase MSK1 by phosphorylation of 4E-BP1 at Thr 36, Thr 45, Ser 64 and Thr 69 in the mouse epidermal JB6 cell line (G. Liu et al., 2002). Serine/threonine kinase PIM2 (proviral integrations of moloney virus) directly phosphorylates Ser 65 and Thr 70 which has been related to embryonic stem cell self-renewal and pluripotency (H. Sun et al., 2017). Lastly, the kinase ATM, found mutated in ataxia telangiectasia patients, is thought to respond to insulin treatment by direct phosphorylation of Ser 111 *in vitro* and cause *in vivo* phosphorylation of 4E-BP1 facilitating its inactivation hence promoting protein translation (D. Q. Yang & Kastan, 2000).

Kinase	Conditions	Phosphorylation site	Ref.
GSK3b	N/A	Thr 37, Thr 46 (possibly Ser 65 and/or Thr 70)	Shin et al., 2013
p38MAPK	Viral infection, TNFa/CHX-mediated apoptosis	N/A	Janzen et al., 2011; Walsh & Mohr, 2004
	UVB	Thr 36, Thr 45, Ser 64, Thr 69	G. Liu et al., 2002
ERK	Ionizing radiation	Ser 65 (in vitro)	Braunstein et al., 2009
PIM2	N/A	Ser 65	Fox et al., 2003
ATM	Insulin treatment	Ser 112	D. Q. Yang & Kastan, 2000
CDC2/CDK1	Paclitaxel (PTX) treatment	Thr 70	Greenberg & Zimmer, 2005; Heesom et al., 2001
LRRK2	Physiological condition	Thr 37, Thr 46	Imai et al., 2008

Table 1.- mTOR-independent kinases reported to phosphorylate 4E-BP1. Corresponding phosphorylation condition and site are listed. Table extracted from Qin et al., 2016.

4.7 Phosphatases involved in 4E-BP1 activity regulation

Although 4E-BP1 phosphorylation has been extensively studied, the process by which 4E-BP1 is dephosphorylated remains mysterious. It is important to keep in mind that it can be difficult to prove that a protein is a real substrate of a phosphatase as interactions between phosphatases and their substrates are transient and they can be challenging to identify, especially using conventional immunoprecipitation of endogenous proteins. Currently, overwhelming evidence suggests that 4E-BP1 is likely

dephosphorylated by PP2A, though direct interaction between 4E-BP and PP2A has yet to be demonstrated (Guan et al., 2007; Janzen et al., 2011; Nho & Peterson, 2011). Moreover, Gardner et al. (2015) reported that dephosphorylation of 4E-BP1 in the presence of glycolytic inhibitor in *ex vivo* rat retinas stimulated with insulin was strongly prevented by inhibition of PP1/PP2A phosphatases with okadaic acid and inhibition of PPM1 phosphatases with cadmium. In another study by J. Liu et al. (2013), phosphatase Mg^{2+}/Mn^{2+} dependent 1G (PPM1G), another member of PPM1 family protein, was also found implicated in the dephosphorylation of the 4E-BP1 at both the Thr 37/46 and Ser 65 sites. Overall, despite the preliminary evidence, more research is needed to determine if 4E-BP is a direct PP2A, PP1 or PPM1G substrate.

4.8 4E-BP1 dysregulation in neurological disorders

Many translational machinery components are implicated in neurodegenerative disorders (Skariah & Todd, 2021). The idea that aberrantly increased bulk protein synthesis results in a mechanism of neurodegeneration shared by different neurodegenerative disorders has been taken into consideration by different groups. Presumedly, increased protein synthesis in these types of neurological disorders could lead to neuronal dysfunction by increasing cellular stress through, for instance, depletion of energy stores and overload of the protein degradation systems (Zheng et al., 2016). Moreover, many cell processes, specifically in neurons, such as synaptic plasticity, require precise temporal and spatial-specific changes in the translation of a specific mRNA, which could be disrupted by increased bulk protein synthesis. In fact, it is well-known that protein synthesis dysregulation can interfere with long-term synaptic plasticity and memory formation (Costa-Mattioli et al., 2009). Therefore, it is not surprising that a genetic increase of total eIF4E levels, and consequent increase in cap-dependent translation, resulted in a mouse model for autism spectrum disorders, with a phenotype that could recapitulate autism-like symptoms like impaired social skills and repetitive behaviours (Santini et al., 2013). Moreover, another group independently replicated the findings by increasing cap-dependent protein synthesis through 4E-BP knock-down (Gkogkas et al., 2012). In a subsequent study performed by this same group, genetic inhibition of eIF4E restored synaptic dysfunction and behavioural alterations in a Fragile X syndrome (FXS) mouse model, caused by a mutation of a single gene *FMR1* coding for Fragile X Mental Retardation Protein (FMPR), which behaves as

a repressor of protein translation (Gkogkas et al., 2014). In the last three citations, the hypothesis of the contribution of aberrant cap-dependent translation in neuron dysfunction was reinforced with the finding that treatment with the cap-dependent translation inhibitor 4EGI-1, an eIF4E/eIF4G interaction inhibitor, reverted synaptic dysfunction and autistic-like behaviour.

4.9 4E-BP1 dysregulation in neurodegenerative diseases

eIF4E – 4E-BP1 axis dysregulation has also been reported in neurodegenerative disorders. For instance, Li et al. (2005) studied the state of various translation control elements in human Alzheimer's disease (AD) cortex samples and reported increased mTOR phosphorylation at Ser 2481 accompanied by a dramatically increased phosphorylation of 4E-BP1 at Thr 70 and Ser 65 and a reduction of 4E-BP1 total levels in neurons that correlated with the progression of neurofibrillary degeneration. In this line, the lentiviral expression of $A\beta_{1-42}$ in the rat cortex induced mTOR signalling activation significantly increasing 4E-BP1 phosphorylation (Algarzae et al., 2012). Moreover, with a different approach but similar results, Baleriola et al. (2014) demonstrated that axonal protein synthesis was induced by the local application of $A\beta_{1-42}$ oligomers which also resulted in a localized inactivation of 4E-BP1.

On the other hand, although increased mTOR levels are detected in human PD brains, controversial results were obtained in different animal models (Crews et al., 2010; Dijkstra et al., 2015). Interestingly, as mentioned before, 4E-BP is inhibited by the most common form of genetic parkinsonism caused by dominant mutations in *LRRK2* (Imai et al., 2008). In agreement, Tain and colleagues (2009) found that overexpression of a mammalian ortholog of 4E-BP1 suppressed the pathological phenotype, including degeneration of dopaminergic neurons, in various *Drosophila* genetic models of PD including *lrrk*, *pink1* and *park* (the *Drosophila* homologs of human LRRK2, PINK1 and PARK2, respectively) mutants. In this study, the authors also obtained similar results by pharmacologically blocking 4E-BP1 activity through inhibition with rapamycin of its negative regulator mTOR.

4.10 4E-BP1 dysregulation in HD

Interestingly, recently published papers from our group reported the increased formation of the eIF4F protein complex and increased 4E-BP1 phosphorylation at Thr 37/46 in line with an aberrant increase in the global translation rate in striatal DARPP-32-positive neurons from the R6/1 mouse model of HD (Creus-Muncunill et al., 2019). However, although we also reported increased activation of the mTOR pathway, 4E-BP1 inactivation appears in earlier stages in R6/1 mice suggesting the involvement of an mTOR-independent mechanism (Creus-Muncunill et al., 2018). In addition, controversial results about mTOR activity status have been reported in different HD animal models varying from upregulated (Pryor et al., 2014), to downregulated (J. H. Lee et al., 2015; Ravikumar et al., 2004) and even unaltered activity levels, which highlights the need for further research in the matter. Interestingly, we described altered 4E-BP1 total protein levels in the human HD putamen post-mortem samples. Unfortunately, data about the phosphorylation status in human tissue was not acquired due to technical difficulties. The further identification of proteins selectively up-translated in the HD context was performed using the PUNCH-P method. This approach revealed an increase in ribosomal proteins that could lead to neuronal dysfunction through different mechanisms (Creus-Muncunill et al., 2019).

4.11 Cap-dependent translation inhibitors: therapeutic strategies targeting regulation of eIF4F assembly

Although it is still an emerging field, cap-dependent translation and eIF4F complex assembly inhibition are considered attractive targets since, as mentioned above, alterations in the PI3K/Akt/mTOR pathway activity, eIF4E overexpression and hyperphosphorylation of 4E-BPs have been associated with neurodegenerative disorders. Interestingly, the interaction of eIF4G with eIF4E is essential for cap-dependent translation initiation resulting in a useful limiting factor for the development of therapeutical strategies. In general, the numerous strategies targeting the eIF4F complex range from the modulation of upstream signalling pathways regulating eIF4E activity or availability to the disruption of the eIF4F complex assembly by preventing eIF4E interaction to capped mRNAs or eIF4G (Lu et al., 2015).

As aforementioned, 4EGI-1, a potent inhibitor of the eIF4E and eIF4G interaction, has been used by several groups to evaluate the consequences of the exaggerated cap-dependent translation in several neurological disorders (Gkogkas et al., 2014; Santini et al., 2013). Interestingly, in a recent paper, our group reported that the intracerebroventricular infusion of 4EGI-1 was able to normalize striatal protein synthesis and significantly ameliorate motor symptoms in R6/1 mice. 4EGI-1 infusions, although not able to restore striatal markers or reduce mHTT aggregation, were able to normalize the translation of ribosomal proteins and restore cyclin D1 protein to WT levels, which serves as a readout 4E-BP1 phosphorylation level. Noteworthy, ATP levels and synaptic plasticity were also restored in the 4EGI-1 treated group suggesting that these alterations rely on aberrantly upregulated translation in the HD context (Creus-Muncunill et al., 2019). Overall, these results support the hypothesis of the role of an aberrant increase in cap-dependent translation as a direct contributor to neuronal dysfunction and behavioural deficits in HD arising as an interesting therapeutic strategy which may be shared between various neurodegenerative disorders. Thus, despite promising results, the precise contribution of cap-dependent translation alterations to the onset and progression of motor deficits needs to be explored in further detail. For this reason, in this Thesis, we evaluated the potential of 4E-BP1 activity modulation, as a key rate-limiting step in cap-dependent translation initiation, on reducing HD striatal pathology and motor-behaviour abnormalities along with disease progression in the R6/1 mice.

As a key upstream regulation pathway of translation initiation, the inhibition of mTORC1 arises as a potential candidate for HD treatment. Although numerous studies describe the neuroprotective effects of mTORC1 inhibitors like rapamycin in the context of various neurodegenerative disorders, they are usually attributed to the restored levels of autophagy, also regulated by mTORC1. For instance, treatment with the canonical allosteric mTORC1 inhibitor rapamycin alleviates proteotoxicity and ameliorates the associated behavioural deficits in multiple transgenic AD and tauopathy mouse models (Caccamo et al., 2010, 2013; Spilman et al., 2010; L. Zhang et al., 2017). Similar results in Aβ and phospho-tau clearance and memory deficits were obtained after treatment with the rapalogs (rapamycin analogs) temosirolimus and everolimus (Cassano et al., 2019; Frederick et al., 2015; Jiang et al., 2014). In this line, mTORC1 inhibition was also found neuroprotective in PD cellular and animal models presenting α-synuclein accumulation (Crews et al., 2010). Moreover, neuroprotective effects have also been

reported on the effect of mTORC1 inhibition in the HD context. On one hand, in cellular models presenting protein aggregates due to the expression of mHTT, catalytic inhibitors of total mTOR and mTOR-specific siRNA were cytoprotective, which the authors attributed to increased autophagy and aggregate clearance (Berger et al., 2006; Roscic et al., 2011). Interestingly, Ravikumar et al. (2004) suggested that the sequestration of mTOR in the mHTT aggregates decreased mTOR activity and induced autophagy, resulting in a protective pathway that could be enhanced by treatment with rapamycin-like drugs. However, rapamycin and rapalogs are not complete inhibitors of mTORC1 and in some conditions, they are not sufficient to block 4E-BP1 phosphorylation (Choo et al., 2008; Ducker et al., 2013; Mi et al., 2015; Y. Zhang & Zheng, 2012). In fact, rapamycin inhibits the phosphorylation of S6 kinase 1, but it is not an effective inhibitor of mTORC1′s phosphorylation of 4E-BPs (Thoreen et al., 2009). Thus, in this Thesis, we explore the potential of other already FDA-approved and tested generic drugs to normalize aberrant cap-dependent translation and ameliorate HD symptomatology by mimicking the effect observed by the 4EGI-1 treatment in the R6/1 mice striatum, which is still an experimental drug. In particular, we evaluate the potential of the commonly prescribed SSRI, sertraline, as a modulator of cap-dependent translation in the HD molecular context. Sertraline's beneficial effects on HD phenotype have been proposed in the N171-82Q HD and R6/2 mice transgenic mouse models of HD, respectively (Duan et al., 2008; Peng et al., 2008). In both studies, sertraline administration improved survival and motor performance and reduced striatal atrophy. The beneficial effects of sertraline in these mice were attributed to enhanced adult neurogenesis and BDNF neurotrophic support. Strikingly, sertraline has also been reported to exhibit antiproliferative activity by inducing a protein synthesis reduction. Polysome profile analysis of sertraline-treated cells showed a reduction in polysome content and an increase in 80S ribosomes. The effect of sertraline on translation was mediated by a decrease in the eIF4F complex levels, altered localization of eIF4E, and increased eIF2α phosphorylation (C. J. Lin et al., 2010). In a more recent study, sertraline has also been found to interact and antagonize the mitochondrial voltage-dependent anion channel 1 (VDAC1), resulting in reduced cellular ATP levels and activation of AMPK, which inhibits its downstream mTOR signalling pathway (Hwang et al., 2021). Taking this into account, in this Thesis we explore the contribution of the normalization of striatal protein synthesis as a molecular mechanism for the observed beneficial effects of sertraline on motor deficits in different mouse models of HD.

Similarly, metformin, an antidiabetic drug which indirectly suppresses mTOR function and Raptor/mTOR interaction through the activation of the AMPK pathway has been reported to show a strong pro-cognitive effect in patients with cognitive impairment. The mechanism of the observed pro-cognitive effect of metformin remains unknown (Nibber et al., 2022). Sanchis et al. (2019), described a neuroprotective effect of metformin on *C. elegans* and the zQ175 knock-in HD mouse model. In this study, metformin reduced early behavioural deficits, mHTT aggregation, loss of neurotrophic support and neuroinflammation, which correlated with AMPK activation and normalized pERK levels. Moreover, in a statistical analysis of a sample population of patients participating in the Enroll-HD database, the use of metformin was associated with better results on cognitive tests and a positive trend towards motor function improvement was observed, although not statistically significant (Hervás et al., 2017). Overall, metformin has been reported to be an indirect negative modulator of cap-dependent protein synthesis and several independent studies propose its neuroprotective role, especially in affecting cognitive dysfunction. Therefore, although our group has only described translational alterations in the striatum, these observations raise the question of whether alterations in cap-dependent translation might also affect other non-striatal brain regions, which are responsible for cognitive disturbances including the hippocampus and the cortex. We also hypothesize that the normalization of cap-dependent translation could be involved in the reported beneficial effects of metformin on HD disease progression.

AIMS

AIM 1: To evaluate the potential of translation modulation as a therapeutic strategy for striatal associated neuropathology and motor behaviour.

1.1 To evaluate the effects of 4E-BP1 modulation in the striatum on motor symptoms and striatal pathology.

1.2 To establish an *in vitro* model to study translational alterations in HD and the use of pharmacological agents to modulate protein synthesis.

1.3 To study the behavioural and biochemical effects of the pharmacological inhibition of 4E-BP1.

AIM 2: To investigate the role of translational alterations in the cognitive deficits observed in HD.

2.1 To analyse whether 4E-BP1 activity and *de novo* protein synthesis rate are altered in HD hippocampus and cortex.

2.2 To study the behavioural, electrophysiological and biochemical consequences of the modulation of translation initiation in HD hippocampus.

METHODS

1. HD MOUSE MODEL

The animal model used throughout this Thesis is the R6/1 transgenic mice model of HD with a B6CBA background obtained from Jackson Laboratory (Bar Harbor, ME, USA). The R6/1 mouse model expresses the N-terminal exon 1 fragment of m*HTT* with a CAG repeat length of 115 CAGs (Mangiarini et al., 1996). Genotyping of the mice was performed by conventional PCR using primers to expand the exon 1 of m*HTT*. Only male R6/1 mice were used in all the experiments, and their non-transgenic WT littermates were chosen as controls. All mice were housed in the Animal Facility of the University of Barcelona. Mice were housed together in numerical birth order in groups of mixed genotypes and a microchip was used for subject identification. The animals were housed with ad libitum access to food and water in a room kept at 19-22°C and 40-60% humidity, under a 12:12 light/dark cycle. All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local animal care committee of the Universitat de Barcelona, following European (2010/63/UE) and Spanish (RD53/2013) regulations for the care and use of laboratory animals.

2. HUMAN POST-MORTEM BRAIN SAMPLES

Samples from hippocampal CA1 region from 11 patients and 7 control individuals were obtained from the Neurological Tissue Bank of the Biobank-Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS; Barcelona, Catalonia) following the guidelines and approval of the local ethics committee (Hospital Clínic of Barcelona's Clinical Research Ethics Committee). See details in Table 2.

ID	Pathological diagnosis	Gender	Age (years)	CAG repeats	PMD (hh:mm)
CS-497	Control	M	82		2:30
CS-1694	Control	M	58		5:00
CS-1858	Control	F	83		7:30
CS-1870	Control	F	97		7:20
CS-1888	Control	F	93		5:30
CS-1937	Control	F	83		7:33
CS-1949	Control	M	86		7:25
CS-1193	HD, VS 3-4	M	55	-	7:00
CS-1294	HD, VS 3	M	53	45 +/- 2	7:00

CS-1334	HD, VS 1	M	73	40 +/- 2	7:00
CS-1438	HD, VS 3	M	85	40	5:30
CS-1630	HD, VS 2	M	76	41	6:00
CS-1638	HD, VS 2	M	72	-	13:10
CS-1758	HD, VS 2-3	M	68	42 +/- 2	6:10
CS-1844	HD, VS 2	F	69	43	15:30
CS-1874	HD, VS 3	M	56	43	4:30
CS-1875	HD, VS 2-3	M	84	39	8:00
CS-1933	HD, VS 2	F	86	40	12:20

Table 2.- Human CA1 post-mortem samples used in this Thesis. Information about the Vonsattel grade (VS), gender, age, CAG repeat length and post-mortem delay (PMD) is provided.

3. HUMAN FIBROBLAST BIOPSY

Patient-derived fibroblast cultures were obtained from sterile and non-necrotic skin biopsy samples from HD patients and control individuals (Table 3) provided by Dr. Pérez from Unitat de Transtorns del Moviment del Servei de Neurologia de l'Hospital de Sant Pau. After the nature, purpose and risks of the study were explained, informed written consent was obtained from each subject before participation in this study.

ID	Diagnosis	Gender	Age (years)	CAG repeats	Stage	Depression
1	Control	F	43	-	0	0
2	Control	F	43	-	0	0
3	Control	F	62	-	0	0
4	Control	F	41	-	0	0
5	Control	M	26	-	0	0
6	Control	F	45	-	0	0
7	HD	F	49	43	1	0
8	HD	F	41	43	1	0
9	HD	F	69	42	1	0
10	HD	F	56	41	1	0
11	HD	F	37	44	1	0
12	HD	F	40	42	1	1
13	HD	F	69	40	2	0
14	HD	M	43	41	2	0
15	HD	F	39	45	2	1
16	HD	M	43	41	2	1
17	HD	M	59	41	2	1
18	HD	F	40	49	2	1

19	HD	M	59	46	3	1
20	HD	F	43	42	3	1
21	HD	M	62	41	3	1
22	HD	F	70	41	3	1

Table 3.- Details from HD patients and control individuals from which skin biopsies were obtained. Information about gender, age (years), CAG repeats, disease stage and major depression diagnosis (1: yes; 0: no) is provided.

4. AAV-MEDIATED 4E-BP1 EXPRESSION VECTORS

A constitutively active form of 4E-BP1 (4E-BP1^{F113A}) was expressed in *in vitro* and *in vivo* models using different kinds of expression vectors all derived from the plasmid pCAGGS-4EBP1 F113A which was a gift from Dr. Angelique Bordey's lab from Yale School of Medicine (Connecticut, EEUU; Addgene plasmid #81122).

For the 4E-BP1^{F113A} immunoprecipitation in STHdh$^{Q7/Q7}$ striatal cells, a polyhistidine-tagged fusion protein (HA-4E-BP1^{F113A}) was produced by cloning the gene of interest from the pCAGGS-4EBP1 F113A into a pNBM470 backbone vector. Correct insertion of the gene was confirmed by plasmid sequencing.

For the *in vivo* studies, the same vector, pCAGGS-4EBP1 F113A, was used to obtain a rAAV2/8 adenoviral vector (BamHI site at 5' and AgeI at the 3'). The rAAV2/8 plasmids and infectious AAV viral particles were generated by the *Unitat de Producció de Vectors* from the Center of Animal Biotechnology and Gene Therapy at the Universitat Autònoma de Barcelona, Catalonia. The empty AVV backbone vector was used as a negative control in all experiments.

5. CELL CULTURES

5.1 Primary striatal neuronal cultures

Primary neuronal cultures were obtained from R6/1 mice striata on day 18 of embryonic development (E18). Tissue dissection was conducted in ice-cold Neurobasal medium (Gibco; 21103-049) and isolated striata were transferred to a tube containing

supplemented Neurobasal with 2% B27 (Gibco; 17504-044) and 1% Glutamax (Gibco; 35050-038), where they were mechanically dissociated using a 1 ml filtered tip. The dispersed cells were counted and seeded onto 6-well plates, for protein extraction, or 12 mm coverslips, for immunocytochemistry, previously coated with 0.1 mg/mL poly-D-lysine (Sigma Aldrich; P0899). Cultures were incubated in supplemented Neurobasal medium at 37ºC and 5% CO_2. Strict sterile conditions were kept throughout the whole procedure as no antibiotics were used. PCR from tail biopsies was conducted to determine the embryos' genotypes.

5.2 Primary human fibroblast cultures

Skin biopsies were rinsed in Gibco Dulbecco's Modified Eagle Medium (DMEM) (Gibco; 41966-029) and mechanically fragmented in 1-2 mm diameter pieces. Tissue pieces were then spread in T25 culture flasks in DMEM with 25 mM glucose (Gibco; 41966-029) supplemented with 10% v/v Fetal Bovine Serum (FBS) (Biological Industries; 040071A), 1% v/v Penicillin/Streptomycin (Gibco; 15140122) and 1% v/v Amphotericin B (Sigma; A-2942). Cell growth was assessed using a phase-contrast microscopy and after 50% confluence was achieved, cells were detached from the flask by trypsinization (2.5%) (Gibco; 1509046) and split into two T75 culture flasks for expansion. Fibroblast cell lines were cultured in the same growth media across experiments.

5.3 STHdh$^{Q7/Q7}$ culture

Conditionally immortalized STHdh$^{Q7/Q7}$ striatal neuronal progenitor cell line expressing endogenous levels of *HTT* with 7 glutamines were obtained and cultured as described elsewhere (Trettel et al., 2000). Concisely, cells were grown at 37ºC and 5% CO2 in DMEM (Sigma-Aldrich; 5671) supplemented with 10% FBS, 1% streptomycin-penicillin, 1 mM sodium pyruvate, 2 mM L-glutamine and 400 µg/ml geneticin (G418 sulfate) (Thermo scientific; 11811-023) as a selection antibiotic.

5.4 Drug screening in primary neuronal cultures

Sertraline and metformin effects on protein synthesis were first evaluated in striatal primary neuronal cultures from R6/1 mice. To do so, 400,000 cells/well were seeded in 6 well-plates previously coated with a poly-D-lysine. Cultures were grown for 14 days

before treatment to allow neuron growth and maturation. At DIV 10, B27 supplement was removed from the medium to avoid interferences with the treatment as it leads to hyperactivation of the mTOR pathway in neuronal cultures as reported (S. W. Park et al., 2014). and confirmed in our experimental conditions (Data not shown). Cells were finally treated at 14 DIV with either the appropriated vehicle or 10 μM of sertraline (Toronto Research Chemicals; S280007) or 1 mM of metformin (Merck; 317240) for 4 h. Experimental conditions were optimized after an extensive experimental set up in which 4 and 24 hours of incubation and different concentrations were tested.

6. MOLECULAR TECHNIQUES

6.1 Total protein extraction

Mouse brain tissue samples from different brain regions were dissected on ice and flash frozen. For total protein extraction, samples were sonicated in ice-cold tissue lysis buffer containing 150 mM NaCl, 1% Triton X-100, 10 mM EGTA, 50 mM Tris-HCl (pH 7.5) supplemented with protease (2 mM phenylmethylsulphonyl fluoride (PMSF), 10μg/μl aprotinin, 1 μg/μl leupeptin) and phosphatase (2 mM sodium orthovanadate) inhibitors.

Alternatively, protein extraction from cultured cells was performed as described elsewhere (Saavedra et al., 2011). Briefly, cell pellets were obtained by trypsinization (2.5%) followed by an ice-cold PBS wash (for proliferating cell cultures) or scraping (for neuronal cultures) and mechanically lysed using a narrow-gauge syringe needle in cell lysis buffer containing 1% Nonidet™ P 40 Substitute (NP40; Sigma-Aldrich; 1175499001), 50mM Tris-HCl (pH 7.5), 150mM NaCl and 10mM EDTA, supplemented with the same protease and phosphatase inhibitors as the tissue lysis buffer described above.

In either case, supernatants were collected after centrifugation (16,500 g for 15 min at 4°C) and total protein concentration was quantified using the Dc Protein Assay Kid (Bio-Rad Laboratories; 5000116).

6.2 Western Blot (WB)

Proteins were reduced and denatured at 100°C for 5 min in SDS sample buffer containing 62.5 mM Tris-HCl pH 6.8, 140 mM β-mercaptoethanol, 2% (w/v) SDS, 10% glycerol and 0.1% (w/v) bromophenol blue. Next, denatured samples were loaded and resolved in denaturing polyacrylamide gels and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell; Dassel, DE). Successful transfer was confirmed by Ponceau staining. Membranes were washed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and unspecific antibody binding was prevented by blocking (TBS-T plus 5% bovine serum albumin (BSA) and 5% powder milk) at room temperature (RT) for 1 h. Primary antibody incubation was conducted overnight (O/N) at 4°C with a gentle agitation.

Antigen	MW (kDa)	Host	Dilution	Source / Identifier
4E-BP1	15-20	Rabbit	1:1000	Cell Signaling / 9452
Cyclin D1	34	Rabbit	1:1000	Abcam / ab134175
STEP (23E5)	46	Mouse	1:1000	Santa Cruz Biotech / sc-23892
DARPP-32 (clone 15)	32	Mouse	1:1000	BD Bioscience / 611520
eIF4E	25	Rabbit	1:1000	Cell Signaling / 9742
Huntingtin (EM48)	>350	Mouse	1:1000	Millipore / MAB5374
4E-BP1 (pThr37/46)	15-20	Rabbit	1:1000	Cell Signaling / 9459
HA-tag	-	Rabbit	1:1000	Cell Signaling / 3724
Puromycin (clone 12D10)	-	Mouse	1:1000	Merck / MABE343
α-Tubulin (loading control)	55	Mouse	1:50000	Sigma Chemical Co. / 6074
Actin (loading control)	42	Mouse	1:10000	MP Biochemicals / 69100

Table 4.- Primary antibodies used for WB and immunoprecipitation analysis. A list of the antibodies is included, along with information on the host species, dilution used, estimated molecular weight of the target protein and source/identifier.

After primary antibody incubation, membranes were properly washed with TBS-T and incubated with the appropriated horseradish peroxidase-conjugated secondary antibody at RT for 1 h. After three washes with TBS-T, chemiluminescence protein detection was performed using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology; sc-2048). Detected protein bands were quantified using a computer-assisted densitometric analysis (Gel-Pro Analyzer, version 4, Media Cybernetics).

Secondary antibody	Dilution	Source / Identifier
Anti-Mouse IgG	1:2000	Promega / W4021
Anti-Rabbit IgG	1:2000	Promega / W4021

Table 5.- Secondary antibodies used for WB blot analysis. A list of the antibodies is included, along with information on the dilution used and source/identifier.

6.3 Cell transfection and immunoprecipitation

pNBM470-HA-4E-BP1 F113A plasmid was transfected into STHdh$^{Q7/Q7}$ cells 24h after seeding using Lipofectamine™ 3000 Transfection Reagent (ThermoFischer Scientific, L3000001) following manufacturer instructions. After 48h, transfected STHdh$^{Q7/Q7}$ striatal cells were collected and protein extraction was performed as described above in ice-cold immunoprecipitation (IP) buffer containing 50 mM Tris-HCl (pH 8.0), 1% IGEPAL, 150 mM NaCl and 50mM NaF. Phosphatase Inhibitor Cocktail Set II (Calbiochem; 524625) and Protease Inhibitor Cocktail (Sigma-Aldrich; 04693116001) were added to avoid protein degradation. 300 µg of protein were incubated O/N at 4ºC on a rotating mixer with anti-HA-Tag antibody (Table 4) or rabbit IgGs (Cell Signalling Technology; 3900) as a negative control. Antibody-antigen complexes were incubated O/N at 4ºC with 50µL of Protein A-Agarose (Santa Cruz Biotechnology; sc-2001) beads and immunoprecipitates were collected by centrifugation (5 min, 4000 rpm at 4ºC) and washed three times with IP buffer, IP buffer/PBS (1:1) and PBS, respectively. Proteins were eluted from the beads by boiling the samples for 10 min at 100ºC in 4% SDS

sample buffer with 10% β-mercaptoethanol. Protein precipitation was then analyzed by WB as detailed previously.

7. SURFACE SENSING OF TRANSLATION (SUnSET)

Global protein synthesis rates were measured by applying variations of the SUnSET method adapted to *in vitro* and *ex vivo* models of HD. This method is based on the ability of the puromycin to label the newly synthesized peptides when administrated at low doses (Santini et al., 2013).

7.1 SUnSET method in cell cultures

In cultured cells, the medium was replaced with fresh medium containing 1 μM puromycin (Sigma-Aldrich; P8833) and incubated at the standard culture conditions for each cell culture type for 30 min. After incubation, protein extraction and WB was performed as described above using an anti-puromycin primary antibody (Table 4).

7.2 SUnSET method in brain slices

Protein synthesis was also measured in corticostriatal slices as described at Santini et al. (2013). Animals were sacrificed by cervical dislocation and the brain was quickly removed and kept in oxygenated Artificial CerebroSpinal Fluid (ACSF) containing 125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$ and 11 mM glucose. 400 μm coronal slices were obtained from WT and R6/1 mice using a conventional vibratome (Leica; VT1000S) and incubated for 1 h in oxygenated ACSF at 32ºC. Next, ACSF was replaced with fresh ACSF containing 5 ug/ml of puromycin for another 45 minutes. Then, the brain region of interest was dissected out on ice and the resulting tissue samples were flash frozen. Finally, protein extraction from tissue samples was performed and lysates were processed for Western blotting as described above.

In both cases, puromycin-labelled nascent peptide chains were detected using an anti-puromycin primary antibody and protein synthesis was determined by measuring total lane signal from 250-25 kDa and normalizing against α-tubulin as a loading control.

8. TRANSLATOME PROFLING

Translatome profiling of hippocampal tissue from R6/1 mice was performed by applying the SUnSET method to fresh hippocampal slices as described in the section "6.4.2 SUnSET method in brain slices" and analyzing the puromycin pull-down by mass spectrometry as described in (del Toro et al., 2020). Briefly, using an electric homogenizer, dissected hippocampal tissue from was homogenized for 1 minute at 4ºC using lysis buffer (50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 supplemented with protease (2 mM phenylmethylsulphonyl fluoride (PMSF), 10µg/µl aprotinin, 1 µg/µl leupeptin) and phosphatase (2 mM sodium orthovanadate) inhibitors. Samples were centrifuged at 3000 rpm for 10 minutes after being incubated on ice for 20 minutes. Protein concentration was determined as described above. Each pull-down required the usage of 1 mg of protein at a final concentration of 2 µg/µl in lysis buffer in a final volume of 500 µl. 2 µg of goat anti-human IgG antibody (Jackson Immunoresearch; 109-005-098) were used in the control pull-down, whereas 2 µg of anti-puromycin antibody (see Table 4) was utilized in the puromycin pull-down. Samples were incubated at 4°C overnight under rotatory agitation. After 24h, 10 µg of Sepharose beads were added to each sample (Amersham CL-4B, 17-01780-01, 50% v/v in lysis buffer) and incubated for 4 hours under rotatory agitation. After being centrifuged for 5 minutes at 3000 rpm, the sepharose beads were washed three times: once with 400 µl of lysis buffer, once with a 1:1 (v/v) lysis buffer:PBS solution, and once with only PBS. Finally, pull-down samples were analyzed through mass spectrometry (MaxQuant run, Proteomic facility, Max Planck Institute of Biochemistry, Martinsried, Germany)

9. IMMUNOCYTOFLUORESCENCE

For immunofluorescence staining, cells were seeded onto 12mm glass coverslips and fixed with 4% PFA for 10 min at RT. PFA fixation was stopped by a PBS wash and a 20 min incubation with PBS containing 0.2 M glycine. To avoid background fluorescence, cells were incubated with 50mM NH_4Cl in PBS for 10 min and permeabilized in blocking buffer (PBS containing 1% BSA, 0.2% gelatin, 0.2% Triton X100) for 30 min at RT. After this blocking step, cells were incubated for 30 min at RT with the primary antibody (Table 6) diluted in blocking buffer. Next, coverslips were washed thrice with PBS for 5 min under gentle agitation and incubated with the appropriated secondary antibody

(Table 7) for 1h at RT. Finally, coverslips were mounted with Dapi-FluoromontG™ (SouthernBiotech; 010020).

10. IMMUNOHISTOFLUORESCENCE

Pentobarbital (60–80 mg/kg) was used to deeply anaesthetize mice. After 5 minutes of intracardial perfusion with ice-cold PBS, the animals were perfused with a 4% PFA solution in 0.1 M sodium phosphate (pH 7.2). After removal, the brains were post-fixed in PFA throughout the night. After cryoprotection with 30% sucrose in PBS containing 0.02% sodium azide, brains were kept at 4°C until further processing. Brains were flash-frozen in 2-methyl butane to obtain serial coronal sections (30 μm thick) with a cryostat. Free-floating slices were kept in PBS with 0.02% sodium azide at 4°C until processing. Autofluorescence was prevented before the immunostaining protocol by a 30 min incubation with 50 mM NH_4Cl after two PBS washes. Tissue was then permeabilized at room temperature for 20 min with PBS containing 0.5% Triton X-100 and blocked for 2 h with PBS plus 0.2% bovine serum albumin, 0.2% glycine, 0.2% lysine, 0.5% Triton X-100, 0.2% sodium azide and 5% normal horse serum (NHS; Pierce Biotechnology. Afterwards, slices were incubated overnight at 4°C with the corresponding primary antibodies (Table 6) in PBS with 0.3% Triton X-100, 0.2% BSA and 0.2% sodium azide. After 24 hours, slices were rinsed twice with PBS (10 min) and incubated for 2 h at room temperature with the appropriate fluorescent secondary antibodies (Table 7). Then, after two more PBS washes, slices were incubated for 10 min with Hoescht 33258 (1:4000; Invitrogen) at room temperature for nuclear staining. Slices were washed again and mounted on silane-coated slides. When dry, coverslips were placed using Mowiol-mouting media (Sigma-Aldrich; 81381).

Antigen	Host	Dilution	Source / Identifier
Puromycin (clone 12D10)	Mouse	1:200	Merck / MABE343
DARPP-32 (clone 15)	Mouse	1:500	BD Bioscience / 611520
HA-tag	Rabbit	1:200	Cell Signaling / 3724
NeuN	Rabbit	1:200	Cell Signaling / 24307

Pyk2	Rabbit	1:500	Sigma-Aldrich/ P3902

Table 6.- Primary antibodies used for immunostaining. A list of the antibodies is included, along with information on the host species, dilution used and source/identifier.

Secondary antibody	Dilution	Source / Identifier
Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	1:200	Jackson ImmunoResearch / 711-545-152
Cy™3 AffiniPure Donkey Anti-Mouse IgG	1:200	Jackson ImmunoResearch / 715-165-150
Cy™3 AffiniPure Donkey Anti-Rabbit IgG	1:200	Jackson ImmunoResearch / 711-165-152

Table 7.-Secondary antibodies used for immunostaining. A list of the antibodies is included, along with information dilution used and source/identifier.

11. IN VIVO TREATMENT APPROACHES

11.1 *In vivo* sertraline treatment

Twelve-week-old WT and R6/1 mice received a daily intraperitoneal injection of sertraline (20 mg/kg) or vehicle (5% Polysorbate 80 in H_2O) for 4 weeks. After 3 weeks of treatment, motor skills were tested through a battery of motor coordination and learning tests including the open field, the accelerating rotarod, the balance beam and the vertical pole. On days in which motor tests and treatment were coincident, injections were administered 1 h prior to starting the test. Mice were sacrificed at 16 weeks of age by cervical dislocation and brain tissue was processed for biochemical analysis. Protein synthesis was measured in a second batch of animals through the application of the SUnSET method on corticostriatal slices as described above.

11.2 Intrastriatal injection of adenoassociated vectors

Twelve-week-old WT and R6/1 mice were deeply anaesthetized with a mixture of oxygen and isoflurane (4-5% for induction and for 1-2% maintenance) and placed in a

stereotaxic apparatus for the intrastriatal injection of rAAV2/8 expressing 4E-BP1^{F113A} or NULL vectors (2 μl; 1.5 x 10^9 genomic copies). Two injections were performed in each striatum at the coordinates detailed in Table 8. Viral vectors were injected using a 10 μl-Hamilton microliter syringe at an infusion rate of 250 nl/min. The needle was left in place for 6 min to ensure complete diffusion of the viruses and then slowly retracted from the brain. Four weeks after the injection a biochemical and behavioural analysis was conducted.

	AP	ML	DV
Anterior injection	+0.8	±1.8	-2.6
Posterior injection	+0.3	±2	-2.6

Table 8.- Stereotaxic coordinates for bilateral intrastriatal injection of AVV-mediated expression of 4E-BP1^{F113A}. Coordinates are indicated in reference to bregma and dorsoventral coordinates on the brain surface are defined as 0,00 mm. AP: *anteroposterior*; ML: *mediolateral*; DV: *dorsoventral*.

11.3 Surgery and 4EGI-1 treatment

Male WT and R6/1 mice at 13 weeks of age were anaesthetized as previously described and placed in the stereotaxic apparatus. Using a scalpel, an incision was made to expose the skull and a guide cannula (Bilaney, Düsseldorf, Germany) with a depth of 2 mm below the pedestal was placed at the following coordinates: - 0.22mm anteroposterior, + 1mm mediolateral. Guide cannulas were fixed into the skull using two surgical screws and acrylic dental cement. Cannula dummies without projection were inserted into the guide cannulas to prevent clogging. Mice were allowed to recover for one week before the start of the pharmacological treatment and behavioural analysis. 4EGI-1 (Merck; 324517) was dissolved in DMSO and infused in a final concentration of 10 μM in Artificial CerebroSpinal Fluid (ACSF) (TOCRIS Bioscience; 3525) (1% DMSO). Mice received daily vehicle or 4EGI-1 (1 μl at 10 μM) at an infusion rate of 500 nl/min through an infusion cannula with a 0.5 mm projection from the guide cannula. Infusion cannula was not removed for another 3 min after the infusion to ensure proper drug diffusion and reflux prevention. 4EGI-1 treatment was administered for 7 days in which a battery of hippocampus-dependent cognitive tests was performed at least one hour after the infusion. Independent animal batches which did not undergo behavioural tests were used for the electrophysiological and proteomic analysis.

12. BEHAVIOURAL ANALYSIS

12.1 Motor coordination and learning assessment

Open field (OF)

Mice were placed in the centre of a non-reflective opaque square arena with a side length and wall height of 40 cm. Light intensity was kept at around 20 lux throughout the arena. Mice were tracked for 10 min while freely moving. After each subject, the arena was carefully cleaned to avoid odor trails. Total distance travelled, rearing time and mean speed were analysed using SMART v3.0 software (Panlab). Time in the centre was analysed after dividing the surface of the arena into central and peripheral zone.

Vertical pool

Vertical pole test was performed using a wooden non slippery pole. In both, 2 training and the testing session (3 trials per session, 1 session per day), mice were placed on the top of the pole facing upwards and with both front limbs holding onto the tip. Animals were trained to completely turn upside down and descend the pole during the two days. During the testing session, time to turn and time to descend were measured and trials in which the animal fell were counted.

Balance beam

Mice were evaluated when walking through an elevated wooden squared beam (50 cm x 1,3 cm) to a save platform. The beam was placed 40 cm above the ground and divided into 5 cm frames. This test consisted of a 2 min training session and a 2 min testing session carried out 4 h apart, on the same day. The number of slips and the frames crossed are measured during the testing session.

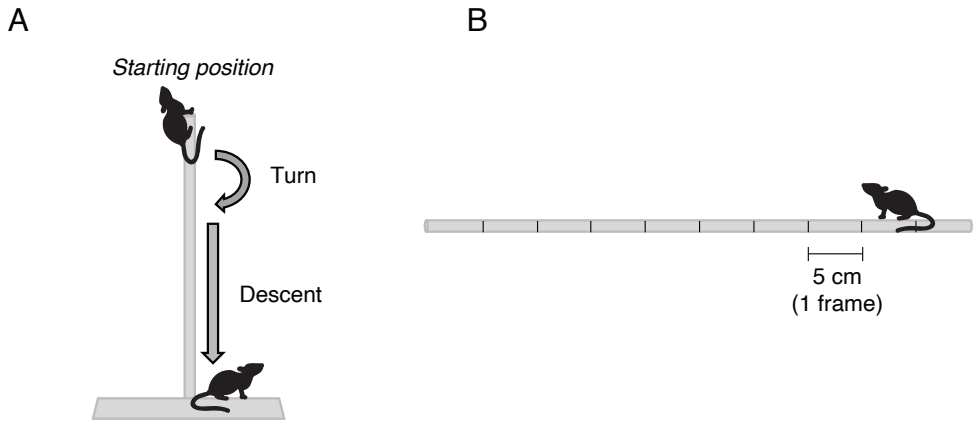


Figure 9. Schematic representation of the (A) vertical pole and the (B) balance beam tests.

Accelerating rotarod

Motor learning and coordination were evaluated using a motorized rod rotating at a gradually increasing speed starting at 4 rpm until reaching 40 rpm in 5 min. Trials where the subject did not walk using all 4 limbs in the rod were not considered. Latency to fall in seconds was analysed in each trial. The test was conducted during 3 consecutive days and 4 trials per day were carried out 1 hour apart. After each subject, the arena was carefully cleaned to avoid odor trails.

12.2 Cognitive function assessment

Novel object location test (NOLT)

Hippocampal spatial memory assessment was conducted on a non-reflective opaque square arena with a side length and wall height of 40 cm. Light intensity was maintained at 40 (± 10) lux in all corners of the arena for preference avoidance. On the first day, during the habituation session, mice were first allowed to freely explore the empty arena without spatial cues or objects for 30 min. In this phase, spontaneous locomotor activity was also evaluated by measuring the distance travelled. After 24h, during the training session, a spatial cue was added on one wall, as well as, two similar objects, placed on two consecutive corners, 9 cm away from the proximal walls. In this session, mice were allowed to explore the objects during 10 min. On the third consecutive day, during the testing session, an object was relocated (new location) in a new corner of the arena

while the placement of the other (old location) and the spatial cue remained the same. During the testing session, the time exploring the object on the new or old location was analysed using SMART v3.0 software. The object preference was calculated as the percentage of time exploring each object from the total time exploring both objects.

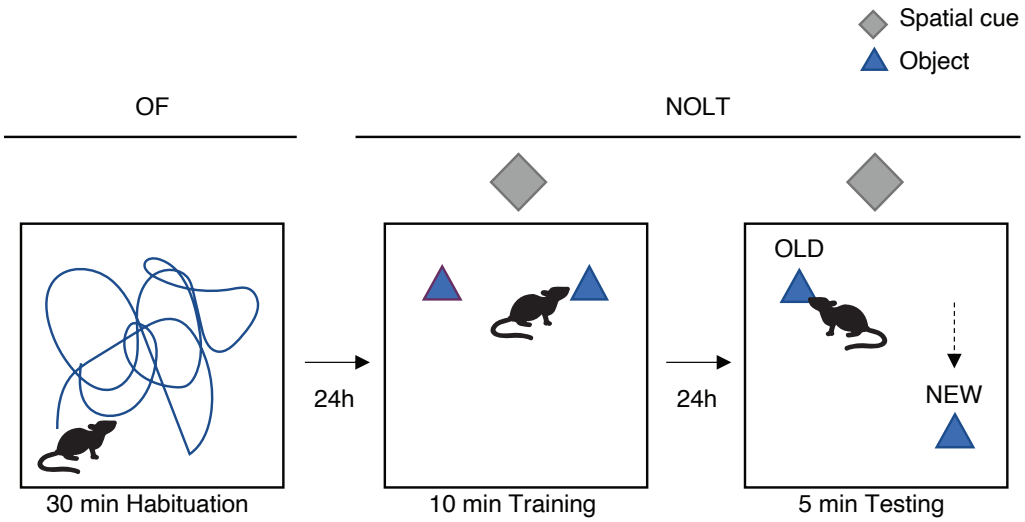


Figure 10. Schematic representation of the NOLT test.

Y-maze spontaneous alternation test

Mice were placed at the end of one arm of a Y-shaped maze with three clear plastic arms at 120º from each other. The animal is allowed to freely explore the 3 arms for 10 min and the number of triads (full arm alternations), and total number of arm entries were analysed. Arm entries were only counted when the animal's 4 limbs have entered the arm. Percentage of spontaneous alternation was calculated as the number of triads/ number of possible triads (number of arm entries -2) * 100.

T-maze

Spatial memory was evaluated through the T-maze Test in which a T-shaped maze consisting in a starting arm and two goal arms (left and right arm) was used. Light intensity was ensured to be the same in both left and right arms for preference avoidance. The test consisted in a training session followed by a testing session conducted 1 h later. During the testing session, the animal was placed at the end of the starting arm and was allowed to freely explore one arm (old arm) for 10 min while the

other remained inaccessible (new arm). For the testing session, the animal was allowed to explore both arms for 5 min and first arm choice and the percentage of time exploring the familiar or new arm were analyzed using SMART v3.0 software. The assignment of the old/new arm was counterbalanced between mice to avoid preference due to uncontrollable variables.

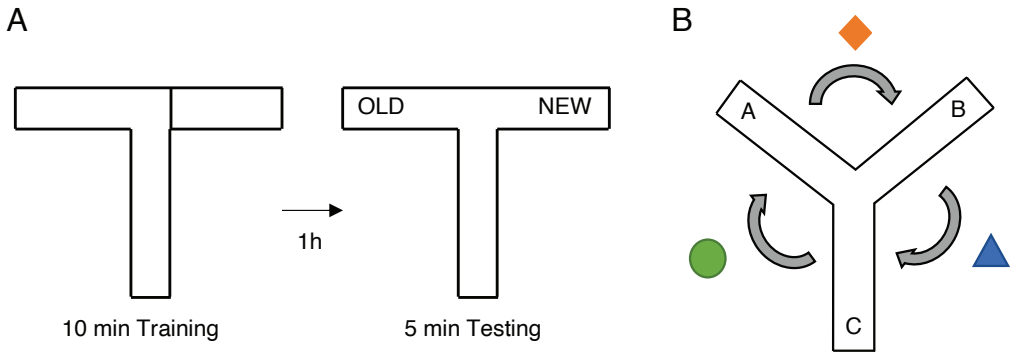


Figure 11. Scheme showing the T-maze (a) and Y-maze spontaneous alternation (b) tests set up.

13. ENROLL-HD DATABASE

Clinical data from the fifth Enroll-HD periodic dataset (PDS5) was analysed to evaluate the potential beneficial effects of sertraline treatment in the disease progression of HD patients in a large cohort. Enroll-HD is a global multi-center longitudinal observational study which results in a clinical research platform designed to facilitate clinical research in HD. PDS5 contains clinical data from 21,116 Enroll-HD participants which is collected annually under local ethical approval. Only HD patients (both Pre-Motor Manifest (PM) and Motor-Manifest (M)) with a minimum of a 3-year follow-up were included in this study. From the participants meeting the set inclusion-criteria n = 63 were treated with sertraline, n = 30 were treated with only sertraline as an antidepressant, n = 208 were treated with other antidepressants but not sertraline and n = 226 were not prescribed any antidepressant pharmacological treatment. In this analysis, a Linear Mixed Effects Models (LMM) was used to evaluate differences in disease time evolution between these groups of patients through the inclusion of time-group interaction term. The model also took into account gender, age, CAG repeats and HD status (PM or M) as potential confounders. Motoric parameters of symptomatic disease manifestation were analysed using the Unified Huntington's Disease Rating Scale (UHDRS)-Total motor score (TMS).

Functionality was also analysed with the UHDRS-Total functional capacity (TFC), the functional assessment scale (FAS) and Independence Scale (IS).

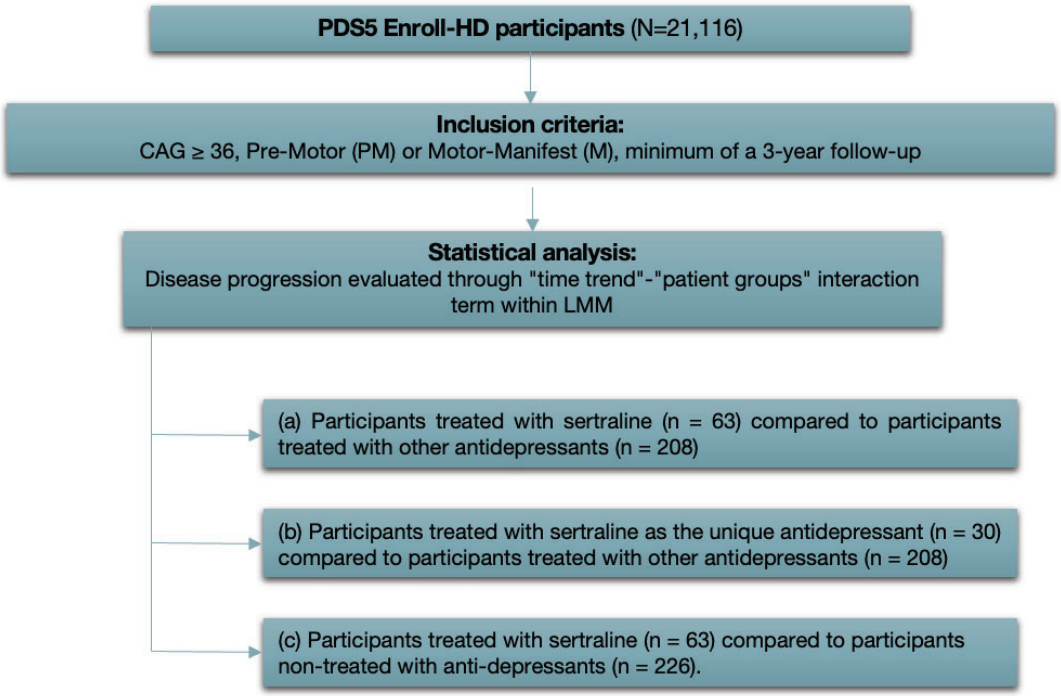


Figure 12.- Schematic workflow of the longitudinal analysis. Enroll-HD database cohort was used to analyse the potential beneficial effects of sertraline treatment in the disease progression of HD patients.

14. ELECTROPHYSIOLOGY

Mice were anaesthetized using isoflurane and euthanized by decapitation. Brains were placed in ice-cold ACSF1 solution (206 mM sucrose, 26 mM NaHCO3, 1.25 mM NaH2PO4, 1.3 mM KCl, 10 mM MgSO4, 1 mM CaCl2, 11 mM glucose, purged with 95% O_2/ 5% CO_2, pH: 7.35). Coronal slices (380um) were then obtained with a vibratome in the same solution. Slices were placed in an incubator beaker containing ACSF2 (119 NaCl mM, 1.25 mM NaH2PO4, 25 mM NaHCO3, 2.5 mM KCl, 2.5 mM CaCl2, 1.5 mM MgSO4, 11 mM glucose, purged with 95% O_2/ 5% CO_2, pH: 7.35) at 34 ºC for 1 h. Subsequently, slices were kept at RT for at least 1 h before starting the recordings. For field excitatory post-synaptic potential (fEPSP) recordings, slices were transferred into a measuring chamber continuously perfused with ACSF2 at 27–29 ºC. The bipolar stimulation and the recording electrode were placed in the Schaffer

collateral pathway and the dendritic branching of the CA1 region, respectively. Stimulation currents between 25 and 155 μA were elicited by a stimulus isolation unit A385 (WPI, Hertfordshire, UK). Input–output (IO) curves were recorded for each slice at 0.03 Hz before baseline recordings for LTP. The stimulation current was then adjusted for each recording to evoke fEPSPs at which the slope was at 50–60% of the maximally evoked fEPSP slope value. LTP was induced by theta-burst stimulation (TBS; 10 theta bursts of four pulses of 100 Hz with an interstimulus interval of 200 ms repeated seven times with 0.03 Hz) after baseline recording for 30 min with 0.03 Hz. fEPSPs were recorded for 1 additional hour with 0.03 Hz after LTP induction. All recordings were amplified and stored using amplifier AxoClamp 2B (Molecular Devices, San Jose, CA). Traces were analysed using Axon pClamp software (Molecular Devices, version 10.6).

15. STATISTICAL ANALYSIS

As stated in the figure legends, statistical analyses were conducted using the Student's t-test for one grouping variable and the one- or two-way ANOVA for multicomponent variables, followed by Bonferroni's post hoc test. All results are expressed as the mean and SEM. A 95% confidence interval was used and values with a $p<0.05$ were considered as statistically significant.

RESULTS

1. STUDY OF THE CONTRIBUTION OF 4E-BP1 IN THE PATHOPHYSIOLOGY OF HD

Previous results obtained in the laboratory correlated the normalization of cap-dependent protein synthesis with the amelioration of the motor symptoms in the R6/1 mice (Creus-Muncunill et al., 2019). In this previous study, inhibition of translation was achieved with an intracerebroventricular infusion of 4EGI-1, a competitive inhibitor of eIF4G-eIF4E interaction (Moerke et al., 2007). These results suggested that the modulation of translation could potentially result in a good therapeutic strategy in the HD striatum. However, since 4E-BP1 activity is regulated through the activity of many upstream kinases which appear altered in HD, amongst them mTOR, it is difficult to determine which of these kinases is significantly contributing to the hyperphosphorylation of 4E-BP1 in the presence of mHTT. For this reason, as the main effector of different pathways regulating the eIF4F complex formation and cap-dependent translation, understanding whether 4E-BP1 phosphorylation alone contributes to the HD phenotype could provide better treatment options. To study this possibility, we used the expression of a constitutively active mutant form of 4E-BP1 (4E-BP1^{F113A}) presenting a mutation in an essential residue within the TOS motif (Phe to Ala, F113A). This mutation prevents 4E-BP1 to be phosphorylated by mTORC1 at residues Thr 37 and Thr 46 (Fig.13A). As a result, 4E-BP1 constitutively binds eIF4E hence, inhibiting protein translation by mimicking the action of all endogenous 4E-BPs (K. M. Choi et al., 2003; Schalm et al., 2003).

1.1 4E-BP1^{F113A} strongly interacts with eIF4E and decreases puromycin incorporation in STHdh$^{Q7/Q7}$ striatal cells

To test whether our construct normalized cap-dependent translation, we analyzed whether overexpressing 4E-BP1^{F113A} in STHdh$^{Q7/Q7}$ striatal cells resulted in increased binding of 4E-BP1 with eIF4E and, consequently, reduced bulk protein synthesis. To this end, STHdh$^{Q7/Q7}$ striatal cells were transfected with a plasmid containing the HA-4E-BP1^{F113A} construct expressing an HA-tagged 4E-BP1^{F113A}. The binding of 4E-BP1 to eIF4E was analyzed by immunoprecipitation 72 h after transfection. As shown in Fig.13C,D, HA-4E-BP1^{F113A} strongly interacted with eIF4E and was accompanied by a reduction in puromycin incorporation levels.

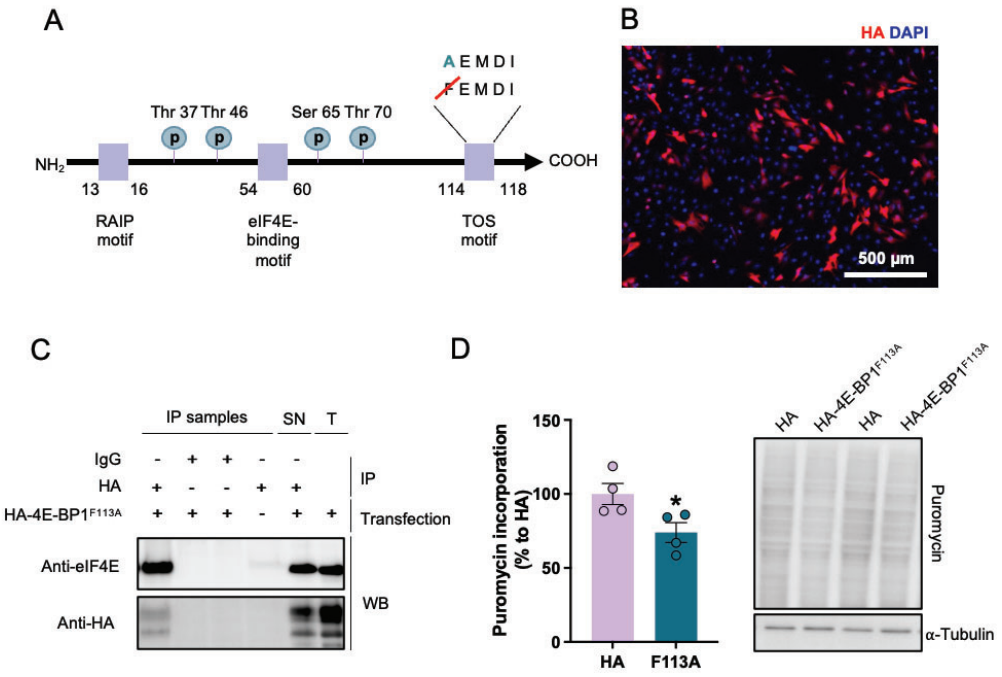


Figure 13.- Overexpression of a constitutively active mutant of 4E-BP1 in STHdh$^{Q7/Q7}$ striatal cells. (A) Schematic model of 4E-BP1^{F113A} showing phosphorylation sites and the point mutation in the TOS motif. (B) Representative image showing HA-4E-BP1^{F113A} plasmid's transfection efficiency through HA tag expression (red). Analysis of (C) HA-4E-BP1^{F113A} binding to eIF4E and (D) puromycin incorporation in STHdh$^{Q7/Q7}$ cells 72 h after transfection with the HA or HA-4E-BP1^{F113A} (F113A) plasmid. Tubulin was used as a loading control. Values are expressed as a percentage of cultures transfected with the control plasmid HA and shown as mean ± SEM. Each point corresponds to the value from an individual culture (n=4). Representative immunoblots are shown. Two-tailed paired Student's t-test, *p<0.05 compared to control plasmid HA. IP: immunoprecipitation; SN: supernatant; WB: Western-blot; T: total lysate.

1.2 4E-BP1^{F113A} striatal expression in WT mice induces an HD-like motor phenotype and striatal atrophy by aberrantly increasing cap-dependent translation

To analyze the contribution of the aberrantly increased inactivation of 4E-BP1 on the R6/1 mice motor phenotype, 10-week-old WT and R6/1 mice were bilaterally injected with AAV-NULL or AAV-4E-BP1^{F113A} into the striatum and behavior was assessed 4 weeks later. Spontaneous locomotor activity was measured on the open field and motor coordination and learning were evaluated by the balance beam and accelerating rotarod, respectively. After the last behavioral test, mice were sacrificed for biochemical analysis (Fig.14A). Unfortunately, a pilot study revealed that no differences were

detected on puromycin incorporation levels as a consequence of 4E-BP1^{F113A} expression in R6/1 mice striatum (Fig.14B).

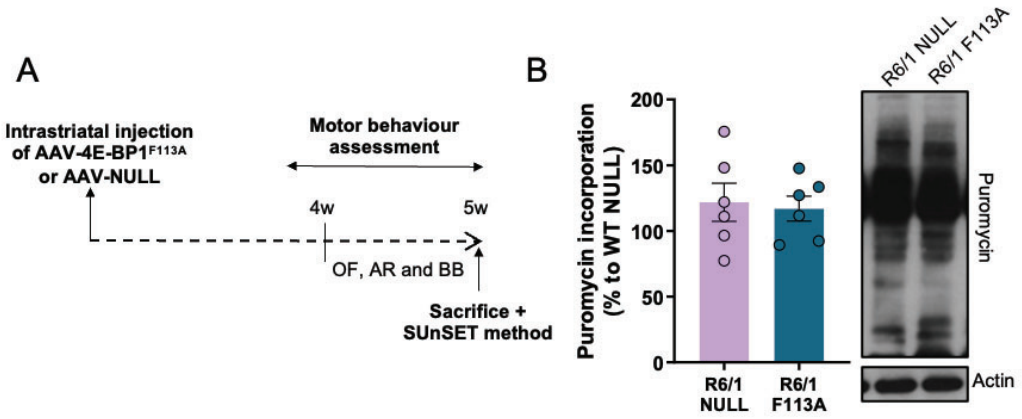


Figure 14.- Intrastriatal injection of AAVs expressing 4E-BP1^{F113A} in the R6/1 striatum does not modify *de novo* translation levels. (A) Schematic representation of the experimental design. R6/1 and WT mice, at 10 weeks of age, were injected bilaterally with AAV-NULL (NULL) or AAV-4E-BP1^{F113A} (F113A) in the striatum and motor behavior was analyzed 4 weeks after injection. (B) Graph shows puromycin incorporation in the striatum of R6/1-NULL (NULL) or R6/1-4E-BP1^{F113A} (F113A) mice 5 weeks after the injection. Representative immunoblots are shown. Data is shown as mean ± SEM and each point corresponds to the value from an individual mouse.

Interestingly, intrastriatal injection of AAV-4E-BP1^{F113A} increased puromycin incorporation in the striatum of WT mice (Fig.15A) and enhanced p4E-BP1 levels (Fig.15B). In addition, although WT-4E-BP1^{F113A} mice showed hyperactive behavior characterized by an increase in the distance travelled during the open field test, we found that 4E-BP1^{F113A} expression induced motor deficits in WT mice (Fig.15C,D,E). Remarkably, in the balance beam test, we observed that the expression of 4E-BP1^{F113A} in WT mice striatum promoted an increase in the slips committed during the test (Fig.15D). Rotarod performance was also altered in the WT-4E-BP1^{F113A} group. Even though latency to fall in the rotarod test progressively increased from the first day in both WT groups, WT mice expressing 4E-BP1^{F113A} fell significantly earlier than the WT-NULL control group (Fig. 15E).

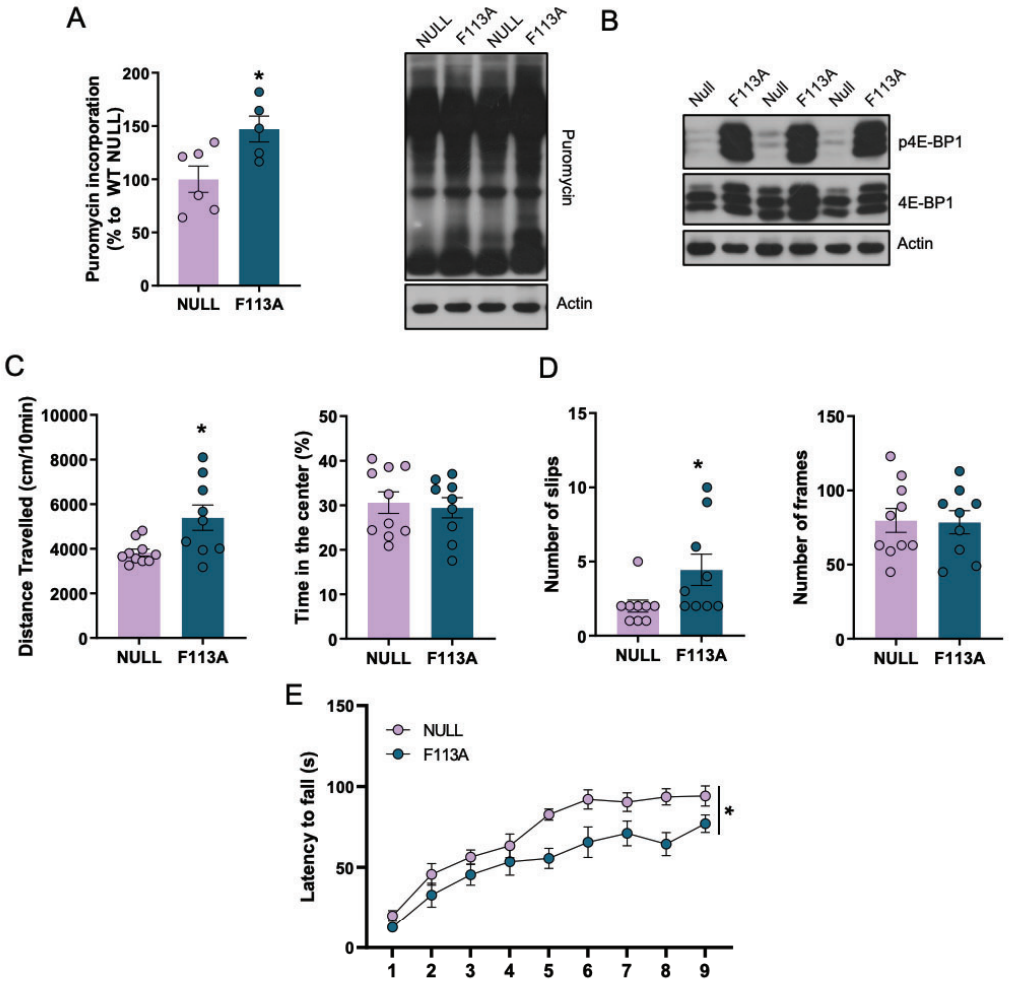


Figure 15.- Intrastriatal injection of AAVs expressing 4E-BP1^{F113A} in WT mice induces motor dysfunction. (A) Graph shows puromycin incorporation in the striatum of WT-NULL (NULL) or WT-4E-BP1^{F113A} (F113A) mice 5 weeks after the injection. Representative immunoblots are shown. (B) Representative immunoblot showing striatal p4E-BP1 and 4E-BP1 levels from the same mice as in A. (C) Open field: Graph showing locomotor activity as the distance travelled (cm) and percentage of time in the center in 10 minutes in an open field arena. (D) Balance beam: Graph shows the number of slips committed and number of frames crossed in the balance beam in 2 minutes. (E) Accelerating rotarod test was performed for four consecutive days (3 trials per day). The latency to fall per test and group is represented as mean ± SEM (WT-NULL, n = 9; WT-F113A, n = 9). In all bar graphs data are shown as mean ± SEM and each point corresponds to the value from an individual mouse. (C,D) Two-tailed unpaired Student's t-test, *p<0.05. (E) Two-way ANOVA with repeated measures followed by Bonferroni's post hoc test, *p < 0.05.

In the same line, we found elevated levels of the protein cyclin D1 (Fig. 16A), which has been reported to be up-translated when 4E-BP1 is inactive in the striatum of WT mice expressing 4E-BP1^{F113A} (Rosenwald et al., 1995). We also examined the levels of the striatal proteins DARPP-32 and STEP46, which are decreased in the HD striatum and

are widely used markers of striatal neurodegeneration and atrophy (Bibb et al., 2000; Saavedra et al., 2011). Interestingly, WB analysis indicated that 4E-BP1^{F113A} expression induced a decrease in the DARPP-32 and STEP46 protein levels (Fig.16A). In agreement, immunohistochemistry analysis corroborated the reduced levels of DARPP-32 and revealed a shrinkage in striatum volume indicating striatal atrophy (Fig.16B). Overall, 4E-BP1^{F113A} expression in the WT striatum induced an unexpected increase in cap-dependent protein translation, perhaps as a result of a compensatory mechanism, and induced striatal neurodegeneration that resulted in an HD-like motor phenotype. Even though our results were unexpected, they reinforce the hypothesis that exaggerated protein translation contributes to HD pathogenesis.

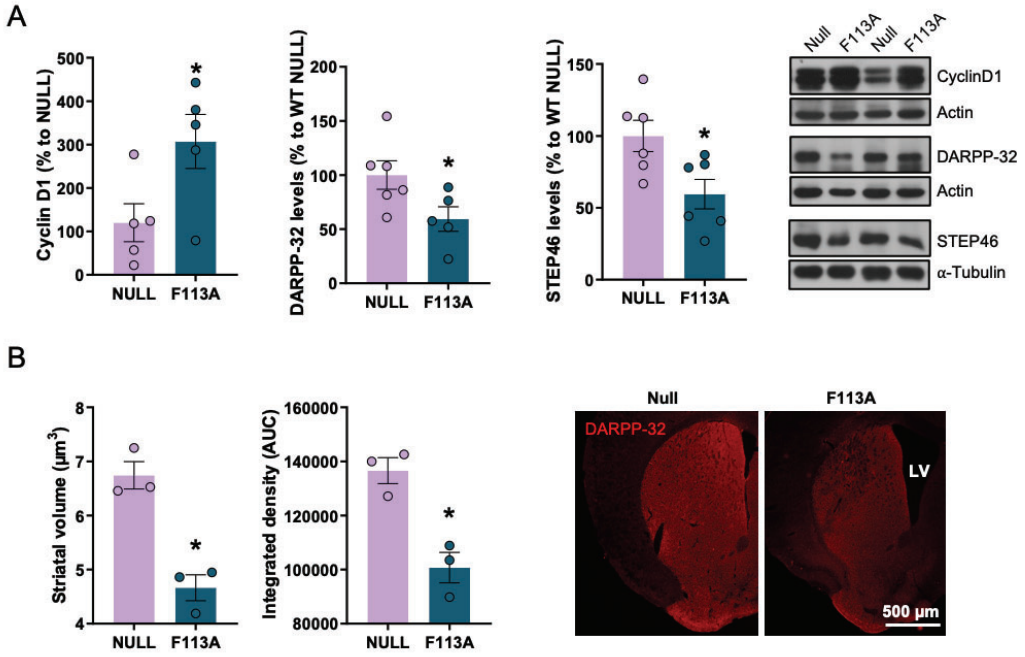


Figure 16.- Intrastriatal injection of AAVs expressing 4E-BP1^{F113A} in WT mice enhances protein translation and induces striatal atrophy. (A) Protein levels of Cyclin D1, DARPP-32 and STEP46 were analyzed by WB in striatal lysates. α-Tubulin and actin were used as loading controls. Values are expressed as a percentage of WT-NULL control group. Representative immunoblots are shown. (B) Graph showing quantification of striatal volume and DARPP-32 expression in WT-NULL or WT-4E-BP1^{F113A} mice. Representative images are shown. LV=lateral ventricle. In all bar graphs data are shown as mean ± SEM and each point corresponds to the value from an individual mouse. Data was statistically analyzed by two-tailed unpaired Student's t-test, *p<0.05.

2. ESTABLISHMENT OF AN *IN VITRO* MODEL TO STUDY HD TRANSLATIONAL ALTERATIONS

Since our results established that normalization of protein translation could ameliorate motor dysfunction in an HD mouse model, in this aim, by testing different *in vitro* models of the disease, we intend to find a model that replicates the exaggerated protein synthesis and inactivation of the translation initiation repressor 4E-BP1 in a dish. Although pharmacological testing on animal models is still necessary to assess the bioavailability, toxicity, and therapeutic efficacy of compounds before being admitted to human clinical trials, *in vitro* disease models are a necessary tool not only for the primary screening of potential new compounds showing neuroprotective effects but also to prevent drugs with insufficient therapeutic efficacy from entering preclinical animal testing (Stevens & Baker, 2009).

2.1 Fibroblasts from HD patients with less than 42 CAG repeats present increased protein synthesis.

Previous research has shown that peripheral tissues including lymphocytes and fibroblasts from HD patients can display alterations that are similar to those described in HD neurons (reviewed in Sassone et al., 2009). Therefore, skin biopsies extracted from HD patients at various stages of the disease progression and from unaffected individuals were used to generate primary fibroblast cultures. Puromycin incorporation and p4E-BP1 at Thr37/46 protein levels were determined by WB. When compared to control fibroblasts, both parameters tended to increase in fibroblasts from HD patients, but the increment was not statistically significant (Fig.17A). Since HD is caused by an expansion of the CAG repeat in exon 1 of the *HTT* gene, we categorized fibroblasts from HD patients based on the number of CAG repeats. We used a scatter plot to determine a CAG threshold by combining the number of CAG repeats (Y axis) and puromycin incorporation levels (X axis) for each individual. The result showed that the threshold was 42 CAG repeats (Fig.17B). Interestingly, our findings revealed an increase in puromycin incorporation levels in fibroblasts from HD patients expressing m*HTT* with <42 CAG repeats, in comparison to control fibroblasts, which was not detected in fibroblasts from patients expressing m*HTT* with ≥42 CAG repeats.

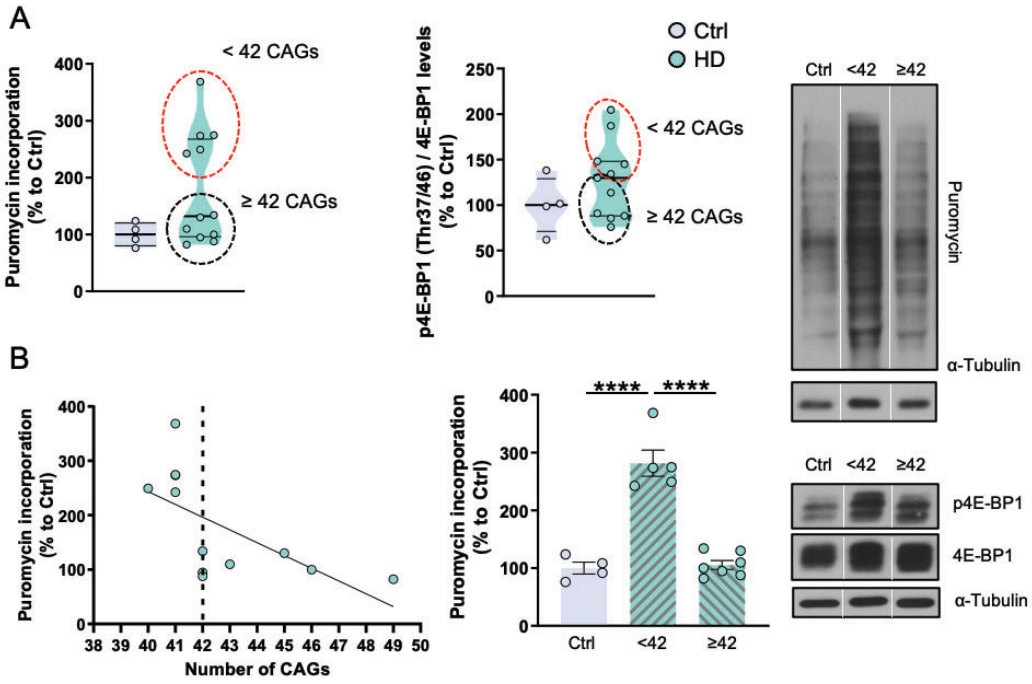


Figure 17.- Protein synthesis is increased in fibroblasts from HD patients expressing mHTT with less than 42 CAG repeats. Puromycin incorporation, p4E-BP1 and 4E-BP1 levels were analyzed by WB in protein extracts obtained from non-affected individuals (Ctrl) and HD patients' primary fibroblast cultures. Tubulin was used as loading control. (A) Violin plot showing puromycin incorporation and p4E-BP1 and 4E-BP1 levels in Ctrl and in HD patients' fibroblasts. (B) Graph showing the correlation between puromycin incorporation and the number of CAG repeats and bar graph with values showing puromycin incorporation grouped according to the number of CAG repeats. Values (obtained by densitometric analysis of WB data) are expressed as a percentage of non-affected individuals and shown as mean ± SEM. Each point corresponds to the value of an individual sample. Representative immunoblots are shown. (A) Two-tailed paired Student's t-test (B) One-way ANOVA with Bonferroni's as a post hoc test, ****$p < 0.0001$.

However, no changes in puromycin incorporation were found after classifying the data based on age, gender, or the presence of depression, one of the main psychiatric symptoms of HD (Duff et al., 2007). Similarly, when data were grouped by disease stage, no differences in puromycin incorporation or p4E-BP1 were found (Fig.18).

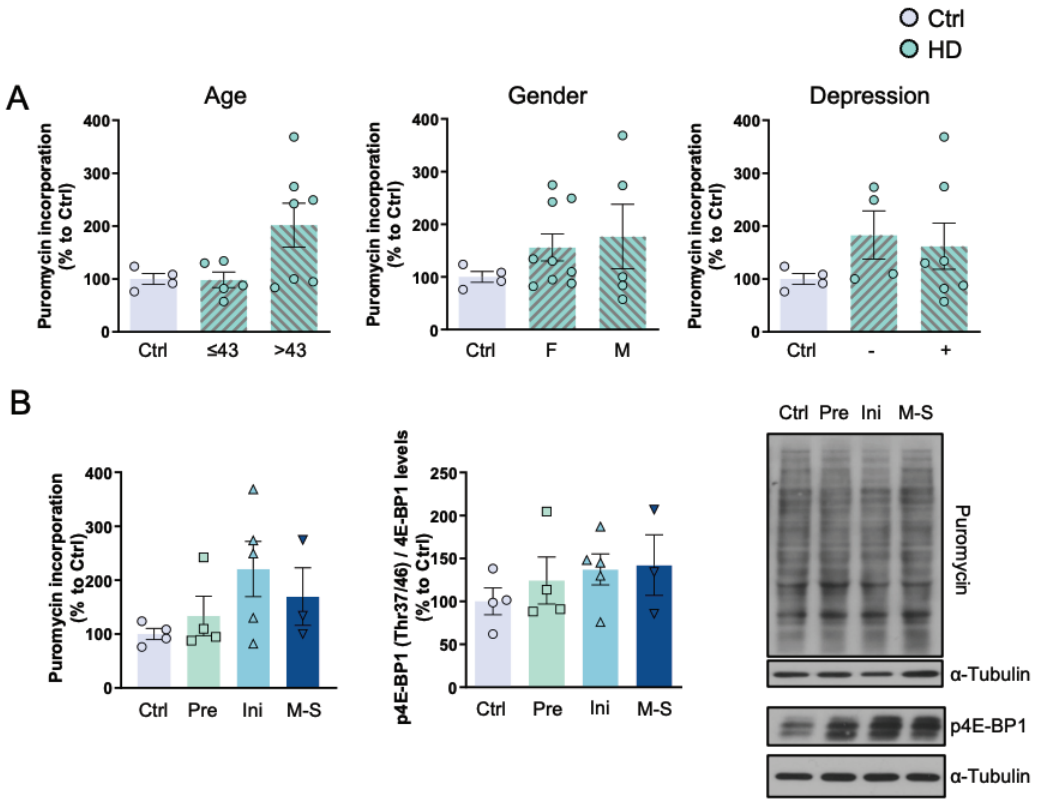


Figure 18.- Analysis of translation in fibroblasts from HD patients. Puromycin incorporation was analyzed by WB and results were classified depending on (A) Age, gender and presence (+) or not (-) of depression. (B) Puromycin incorporation and p4E-BP1 levels were analyzed by WB in human fibroblasts and classified depending on the disease stage (Ctrl: non-affected individuals; Pre: Pre-manifest; Ini: Initial; M-S: Moderate-Severe). Representative immunoblots are shown. (A-B) Values (obtained by densitometric analysis of WB data; α-tubulin used as loading control) are expressed as a percentage of controls (non-affected individuals; Ctrl). Data are shown as mean ± SEM and each point corresponds to the value from an individual.

2.2 Primary neuronal striatal cultures from R6/1 mice present increased protein synthesis

Although human fibroblast primary cultures provide a very authentic molecular context because of their human origin, their proliferative activity might be masking protein synthesis alterations that otherwise may be more evident in non-dividing cells such as neurons (Tanenbaum et al., 2015). In agreement, STHdhQ$^{111/Q111}$ cells, immortalized murine neural progenitors expressing m*HTT*, which present a high mitotic rate, were recently reported to exhibit a decreased global translation rate (Eshraghi et al., 2021). In our lab, we obtained similar results concerning STHdh$^{Q111/Q111}$ when measuring

puromycin incorporation (Data not shown). For this reason, we hypothesized that proliferating cell lines were not fit for an *in vitro* model to study translational alterations. Thus, for this reason, we analyzed protein synthesis alterations in striatal neuronal cultures expressing m*HTT in vitro*. To do so, puromycin incorporation and p4E-BP1 protein levels were measured in striatal primary neurons derived from WT and R6/1 mice at 14 DIV. Both, puromycin incorporation (Fig.19A,C) and p4E-BP1 levels (Fig.19B), were increased in R6/1 mouse striatal primary neurons in comparison to the WT ones. These results suggest that primary striatal neuronal cultures from R6/1 mice can be used for the primary screening of potential new compounds to assess their ability to modulate protein translation in an HD-like molecular context.

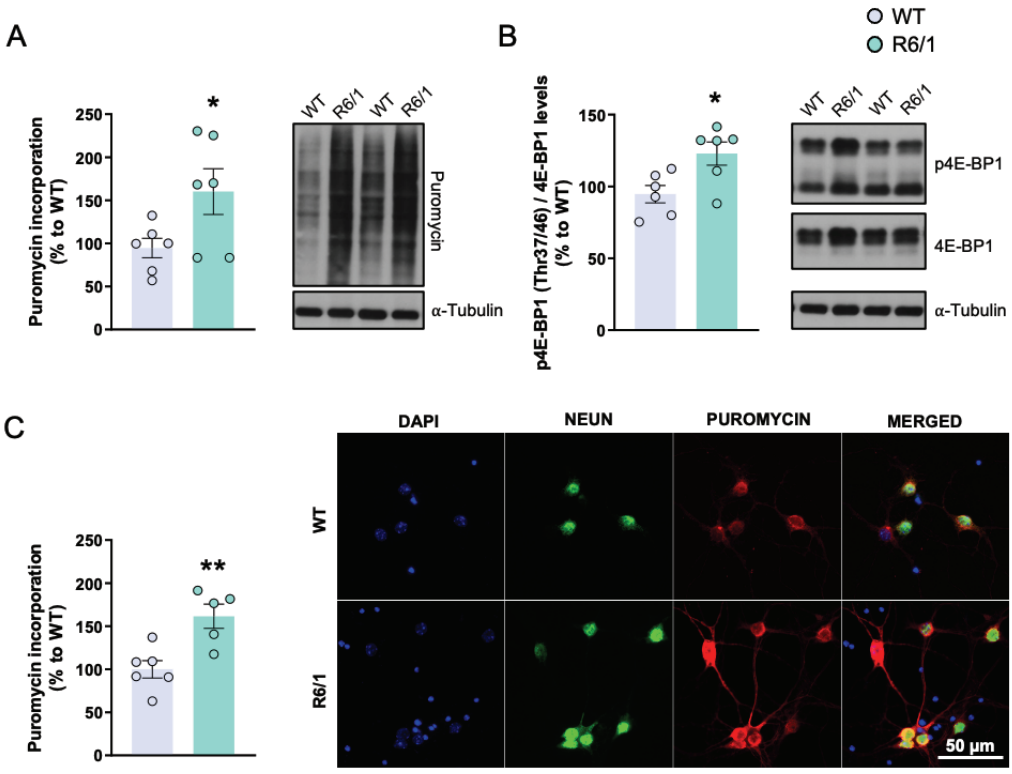


Figure 19.- Puromycin incorporation and p4E-BP1 levels are increased in striatal R6/1 mouse primary cultures. Puromycin incorporation was analyzed by (A) WB and (C) immunocytochemistry, and (B) p4E-BP1 levels by WB, in WT and R6/1 mice striatal primary cultures at DIV 14. Tubulin was used as a loading control. In graphs, values are expressed as a percentage of WT cultures and shown as mean ± SEM. Each point corresponds to the value from an individual culture. Representative immunoblots (A-B) and images (C) for each condition are shown. Two-tailed paired Student's t-test, *p<0.05, **p<0.01 compared to WT mice.

3. STUDY OF THE EFFECT OF THE PHARMACOLOGICAL INHIBITION OF TRANSLATION ON HD BRAIN

Our group previously described that rescue of striatal protein synthesis levels ameliorates motor dysfunction in R6/1 mice by taking advantage of 4EGI-1, a commercial drug that disrupts the eIF4E-eIF4G interaction (Creus-Muncunill et al., 2019). However, this compound is only used for research purposes and has not been approved by the FDA. Moreover, it does not cross the blood-brain barrier, which implies an intracerebral administration. In this study, our goal is to identify an FDA-approved and extensively used drug that could slow the progression of motor dysfunction by restoring protein translation alterations with minimal side effects in HD. To this purpose, we investigated the effect of the antidepressant sertraline, a widely used SSRI that exhibits antiproliferative activity by targeting the mTOR signalling pathway (Hwang et al., 2021; Lin et al., 2010). The neuroprotective role of sertraline has been explored by an independent group in the R6/2 and N171-82Q HD transgenic mouse models, which reported reduced brain atrophy, and improved survival and motor performance although its effects were attributed to enhanced adult neurogenesis and BDNF levels (Duan et al., 2008; Peng et al., 2008). However, the role of sertraline and its impact on 4E-BP1 inactivation and cap-dependent translation regulation in the HD molecular context are unknown and warrant further investigation.

3.1 *In vitro* sertraline treatment normalizes translation in striatal primary neurons from R6/1 mouse model

Several independent groups have shown that sertraline treatment decreases 4E-BP1 phosphorylation and protein synthesis levels (Duan et al., 2008; Peng et al., 2008). To further confirm that in an HD molecular context, we took advantage of the primary neuronal cultures from R6/1 mice striata as an *in vitro* model which, as reported in the previous aim, recapitulates the alterations in protein synthesis found in the R6/1 striatum. Different drug concentrations (10, 25, 50 μM) and incubation periods (4 and 24 h) were tested based on previous studies, and drug concentration of 10uM for a period of 4h was determined as the ideal condition for our purposes. Striatal primary neuronal cultures from R6/1 mice were treated at DIV 14 with either vehicle (DMSO) or 10 μM of sertraline for 4 h. B27 supplement was removed from the medium at DIV 10 to avoid interferences with the treatment as it has been shown to hyperactivate the

mTOR pathway (S. W. Park et al., 2014). As expected, the addition of sertraline to R6/1 mouse striatal primary neurons decreased both puromycin incorporation and p4E-BP1 levels (Fig.20).

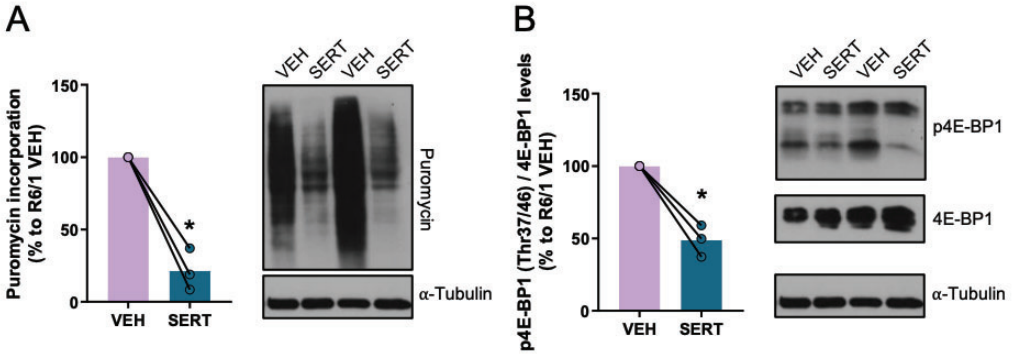


Figure 20.- Treatment with sertraline decreases protein synthesis in R6/1 mouse striatal primary cultures. R6/1 mice striatal primary cultures were treated with sertraline (SERT; 10μM) or vehicle (VEH; DMSO) at DIV 14 during 4 h. (A) Puromycin incorporation and (B) p4E-BP1 levels were analyzed by WB. α-Tubulin was used as a loading control. In graphs, values are expressed as a percentage of the vehicle-treated cultures from the same embryo. Each point corresponds to a pull from at least 2 embryos from 3 independent experiments. Representative immunoblots for each condition are shown. Two-tailed paired Student's t-test, *p<0.05, compared to cultures treated with vehicle from the same embryo.

3.2 Treatment with sertraline prevents motor dysfunction in R6/1 mice and normalizes protein synthesis in the striatum

Based on the above observations, our next aim was to explore in the R6/1 mouse model the beneficial effects of sertraline reported in other transgenic mouse models of HD, and the contribution of the inhibition of cap-dependent translation and 4E-BP1 phosphorylation in them. Thus, we chronically treated WT and R6/1 mice with vehicle or sertraline and motor behavior was analyzed after 3 weeks of treatment by using the open field, the accelerating rotarod, the balance beam and the vertical pole tests (Fig.21). Strikingly, sertraline treatment improved motor skills in R6/1 mice, as shown in all tests analyzed, while it did not have any effects in WT mice. Anxiety behavior was not affected by the treatment, as evaluated by the percentage of time spent in the center of the open field arena (Fig.21A).

In more detail, sertraline promoted normalization of both parameters tracked for the evaluation of spontaneous locomotor: total distance travelled and mean speed (Fig.21A). Evaluation of motor coordination using the vertical pole and the balance beam

tests revealed an improvement of the time to turn and slips committed per frame, respectively, in sertraline-treated R6/1 mice compared to vehicle-treated R6/1 mice (Fig.21B,C). In addition, sertraline treatment resulted in an improvement of motor coordination in the accelerating rotarod test in R6/1 mice, although still not reaching WT performance levels mice (Fig. 21D).

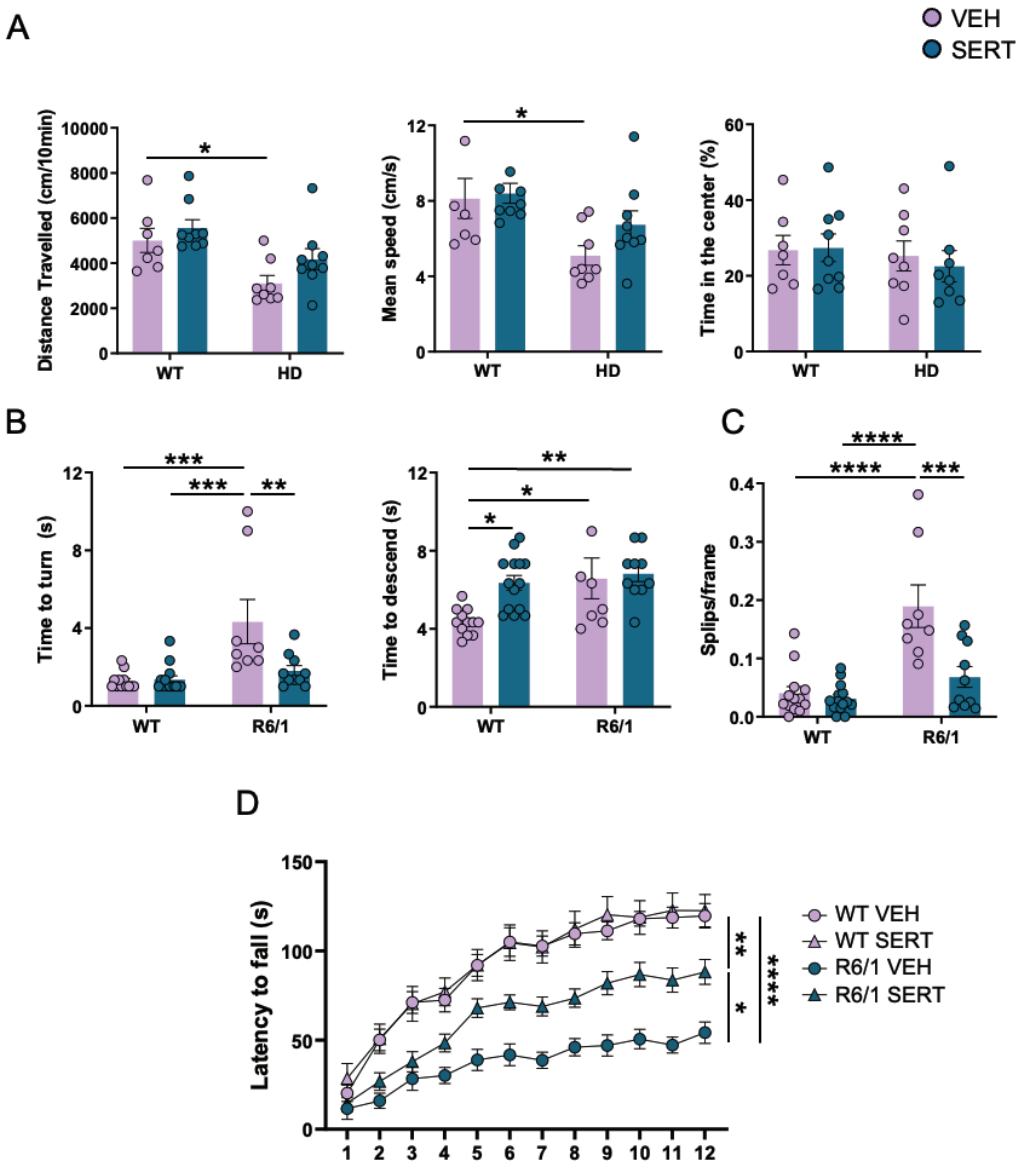


Figure 21.- Sertraline treatment prevents motor deficits in R6/1 mice. WT and R6/1 mice were intraperitoneally injected with vehicle (VEH; H_2O 5% Polysorbate 80) or sertraline (SERT; 20 mg/Kg) from 12 to 16 weeks of age. Motor behavior was assessed during the last week of treatment. (A) Open field: Locomotor activity was measured as the distance travelled (cm) and mean speed and anxiety levels as the time spent in the center in 10 minutes in an open field arena. (B) Vertical pole: time (in seconds, s) to turn (left) and time to descend (right) were recorded after placing the mice facing upwards at the pole. The

average of three trials is plotted. (C) Balance beam: Graph shows the number of slips committed per frame during 2 minutes. (D) Accelerating rotarod test was performed for three consecutive days (3 trials per day). The latency to fall per test and group is represented as mean ± SEM (WT VEH and WT SERT, n = 19; R6/1 VEH, n = 14; R6/1 SERT, n = 17). In all bar graphs, data are shown as mean ± SEM and each point corresponds to the value from an individual mouse. Two-way ANOVA with Bonferroni's as a post hoc test, *p < 0.05, ** p < 0.01; ***p < 0.001; ****p < 0.0001.

To investigate whether sertraline treatment affected protein synthesis, mice were killed 1 hour after the last sertraline injection and protein synthesis was evaluated in the striatum using the SUnSET method. As previously described in Creus-Muncunill et al. (2019), R6/1 mice treated with vehicle presented increased puromycin incorporation and p4E-BP1 protein levels in the striatum, indicating exaggerated protein synthesis, which was normalized by sertraline treatment (Fig.22). In agreement with the results from the behavioral tests, sertraline did not have any effects on WT mice striatum at the level of 4E-BP1 phosphorylation and puromycin incorporation (Fig.22).

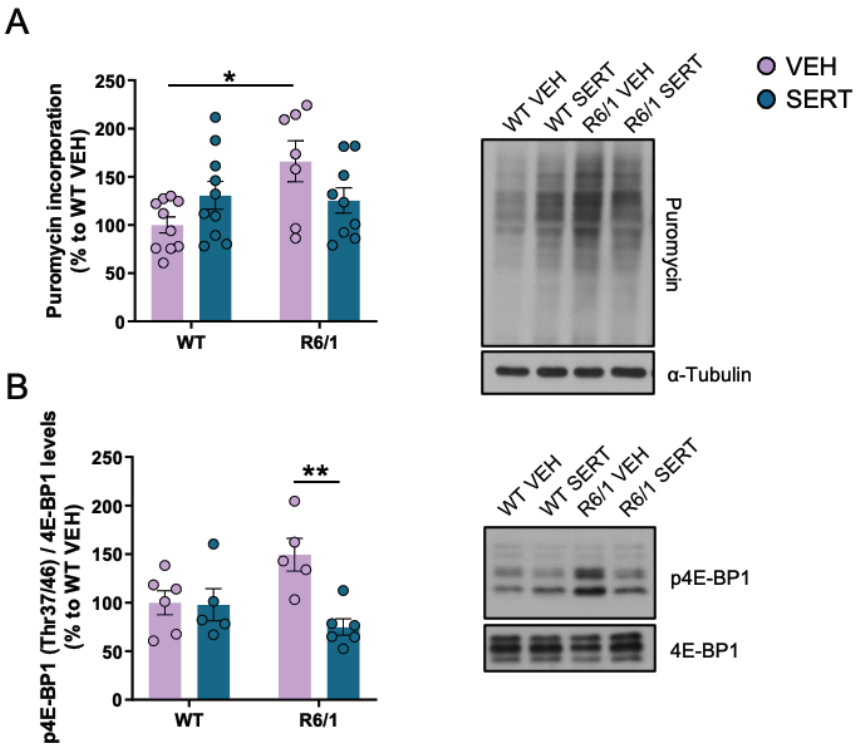


Figure 22.- Sertraline treatment normalizes exaggerated protein synthesis in the R6/1 mice. (A) Puromycin incorporation and (B) Thr 37/46 4E-BP1 (p4E-BP1) phosphorylation levels were examined by WB in the striatum at the end of the sertraline treatment. Tubulin was used as a loading control. Values are expressed as a percentage of WT mice treated with vehicle. Representative immunoblots for each condition

are shown. In all bar graphs, data are shown as mean ± SEM and each point corresponds to the value from an individual mouse. Two-way ANOVA with Bonferroni's as a post hoc test, *p < 0.05, ** p < 0.01.

We observed no effects of the sertraline treatment on the protein levels of the striatal marker DARPP-32, a classical marker for striatal neurodegeneration (Fig.23A). In line with other studies evaluating the effects of sertraline on HD models (Duan et al., 2008; Peng et al., 2008), treatment did not modify insoluble forms of mHTT (Fig.23B), a cap-dependent translated protein, suggesting that behavioral improvements are not related to changes in mHTT levels and aggregation (King et al., 2008).

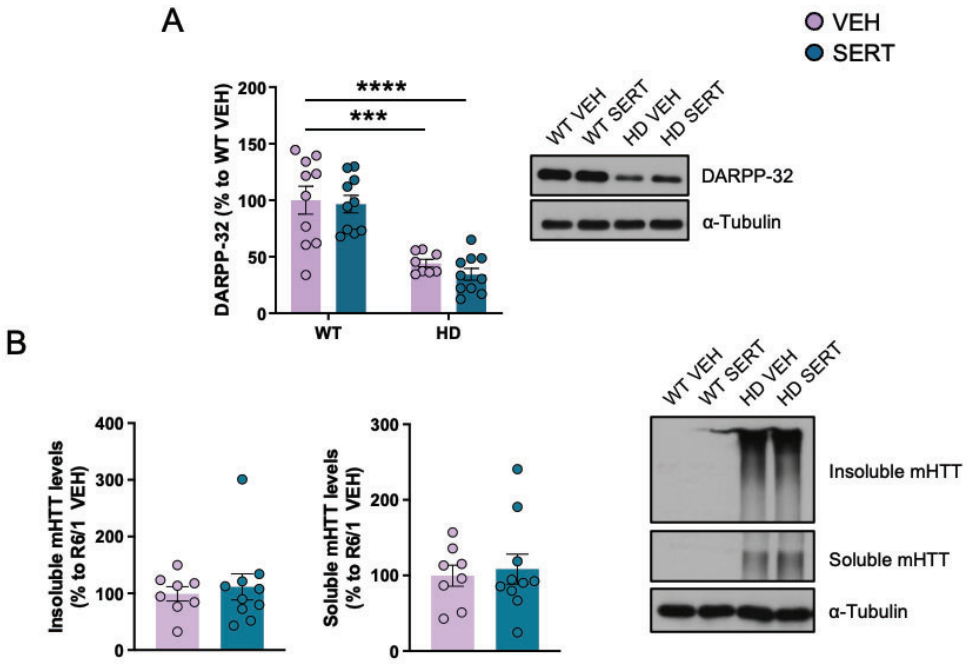


Figure 23.- Sertraline treatment does not modify DARPP-32 striatal marker or mHTT aggregation levels. (A) Striatal DARPP-32 protein levels were analyzed by WB in WT and R6/1 mice treated with vehicle (VEH) or sertraline (SERT). Results are expressed as a percentage of vehicle-treated WT mice. Representative immunoblots are shown. (B) mHTT protein levels were analyzed by WB of protein extracts obtained from the striatum of R6/1 mice treated with vehicle (VEH) or sertraline (SERT) by using the EM48 antibody. Insoluble mHTT was detected in the stacking gel. Representative immunoblots are shown. In all graphs, values are shown as mean ± S.E.M. (A) Two-way ANOVA with Bonferroni's as a post hoc test, ***p < 0.001, **** p < 0.0001.

The findings highlight the potential of sertraline as a pharmacological strategy for HD by improving motor function and restoring normal protein synthesis in the R6/1 mouse model.

3.3 Sertraline treatment slows down the functional performance decline in HD patients in a longitudinal analysis

Since sertraline treatment revealed positive effects on motor function in different preclinical studies including ours and two independent studies using different transgenic mouse models (Duan et al., 2008; Peng et al., 2008), we investigated whether sertraline treatment might influence the HD progression course in terms of motor and functional outcomes. Depression is one of the most prevalent psychiatric symptoms in HD and therefore, SSRIs, among other therapies, are often prescribed as symptomatic approaches (Slaughter et al., 2001). Thus, we took advantage of the Enroll-HD database, the largest HD observational study conducted worldwide to analyze whether sertraline treatment, prescribed to HD patients as a symptomatic therapy for depression, might influence the disease progression course in terms of motor and functional outcomes. In this study, we analyzed longitudinal data of up to seven years follow-up from the Enroll-HD database to evaluate the potential impact on disease progression between patient groups.

From the total 21,116 Enroll-HD participants, only HD patients (both Pre-Motor Manifest (PM) and Motor-Manifest (M)) with a minimum of a 3-year follow-up were included in this study. To rule out the possibility that the use of other antidepressants with differing mechanisms(e.g. tricyclic antidepressants) might be a confounding factor in our control group, we performed 3 comparisons between: (a) HD patients treated with sertraline with or without other antidepressants (n = 63) and HD patients treated with other antidepressants but not sertraline (n = 208); (b) HD patients treated with sertraline as the unique antidepressant (n = 30) and HD patients treated with other antidepressants (n = 208) and (c) HD patients treated with sertraline with or without other antidepressants (n = 63) and HD patients non-treated with anti-depressants (n = 226).

As different measurements of the same patient are collected throughout time, from an annual clinic visit, a Linear Mixed Effects Model (LMM) (Verbeke & Molenberghs, 2000) was used in this study to control the correlation structure of the data and evaluate differences in disease progression between these groups of patients. To assess the difference in disease time evolution between different patient groups, the model includes time as a linear effect and a time-group interaction term. This interaction term describes whether there are two different slopes of evolution depending on the groups, i.e. if the evolution of the disease over time is significantly different between the groups

considered. Considering the slope rather than the mean difference between groups at each time point avoids the baseline mean effect since treated patients are already more severely affected at baseline. The model also includes gender, age, CAG repeats, and HD status (PM or M) as potential confounders. The response variables considered were: the Total Motor Score (TMS) component of the Unified Huntington's Disease Rating Scale (UHDRS), used to examine the motoric parameters of symptomatic disease manifestation; and the UHDRS-Total functional capacity (TFC), the functional assessment scale (FAS), and the Independence Scale (IS), used to analyze functionality.

Results show no significant differences on the decline depending on the medication taken compared to the control group from the three comparisons described above. However, functionality assessments were significantly higher in the sertraline-treated groups. From the first comparison (a), we found that patients treated with sertraline presented significantly higher TFC ($p < 0.0004$), FAS ($p < 0.003$) and IS ($p < 0.0003$) scores compared to the HD control group treated with other antidepressants (Fig.24A). On the other hand, the second comparison (b) suggested that sertraline treatment alone is less efficient than when administered together with other antidepressants on slowing down the progression of the functional impairment since only the IS score was significantly higher in the sertraline-treated group ($p < 0.0172$) (Fig.23B). In the third comparison (c), sertraline treatment slowed the decline of the TFC ($p < 0.0172$) and IS ($p < 0.0307$) scores compared to the HD-control group non-treated with antidepressants (Fig.23C).

In summary, we identified group differences in terms of functionality scores in some treatment groups. We observe a clear beneficial effect of sertraline treatment slowing down the functional performance decline in the HD cohort. Unfortunately, in our data, we did neither observe clear beneficial nor detrimental effects of sertraline on motor function longitudinally which might be explained by the small sample size. Our data supports our hypothesis of a potential neuroprotective effect of sertraline on disease progression in terms of functionality although we could not prove an amelioration of the pure motor score TMS. Overall, we identify a potentially novel indication for an approved antidepressant drug (.i.e. sertraline) for the treatment of HD.

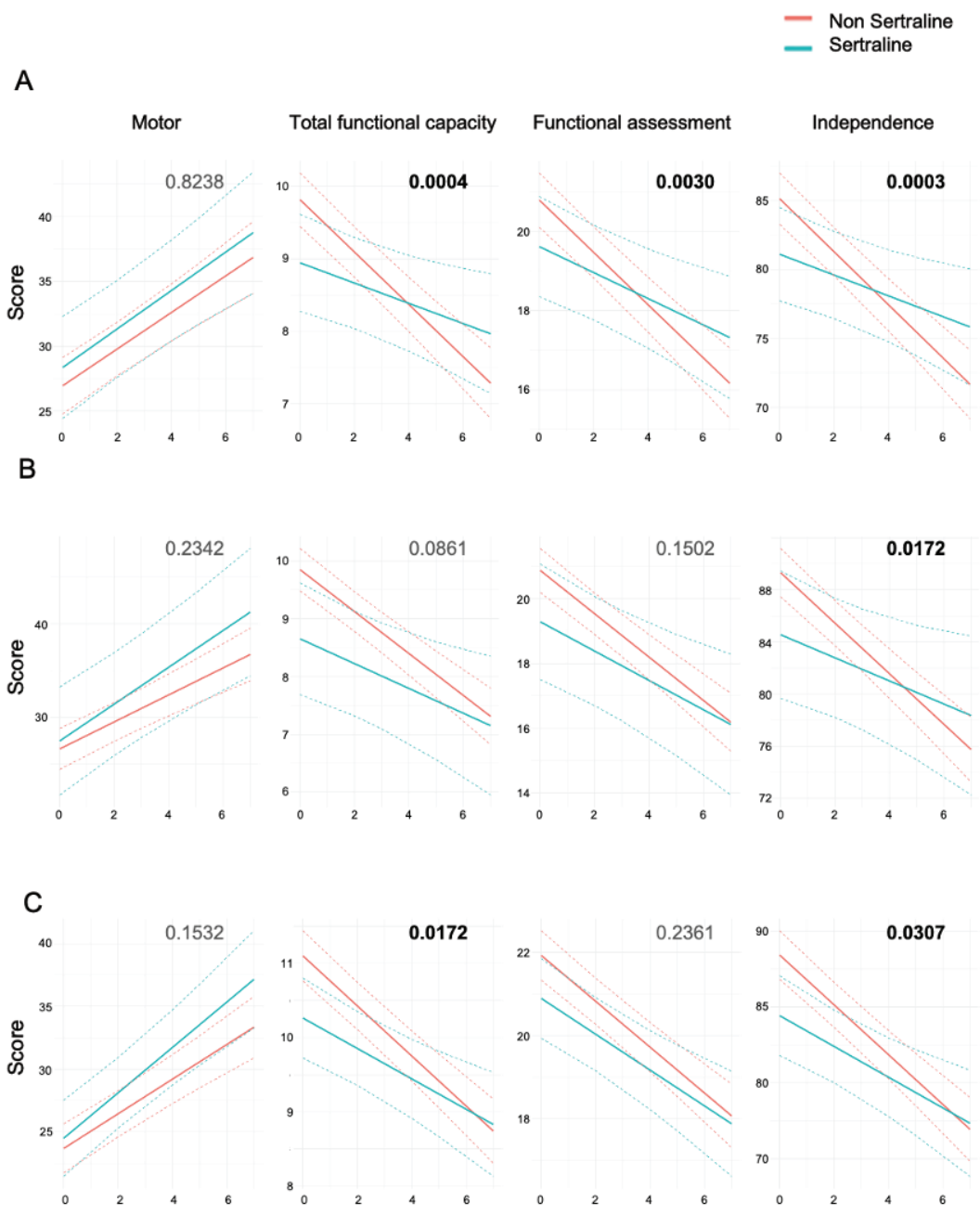


Figure 24.- Progression of motor, total functional capacity, functional assessment, and independence performance over seven years. (A) Comparison between HD patients treated with sertraline +/- other antidepressants (n = 63) and HD patients treated with other antidepressants (n = 208). (B) Comparison between HD patients treated with sertraline as the unique antidepressant (n = 30) and HD patients treated with other antidepressants (n = 208). (C) Comparison between HD patients treated with sertraline +/- other antidepressants (n = 63) and HD patients non-treated with anti-depressants (n = 226). Statistical Analysis: Linear Mixed Effects Model (LMM) was applied to assess differences in disease time evolution between patient groups through the inclusion of time-group interaction term. The model also includes gender, age, CAG repeats, and HD status (PM or M) as potential confounders.

4. STUDY OF PROTEIN TRANSLATION ALTERATIONS THROUGHOUT THE HD BRAIN

4.1 4E-BP1 is inactivated in a region-specific manner in the R6/1 mice brain

Our group previously demonstrated an increase in the phosphorylation levels of 4E-BP1 in the striatum of R6/1 mice at 12 weeks of age and onwards (Creus-Muncunill et al., 2019). However, we did not evaluate whether this alteration was present in other brain regions. To that end, we analyzed by WB total lysates obtained from WT and R6/1 mice hippocampus and cortex from 8 to 30 weeks of age. We detected an increase in protein levels of p4E-BP1 at Thr 37/46 in the hippocampus and cortex of R6/1 mice from early stages of the disease (Fig.25A,B). Nevertheless, in contrast to the results obtained in the striatum, we did not detect changes in total levels of 4E-BP1 in comparison with control animals (Fig.25C,D).

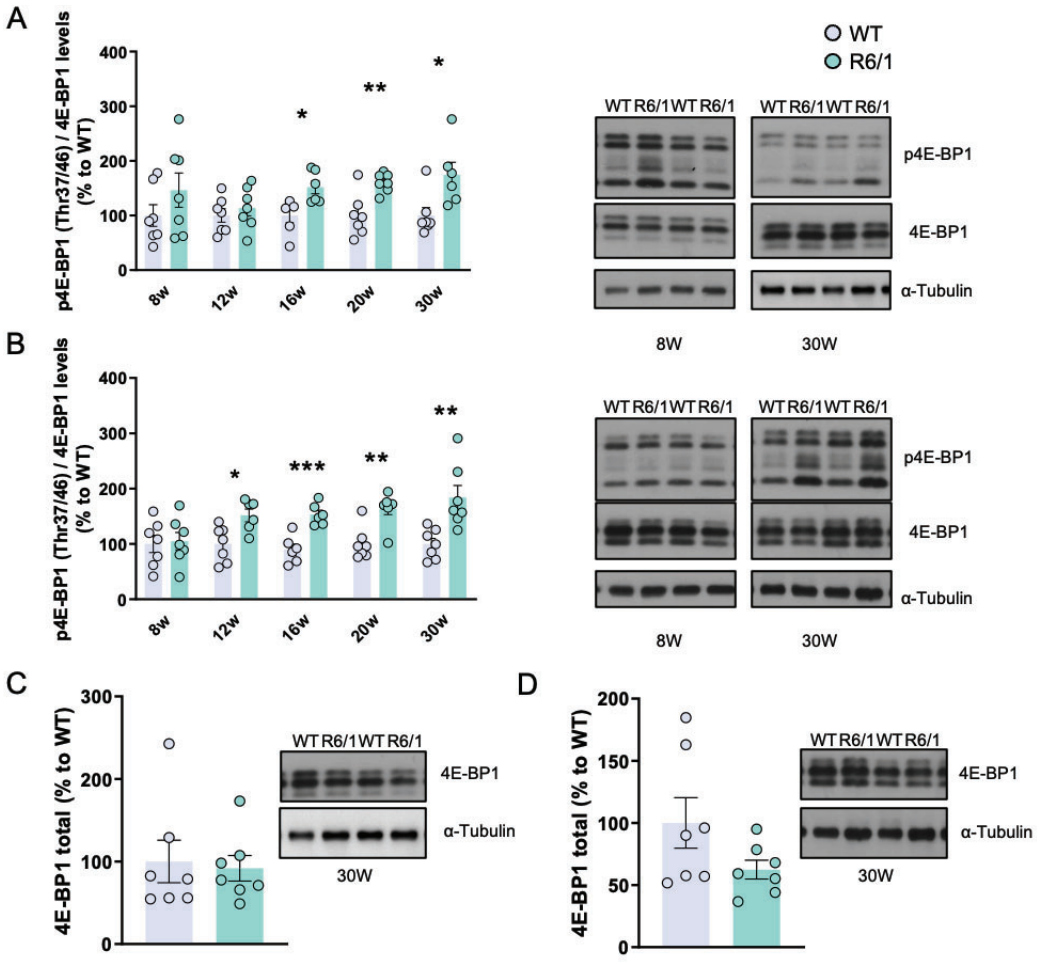
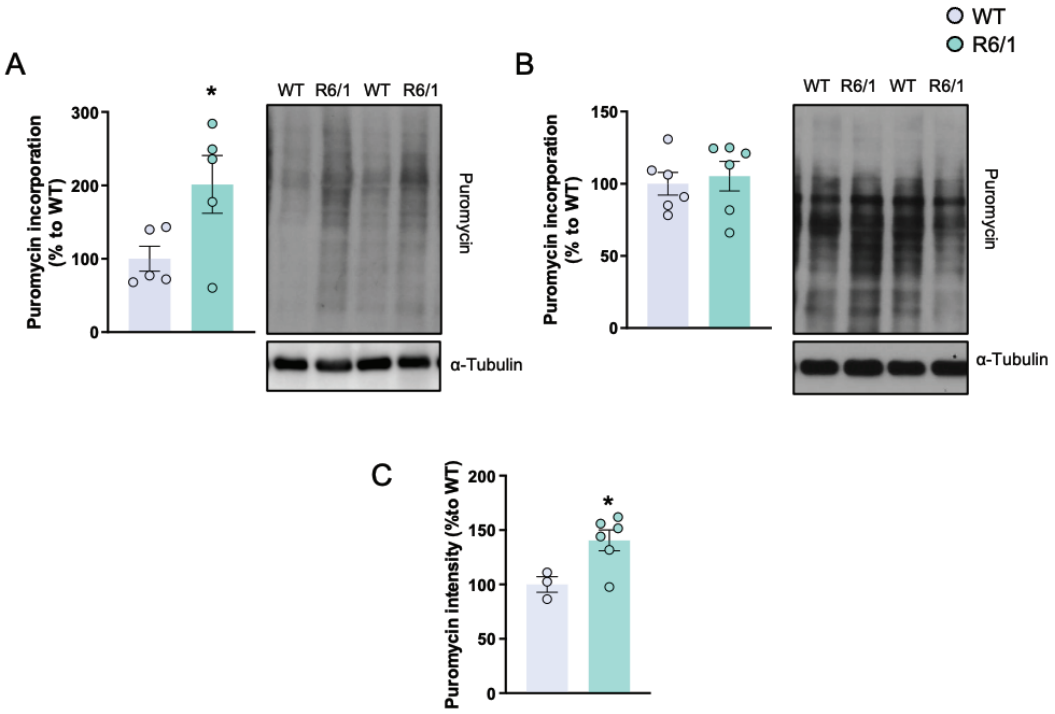


Figure 25.- 4E-BP1 is inactivated in the hippocampus and cortex of R6/1 mice. Total and phosphorylated 4E-BP1 at Thr 37/46 levels were analysed by WB of protein lysates obtained from the (A, C) hippocampus and (B, D) cortex of WT and R6/1 mice at different stages of the disease progression (W, weeks). Values obtained by densitometric analysis of WB data are expressed as a percentage of WT (p4E-BP1/4E-BP1 and 4E-BP1/α-tubulin ratios) and shown as mean ± SEM (n = 7 for cortex and hippocampus). Representative immunoblots are shown. Each point represents data from an individual sample. *P < 0.05, **P < 0.01, ***P < 0.001 compared with respective controls (Student's t test).

4.2 The hippocampus of the R6/1 mouse model shows increased *de novo* protein synthesis

To prove that increased inactivation of the translation repressor 4E-BP1 results in increased protein translation in the R6/1 mouse hippocampus and cortex, we applied the SUnSET method in coronal slices from 16 weeks old WT and R6/1 mice. We found that R6/1 hippocampal, but not cortical, cells presented an increase in the incorporation of puromycin when compared with WT littermates, showing that the increase in de novo cap-dependent translation in HD is not only restricted to the striatum (Fig.26A,B). Specifically, Pyk2-positive cells were the main population that incorporated puromycin, indicating that increased translation in the HD hippocampus occurred mainly in CA1 hippocampal neurons (Fig.26C).



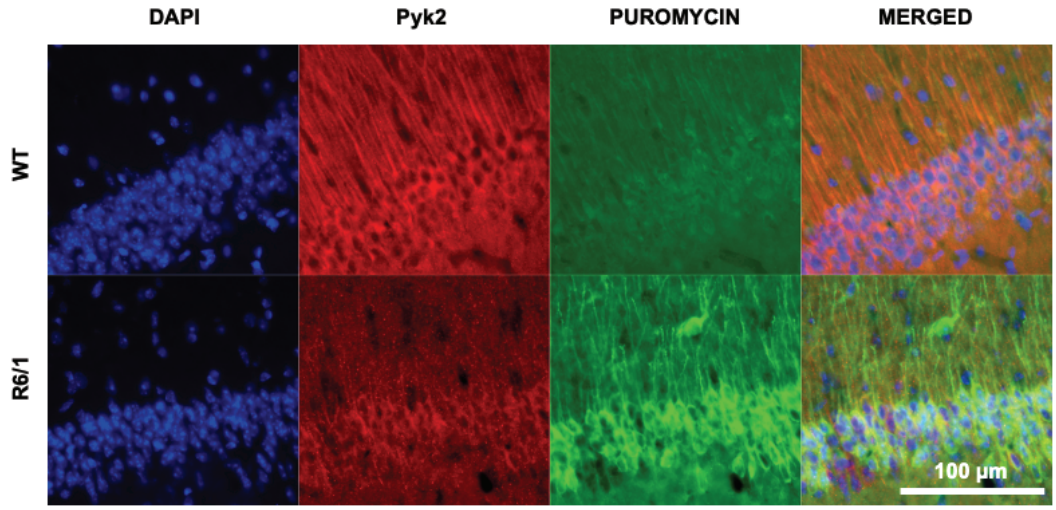


Figure 26.- Protein synthesis is increased in Pyk2-positive hippocampal CA1 neurons in the R6/1 mice. Puromycin incorporation was analyzed by WB of protein extracts obtained from (A) hippocampal and (B) cortical slices of 16-week-old WT and R6/1 mice incubated for 45 min with puromycin. Values obtained by densitometric analysis of WB data (puromycin incorporation/α-tubulin ratio) are expressed as a percentage of WT and shown as mean ± SEM (n = 5-6). Tubulin was used as a loading control. Representative immunoblots are shown. (C) Quantitative analysis of the puromycin intensity in Pyk2-positive CA1 hippocampal neurons. Representative images showing puromycin and Pyk2 immunostaining in the hippocampus of 16-week-old R6/1 mice, 45 min after the infusion puromycin (3 µl of puromycin solution (9 mg/ml, 10% DMSO/90% saline)) in the lateral ventricle. Values are expressed as a percentage of WT and shown as mean ± SEM (n = 3–6). *p < 0.05 compared with WT hippocampus (Student's t-test).

To investigate whether this dysregulation also occurs in humans we examined the CA1 hippocampal region of HD patients. Unfortunately, in human post-mortem samples, we could not detect any phosphorylated form of 4E-BP1, probably due to excessive *post-mortem* delay (see Table 1), as previously described for other protein phosphorylations (J. Li et al., 2002). Furthermore, and in agreement with the results in the R6/1 mice hippocampus, we could not detect altered 4E-BP1 total protein levels in the CA1 hippocampal region of HD patients compared with controls (Fig.27).

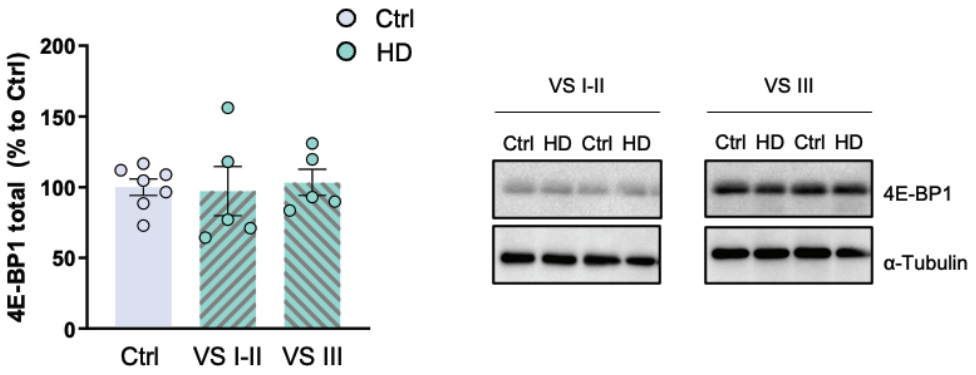
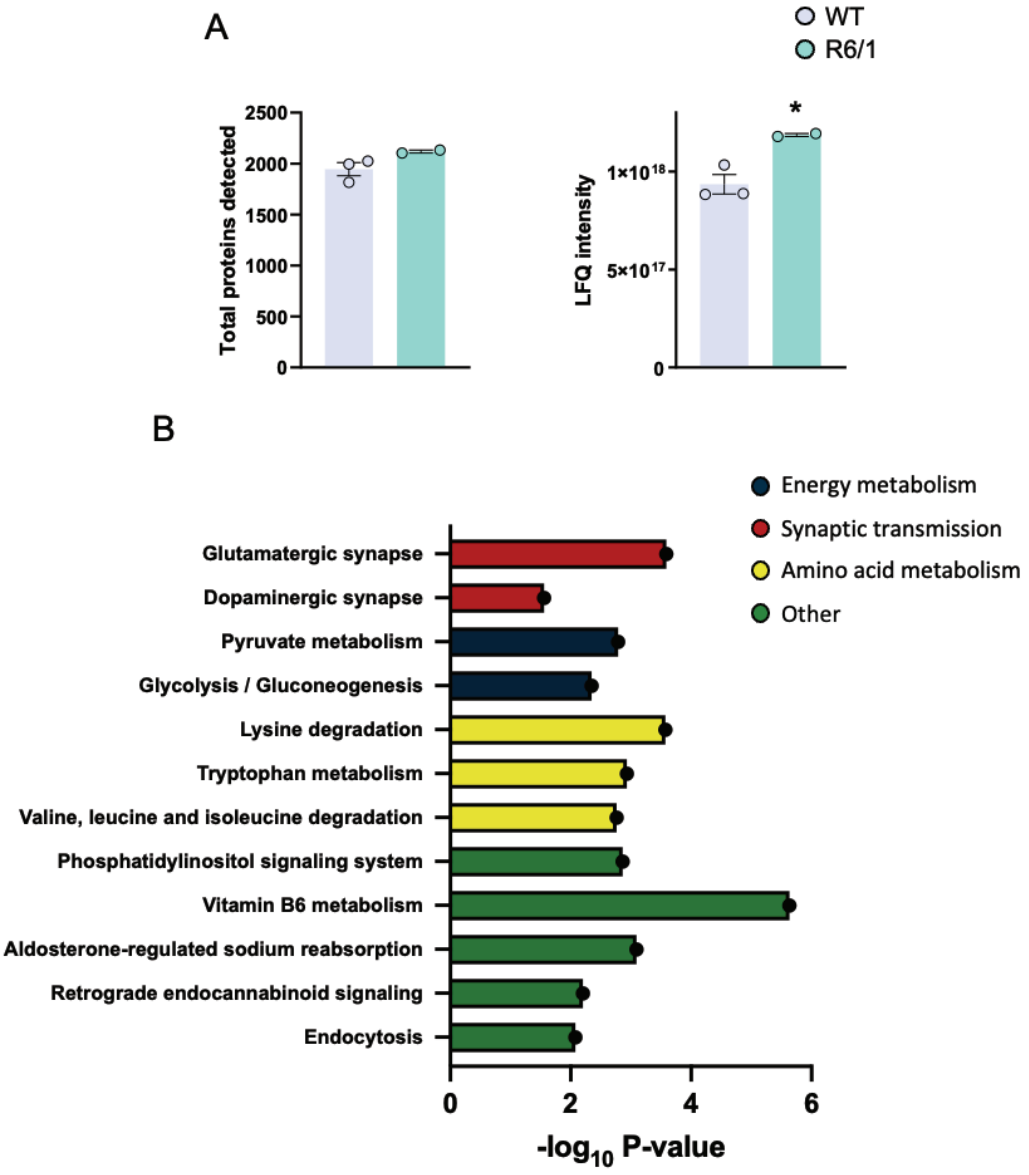


Figure 27.- 4E-BP1 total protein levels are not altered in the hippocampus of HD patients. Total 4E-BP1 levels were analyzed by WB of protein lysates obtained from the CA1 hippocampal region of HD patients at different disease stages (VS, Vonsattel grade) and control individuals (Ctrl). Values obtained by densitometric analysis of WB data are expressed as a percentage of controls (4E-BP1/α-tubulin ratio) and shown as mean ± SEM (n = 5-7). Representative immunoblots are shown. Each point represents data from an individual.

Following the observation of an increase in puromycin incorporation and 4E-BP1 inactivation in the hippocampus of the R6/1 mice, we hypothesise that hippocampal cells undergo a drastic change in the translatome that would be neurotoxic by creating a distinct and more stressful environment. Since 4E-BP1 inactivation increases the translation of a specific subset of transcripts (Avni et al., 1997), we inquired as to whether any particular proteins were over-synthesized in the HD molecular context. Using a puromycin-based labelling approach, we are able to identify and compare the purified nascent proteins under various physiological and pathological conditions with both spatial and temporal resolution. In this experiment, hippocampal slices from 15-weeks-old WT and R6/1 were incubated in ACSF with puromycin in the same concentration used for the SUnSET method for 30 min. Afterwards, puromycin-labelled newly synthesized peptides translated during the incubation period were purified by immunoprecipitation using an anti-puromycin primary antibody and identified by LC-MS/MS. From 1832 proteins detected, we identified 99 proteins differently expressed, 59 up- and 40 down-translated in the HD hippocampus. The number of total proteins detected, and the sum of LFQ intensity were considered a quantitative measurement of the bulk protein synthesis rate, which in agreement with our previous results, is increased in R6/1 mice hippocampus (Fig.28A). Interestingly, KEGG analysis identified various selectively affected pathways. Only significant pathways (p-value < 0.01) involving more than 3 proteins are shown in Fig.28B. Overall, translatome profiling of the R6/1 hippocampus at 16 weeks of age, a timepoint in which we described an

aberrant inactivation of the cap-dependent translation repressor 4E-BP1, revealed a remarkable increase in the translation of proteins associated with metabolic pathways for energy production and nutrient utilization such as glycolysis/gluconeogenesis, pyruvate metabolism and several involved in amino acid metabolism. This was also accompanied by an enrichment of synaptic transmission pathways, such as the glutamatergic synapse which included mainly down-regulated proteins suggesting that dysregulated translation of proteins involved in synaptic transmission could be contributing to the synaptic dysfunction observed in the R6/1 mouse model.



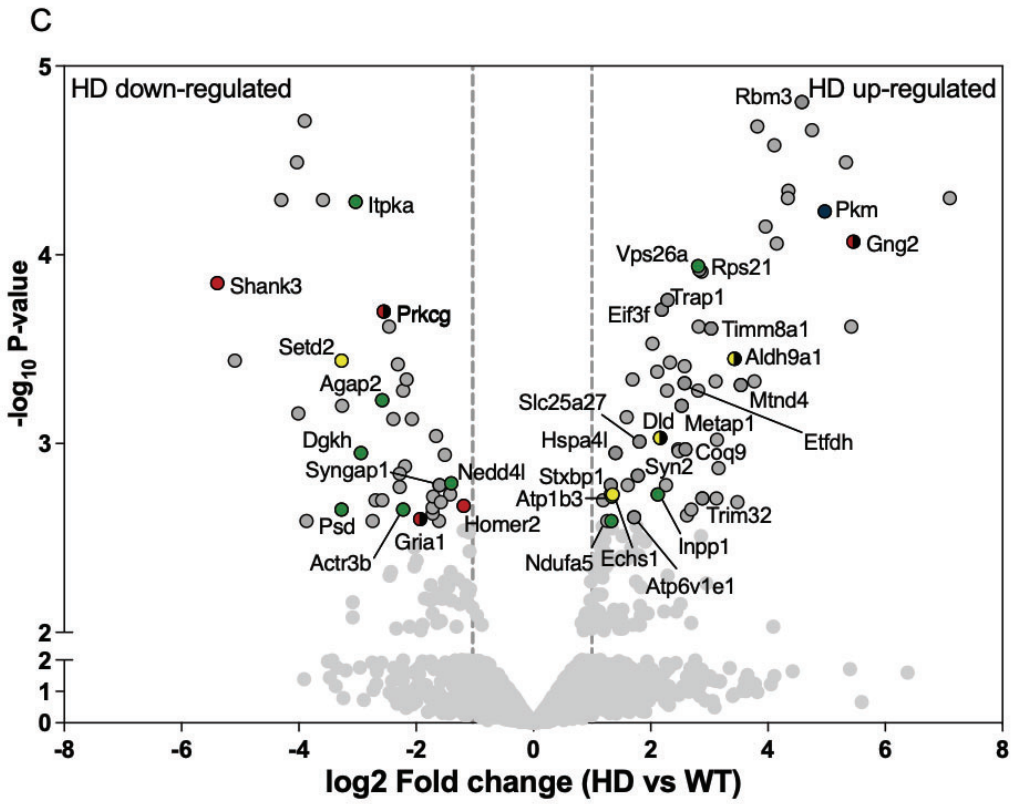


Figure 28.- LC-MS/MS analysis of the puromycin-labelled purified nascent peptides in the R6/1 mouse hippocampus. (A) Total number of proteins detected per genotype and summatory of LFQ intensity per genotype. *p < 0.05 compared with WT hippocampus (Student's t-test). (B) Bar graph showing the KEGG pathway enrichment analysis of the differently expressed proteins. Pathways involving more than 3 proteins and p < 0.01 are showed. (C) Volcano plot of downregulated and upregulated proteins proteins of R6/1 mice hippocampus compared to WT mice. Cut-offs of ±0.5-fold change and p < 0.05. (n = 3-2 mice/group).

5. TO STUDY THE CONSEQUENCES OF THE MODULATION OF TRANSLATION INITIATION IN THE HD HIPPOCAMPUS

In previous results from our group, the eIF4E/EIF4G interaction inhibitor 4EGI-1 was used to study the contribution of the alterations in the eIF4F complex formation on the motor deficits in the R6/1 mice (Creus-Muncunill et al., 2019). Since in the previous aim we found increased inactivation of 4E-BP1 and cap-dependent protein synthesis in the R6/1 hippocampus, in this study, we use the same strategy to explore the contribution of this alteration in the hippocampal-dependent learning and memory deficits.

5.1 4EGI-1 administration improves hippocampus-dependent spatial memory in R6/1 mice

For this purpose, we infused vehicle (ACSF) or 4EGI-1 to 16-week-old WT and R6/1 for 7 consecutive days through a cannula placed in the lateral ventricle. During this period, we performed a battery of spatial memory assessment tasks and on the last day of treatment mice were sacrificed for biochemical analyses (Fig.29A). Hippocampal-dependent cognitive deficits in WT and R6/1 mice receiving vehicle or 4EGI-1 were assessed by subjecting the mice to the novel object location test (NOLT), the Y-maze spontaneous alternation test and the T-maze spontaneous alternation task (T-SAT). In the NOLT, mice were first habituated for 30 min to the open field arena. Locomotor activity and anxiety levels were analysed by the distance travelled and centre/periphery ratio, respectively, during the first 10 min. Locomotor activity was decreased in the R6/1 mice, as expected, but recovered with 4EGI-1 administration, in line with the amelioration of the motor performance described by Creus-Muncunill et al. (2019) (Fig.29B). No significant differences were observed in anxiety levels in any of the conditions analysed (Fig.29C). Mice were subjected to a training session in the presence of two identical objects 24 h after the habituation session. During the training session, mice explored similarly both objects indicating no object preference (data not shown). On the third day of the NOLT test, one of the objects was moved to a new location and the percentage of object preference was analysed. As described before, WT but not R6/1 mice explored more the object in the new location with respect to the familiar one (Alcalá-Vida et al., 2021). Interestingly, R6/1 mice treated with 4EGI-1 also presented an increased preference for the object in the new location indicating a recovery of the spatial memory (Fig.29D).

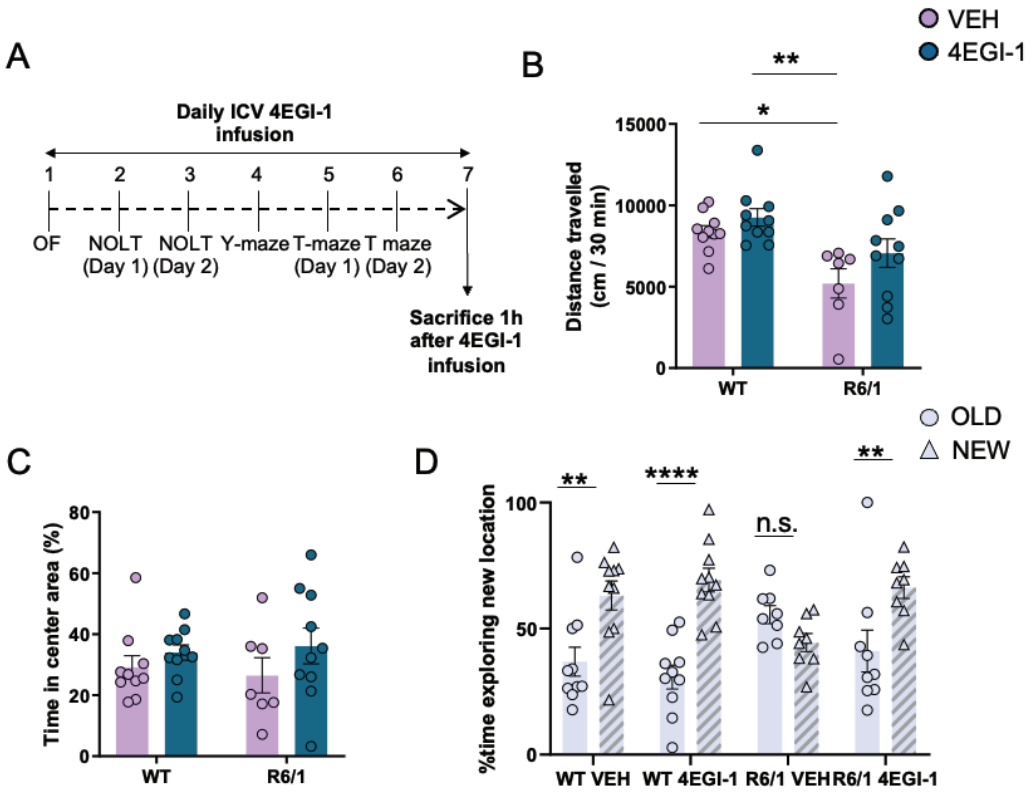


Figure 29.- 4EGI-1 treatment improves hippocampus-dependent spatial memory. (A) Timeline of the experimental approach used to evaluate 4EGI-1's effect on R6/1 mice cognitive function. Each test was conducted on the indicated day of treatment. ICV: Intracerebroventricular; OF: Open Field; NOLT: Novel Object Location Test. (B-C) Open field: Graph shows the distance travelled and percentage of time spent in the central area/center of the arena during the first 10 min of the NOLT habituation. (D) NOLT: preference for an object in the original location (Old) and an object in a new location (New) was quantified 24 h after training. Graphs show the percentage of object preference in WT and R6/1 mice from the total time exploring both objects. In all bar graphs, data are shown as mean ± SEM and each point corresponds to the value from an individual mouse. (B-C) Two-way ANOVA with Bonferroni's as a post hoc test. (D) Two-tailed unpaired Student's t-test compared to the old location. *$p < 0.05$; ** $p < 0.01$; ****$p < 0.0001$.

To further characterize the cognitive amelioration concerning short-term memory, mice were subjected to the Y-maze spontaneous alternation test and the T-SAT. In the first, we observed no differences between both genotypes, as described previously in Liu et al. (2019), however, R6/1 show a clear tendency to present a worsened spontaneous alternation index compared to WT (Fig.30A). 4EGI-1 treatment neither alleviated nor worsened spontaneous alternation behaviour impairment in any genotype. Locomotor activity was also analyzed in this test by the total number of arm entries. In agreement with the results obtained in the open field test, R6/1 mice showed a reduction in the

spontaneous locomotor activity that was recovered by 4EGI-1 administration (Fig. 30B). Lastly, in the T-SAT task, we evaluated the time spent by the mouse exploring the new arm 1 hour after the training session, in which the mouse was not allowed to enter one of the arms. As previously reported, vehicle-treated R6/1 mice did not exhibit a preference for the familiar arm (Garcia-Forn et al., 2018; Giralt et al., 2017). However, interestingly, 4EGI-1 treatment significantly increased the time spent in the novel arm, suggesting an improvement in spatial memory (Fig.30C).

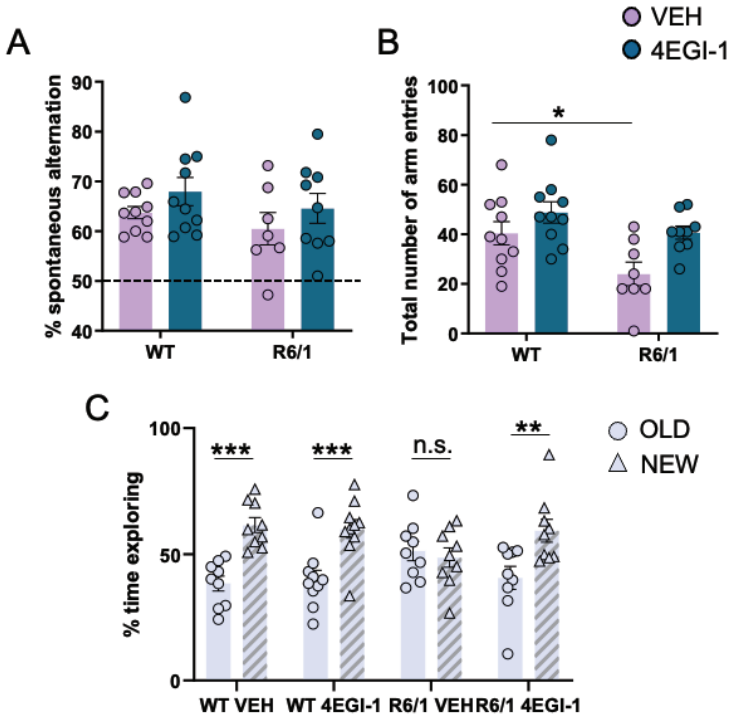


Figure 30.- 4EGI-1 treatment normalizes hippocampus-dependent spatial memory. Y maze spontaneous alternation test: (A) Graph shows the percentage of spontaneous alteration and (B) total number arm entries. (C) T-SAT: The percentage of preference for both arms was quantified 1 h after training. Graphs show the percentage of arm preference in WT and R6/1 mice from the total time exploring both arms. In all bar graphs, data are shown as mean ± SEM and each point corresponds to the value from an individual mouse. Two-tailed unpaired Student's t-test compared to (A) chance level (50%) (C) or the old arm. (B) Two-way ANOVA with Bonferroni's as a post hoc test. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

5.2 4EGI-1 administration slightly ameliorates LTP deficits in hippocampal slices

Since changes in protein translation have been linked to poor synaptic plasticity (Santini et al., 2013, 2017), we hypothesized that enhanced protein translation in the HD hippocampus would contribute to the hippocampal synaptic plasticity impairment

described in R6/1 mice. To address this hypothesis and determine whether the cognitive improvement shown in 4EGI-1-treated R6/1 mice was accompanied by an improvement in the hippocampal synaptic plasticity, we assessed hippocampal CA3-CA1 LTP in 15 weeks-old WT and R6/1 mice following treatment with 4EGI-1. Our data confirmed that hippocampal CA3-CA1 LTP was significantly reduced in adult 15 weeks-old R6/1 mice compared to WT mice, as reported by other groups (Giralt et al., 2017). 4EGI-1 administration, although without reaching significant values, slightly increased the LTP effect in the R6/1 group compared to the vehicle-treated mice (Fig.31).

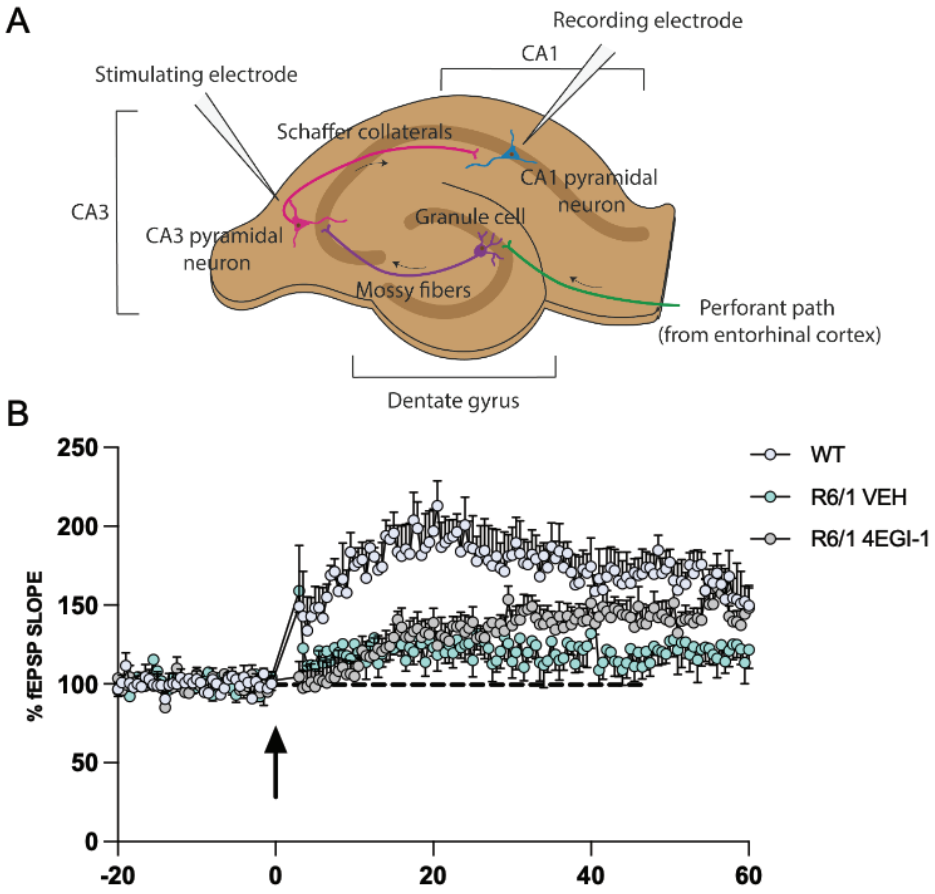


Figure 31.- 4EGI-1 administration slightly ameliorates LTP deficits in R6/1 mice. (A) Schematic representation of the electrode placement for CA3-CA1 LTP recordings. (B) Time course of fEPSP slope (CA3-CA1 LTP) in WT and R6/1 mice hippocampal slices. Arrow indicates theta-burst stimulation (TBS). N = 8–5 mice per group (1-2 slices per mouse).

5.3 Analysis of changes in the translatome induced by 4EGI-1 administration

To monitor the effects of 4EGI-1 treatment on the R6/1 mouse hippocampus translatome, we applied the same puromycin-based labelling experimental approach

used to compare translational alterations between naïve WT and R6/1 mouse hippocampal tissue (Fig.28). Similarly, the sum of the LFQ intensity and the total number of identified proteins were regarded as a quantitative indicator of the rate of bulk protein production. As expected, we observed enhanced translation levels in vehicle-treated R6/1 mice relative to vehicle-treated and 4EGI-1-treated WT animals, similar to that observed in naive mice (Fig.32A). These findings show that 4EGI-1 administration normalizes bulk protein synthesis and improves cognitive performance in the R6/1 mouse model. Interestingly, 20 aberrantly translated peptides in the R6/1 mouse compared to the WT mouse were restored under 4EGI-1 treatment, most of them (18 proteins) through down-regulation (Fig.32B). Specifically, we found the recovery of several proteins associated with response to metabolic and oxidative stress and components of the translational machinery such as the initiation factor eIF3F and the aminopeptidase Metap1. Importantly, Rbm3, a stress-response mRNA binding protein known to enhance global protein and phosphorylate 4E-BPs that we found upregulated in the HD hippocampus (Fig.28C) was restored by 4EGI-1 treatment, hence, it arises as a possible candidate for the cap-dependent translation dysregulation in HD.

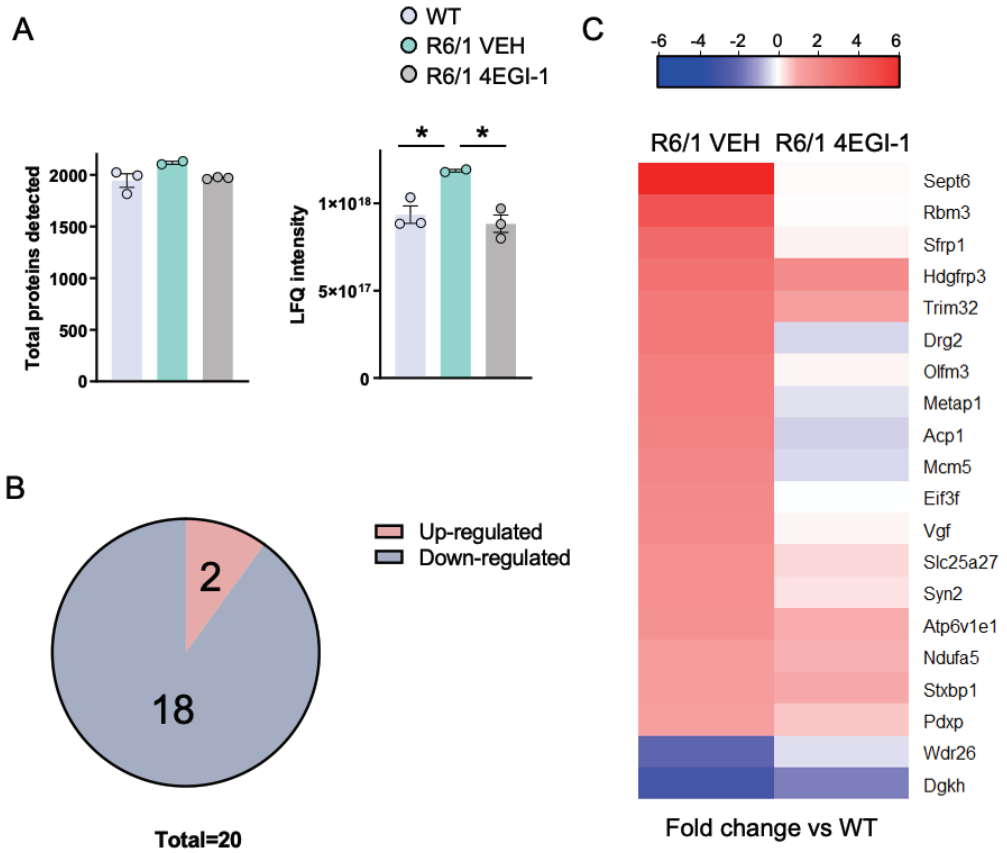


Figure 32.- Analysis of the translatome changes induced by 4EGI-1 treatment in the R6/1 mouse hippocampus. (A) Total number of proteins detected and summatory of LFQ intensity per condition. *$p<0.05$ compared with WT hippocampus (One-way Anova followed by Bonferroni post hoc test. (B) Pie chart showing down-regulated (blue) and up-regulated (red) proteins in the 4EGI-1-treated R6/1 mice hippocampus compared to the vehicle-treated 4EGI-1. (C) Heat map showing dysregulated proteins in the R6/1 mouse hippocampus that were normalized by 4EGI-1 treatment compared to WT mice. Cut-offs of ±0.5-fold change and $p<0.05$. ($n = 2$ mice/group).

6. METFORMIN INHIBITS PROTEIN SYNTHESIS THROUGH 4E-BP1 ACTIVATION IN CELLS EXPRESSING mHTT

Emerging new research suggests that the antihyperglycemic metformin, a first-line treatment for type 2 diabetes, may be neuroprotective in a variety of neurological pathologies. Despite controversial results in other neurodegenerative disorders such as AD and PD, multiple reports have recently reported metformin's therapeutic effects in cellular and animal models of HD suggesting that its therapeutic efficacy might be dependent on the disease context (Tang, 2019). Interestingly, metformin has been shown to act as a mTORC1 inhibitor through activation of the Ser/Thr kinase AMPK which would result in a 4E-BP1 activation and a global decrease in protein synthesis (Jang et al., 2021). Since we previously linked the normalization of protein synthesis and 4E-BP1 phosphorylation levels with an amelioration of the motor and cognitive phenotype in the R6/1 mouse, our aim in this part of the Thesis was to explore the correlation between metformin's effect on protein synthesis and the previously reported HD phenotype improvement. To do so we investigated the capacity of metformin to normalize aberrantly increased cap-dependent protein synthesis in an HD molecular context.

6.1 Metformin reduces protein synthesis levels and p4E-BP1 levels in primary cultures of mouse striatal neurons

We used primary neuronal striatal cultures derived from R6/1 mice striata as an *in vitro* model to confirm that metformin treatment results in a decreased 4E-BP1 phosphorylation and protein synthesis. Striatal primary neuronal cultures from R6/1 mice were incubated at 14 DIV with either vehicle or 1 mM of metformin for 24 h. B27 supplement was also removed from the medium at DIV 10 to avoid interferences with the treatment. As expected, the addition of metformin to R6/1 mouse striatal primary neurons decreased both puromycin incorporation and p4E-BP1 levels (Fig.33).

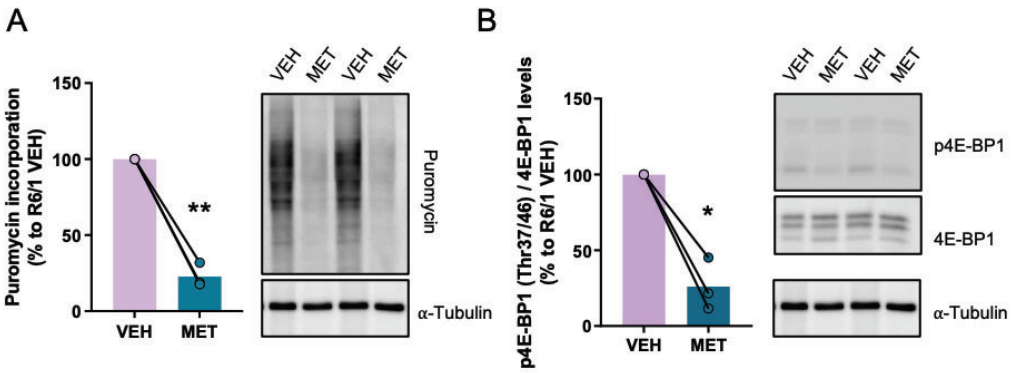


Figure 33.- Treatment with metformin decreases bulk protein synthesis rate. R6/1 mice striatal primary cultures were treated with metformin (MET; 1mM) or vehicle (VEH) at DIV 14 for 24 h. (A) Puromycin incorporation and (B) p4E-BP1 levels were analyzed by WB. α-Tubulin was used as a loading control. In graphs, values are expressed as a percentage of the vehicle-treated cultures from the same embryo. Each point corresponds to a pull from at least 2 embryos from 3 independent experiments. Representative immunoblots for each condition are shown. Two-tailed paired Student's t-test, *p<0.05, **p<0.01, compared to cultures treated with vehicle.

6.2 Ex vivo metformin treatment decreases translation in R6/1 mouse hippocampus

To further confirm metformin's inhibitory effect on global protein synthesis, we applied the SUnSET method *ex vivo* in coronal cortico-striatal and hippocampal slices treated with metformin or vehicle from 16 weeks-old WT and R6/1 mice. Mouse brains were divided into two hemispheres, each one randomly treated either with vehicle or metformin (see Fig.34A for experimental design schematic representation). Slices were incubated for 1h in oxygenated ACSF with the control solution or with 10mM metformin as described in (Bhat et al., 2017). Then, puromycin was added for 45 minutes, and the striatum and hippocampus were dissected and processed for WB. This experimental design and time point were chosen so they replicate the conditions established for the previous experiment in which we measured the puromycin incorporation in cortical and hippocampal slices from WT and R6/1 mice (Fig.27). Quantitative WB of newly synthesised peptides did not reveal a clear difference between control (vehicle-treated) and treated WT striatal nor hippocampal slices at this concentration. Interestingly, we did find a significant decrease in translation levels in the hippocampus of R6/1 mice (Fig.34C). In the R6/1 mouse striatum, although we observed a tendency to decrease it did not reach statistical significance (Fig.34B)

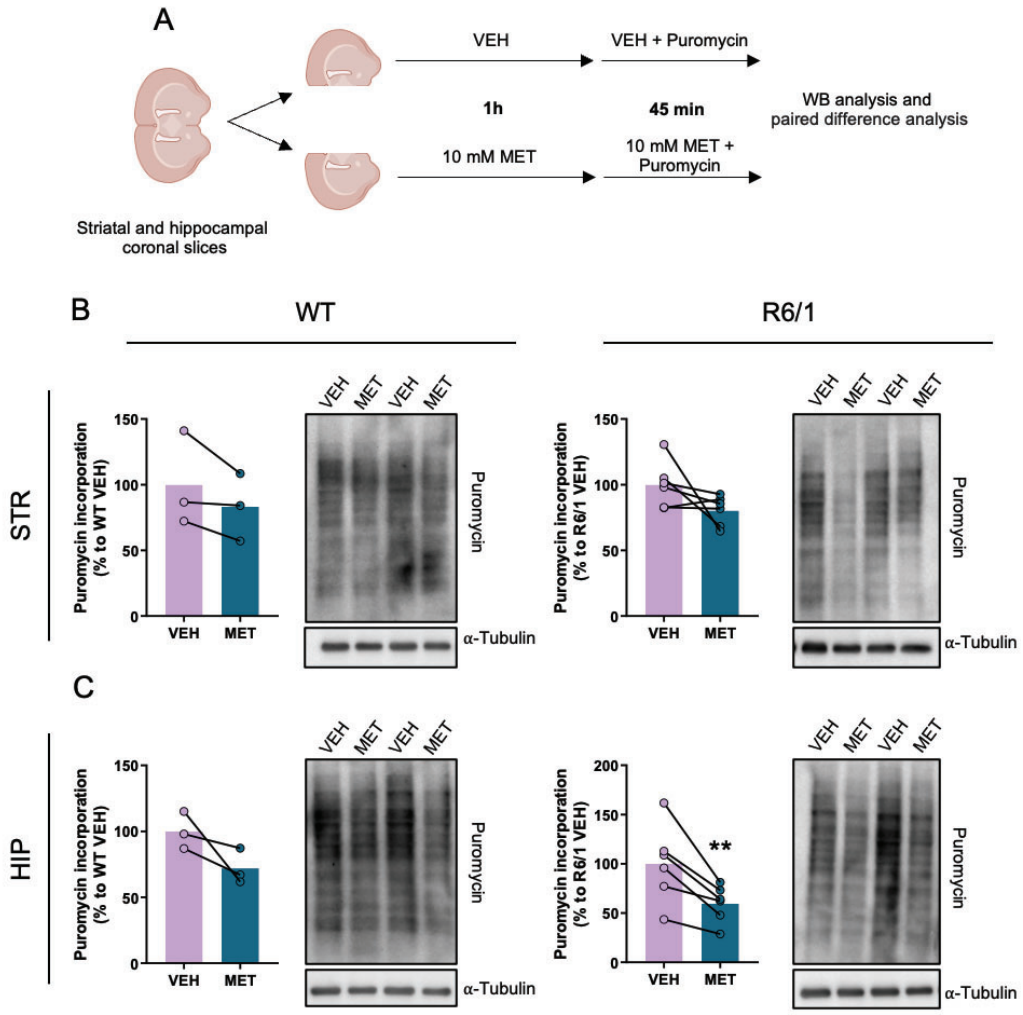


Figure 34.- Treatment with metformin decreases puromycin incorporation in R6/1 mouse hippocampal slices. (A) Schematic representation of the experimental design. (B) Striatal and (C) hippocampal slices from WT and R6/1 mice were treated with metformin (MET; 10 mM) or vehicle (VEH; H_2O) for 1h before puromycin incorporation. Puromycin incorporation and p4E-BP1 levels were analyzed by WB from protein extracts obtained from the dissected hippocampal tissue. α-Tubulin was used as a loading control. In graphs, values are expressed as a percentage of the vehicle-treated hemisphere from the same mice as an internal control. Each point corresponds to the value from an individual hemisphere. Representative immunoblots for each condition are shown. Two-tailed paired Student's t-test, **p<0.01, compared to the vehicle-treated hemisphere.

DISCUSSION

Previous results from our group reported the increased formation of the eIF4F protein complex and increased 4E-BP1 inactivation in line with an aberrant increase in de novo cap-dependent translation in striatal neurons from the R6/1 mouse model of HD (Creus-Muncunill et al., 2019). Interestingly, intracerebroventricular infusion of 4EGI-1, a potent inhibitor of the eIF4E and eIF4G interaction, was able to normalize striatal protein translation and significantly ameliorate motor symptoms in R6/1 mice. Overall, these results support the hypothesis of the role of an aberrant increase in cap-dependent protein translation as a direct contributor to neuronal dysfunction and behavioral deficits in HD. Despite these promising results, the precise contribution of cap-dependent translation alterations to the onset and progression of motor deficits needs to be explored in further detail. For this reason, in this Thesis, we evaluated the potential of 4E-BP1 activity modulation, as a key rate-limiting step in cap-dependent translation initiation, on reducing HD striatal pathology and behavioral abnormalities along with disease progression in the R6/1 mice.

4E-BP1 DYSREGULATION CONTRIBUTION TO HD PATHOLOGY

EIF4F complex is highly regulated under normal physiological conditions through different mechanisms like post-transcriptional modifications, phosphorylation and binding of inhibitory proteins including 4E-BPs. The aberrant function of these mechanisms is believed to be an important contributor to neurological disorders' molecular pathology (Gkogkas et al., 2013; Martin et al., 2014; Santini et al., 2013, 2017). In previous studies from our group, 4E-BP1 was found hyperphosphorylated at Thr 37/46 in the striatum of the R6/1 mice from an early stage of the disease, starting at 8 weeks of age, before the appearance of the motor symptoms (Creus-Muncunill et al., 2019). Although unpublished results from our group also found increased phosphorylation in other 4E-BP1 sites such as Ser 65/70, Thr 37/46 was used in this Thesis to determine 4E-BP1 activity since it corresponds to the priming event which is followed sequentially by the other phosphorylation events. Remarkably, this alteration was also found in other animal models including the $Hdh^{Q7/Q111}$ knock-in mouse (Creus-Muncunill et al., 2019). Even though it was not possible to detect 4E-BP1 phosphorylation in post-mortem brain samples, a decrease in the 4E-BP1 total protein levels was observed in putamen from HD patients (Creus-Muncunill et al., 2019). Although the direct causative relationship between the 4E-BP1 hyperphosphorylation and an increase in the global translation rate is still not proven, Creus-Muncunill et al.

(2019) provided strong evidence about the existence of this loss of translational control in the striatum of the R6/1 mice. Among them, we find an increased protein interaction between eiF4E and eiF4G, anteriorly proposed as a readout of translation initiation levels (Pause et al., 1994), and a clear increase in the puromycin incorporation when applying the SUnSET method, hence, an increase in the newly synthesized peptides during the incubation period. Taking a further step, 4EGI-1, a repressor of cap-dependent translation by disrupting the assembling of the initiation complex, provided strong proof that excessive translation initiation and eIF4F complex formation were responsible, at least partially, for the R6/1 mice motor phenotype. In this Thesis, we extended this work and described increased inactivation of 4E-BP1 in the cortex and hippocampus of the R6/1 mice as well as increased *de novo* protein synthesis in the latter that when normalized through 4EGI-1 intracerebroventricular administration promted an improvement on the hippocampal-dependent cognitive deficits. Overall, these data support the promising role of cap-dependent translation machinery regulation as a potential therapeutic target for HD.

Despite being one of the most studied substrates of mTORC1, we lack evidence to corroborate that mTOR hyperactivation is the mechanism responsible for the 4E-BP1 inactivation in the R6/1 mouse striatum. First, mTOR hyperactivation does not occur until late stages of the disease, at 12 weeks of age. In addition, S6K1, another important mTORC1 substrate widely used to monitor its kinase activity, appears unaltered in the R6/1 mouse striatum suggesting the involvement of an mTOR-independent mechanism (Creus-Muncunill et al., 2018). Furthermore, mTOR activity dysregulation seems to be region-specific since mTORC2, which does show an enhanced activity, appears hyperactive only in the striatum (Creus-Muncunill et al., 2018) while 4E-BP1 activity is altered in other affected brain regions like the hippocampus and the cortex (present results). The study of mTORC1 activity in other HD models have obtained contradictory results suggesting that it is largely context-dependent. For example, Lee et al (2015) described decreased pS6/S6 ratio in the N171-82Q mice striatum that when reverted by overexpression of a catalytically active form of Rheb, un upstream mTORC1 activator, exerted neuroprotective effects and decreased mHTT aggregates through enhanced autophagy-related factors. In contrast, Pryor et al (2014) reported a Rheb-dependent increase in mTORC1 signaling in HD cellular models in a context of amino acid stimulation and even demonstrate that mTORC1 hyperactivation through TSC1 depletion was detrimental for the same mouse model used in Lee et al. In the same line,

Pérez-Sisqués et al. (2021) downregulated REDD1, an upstream inhibitor of mTORC1 and reported cognitive deficits improvement and decreased neuroinflammation and microgliosis.

Despite the contradictory results in the upstream modulation of the mTOR pathway, 4EGI-1 treatment provided strong evidence that exaggerated translation initiation and formation of the eIF4F complex contributed to the motor deficits, energy failure and synaptic dysfunction in the R6/1 mouse (Creus-Muncunill et al., 2019). Hence, although mHTT could stimulate translation through alternative pathways, mTOR inhibition, if capable of activating the translation repressor 4E-BP1, might still be sufficient to exert a beneficial effect on HD phenotype. However, since 4EGI-1 has been reported to show minor off-target effects, a better understanding of the contribution of the 4E-BP1 inactivation and enhanced cap-dependent translation on HD striatal pathology would help to provide better treatment options (Fan et al., 2010). For this reason, in this Thesis, we asked whether the recovery of 4E-BP1's activity was sufficient to ameliorate striatal pathology in the R6/1 mouse model. To do so, we obtained adeno-associated viruses to overexpress a 4E-BP1 catalytically active form, 4E-BP1^{F113A}, in the striatum of WT and R6/1 mice. Unfortunately, 4E-BP1^{F113A} overexpression did not normalize protein synthesis in the striatum of R6/1 mice but instead, we detected an increase in de novo protein synthesis in the WT mice that led to an HD-like phenotype. Specifically, we observed a worsening in the performance on the rotarod and the balance beam tests. In accordance, we detected reduced levels of DARPP-32 and shrinkage in the striatal volume of the 4E-BP1^{F113A} injected mice. Moreover, we detected a very significant overexpression of the phosphorylated form of 4E-BP1 when blotting with the Thr37/46 phosphorylation specific 4E-BP1 antibody. However, although there are some controversial results published on the effect of this Phe113 mutation to Ala to 4E-BP1 phosphorylation, it is generally accepted that despite not completely abolishing Thr37/46, Ser65 and Thr70 insulin-induced phosphorylation, it completely prevents eiF4E release from 4E-BP1 (Beugnet et al., 2003a; K. M. Choi et al., 2003). In addition, independent groups have shown that 4E-BP1^{F113A} expression alone is sufficient to inhibit cap-dependent translation in transfected cultured cells supporting our results in STHdh$^{Q7/Q7}$ cells. For instance, Lin et al. (2016) used a dual luciferase assay to demonstrate that 4E-BP1^{F113A} expression inhibited cap-dependent translation by 63% in Neuro2a cells. Hence, our unexpected results could be due to the required longer time points for *in vivo* overexpression and behavioral analysis experiments. In this line,

we hypothesize that chronically suppressing eIF4E activity and protein translation initiation drives a compensatory response, resulting in elevated phosphorylation of both endogenous and mutant 4E-BP1 protein levels. Moreover, the *in vivo* approach of the SUnSET method is a whole tissue measurement perhaps masking some non-cell autonomous effects on the surrounding uninfected cells that could be contributing to the resulting increase in puromycin incorporation. Altogether, despite the unexpectedness of our results, they support the idea that enhanced protein translation is a putative new molecular mechanism underlying HD pathogenesis.

SUnSET METHOD ON R6/1 PRIMARY STRIATAL NEURONAL CULTURES AS A SCREENING ASSAY FOR THE IDENTIFICATION OF TRANSLATION MODULATORS

Although mTORC1 inhibition can be achieved by classical allosteric inhibitors like rapamycin or its analogues, called rapalogs, they mainly affect the phosphorylation of its other substrate S6K and only weakly inhibit 4E-BP1 phosphorylation (Choo et al., 2008; Livingstone & Bidinosti, 2012). The importance of the mTOR/4E-BP1 axis in cancer prompted the generation of ATP-competitive inhibitors of mTOR kinase (pan-mTOR active-site inhibitors), which inhibit both mTORC1 and mTORC2. Unluckily, mTORC2 inhibition reduces AKT activation due to feedback inhibition which carries undesirable side effects like induction of hyperglycemia and jeopardizes its clinical utility (Graham et al., 2018; Hagiwara et al., 2012; Powles et al., 2016). As a result, new research is directed towards mTORC1-specific inhibitors that have the potential to be more powerful tumor growth inhibitors than pan-mTOR inhibitors while also being less hazardous (B. J. Lee et al., 2021). Since mTORC1 inhibition is still a hot topic in biomedicine research without much success in clinical trials, the development and application of *in vitro* experimental models that replicate cap-dependent translation dysregulation are essential for the initial screening of potential new compounds with therapeutic potential. However, the HD-like phenotypic traits reproduced varies on each of the cellular models.

Although mouse model-derived cell lines were first used in HD research due to their easy accessibility and manipulation, we found that STHdh$^{Q111/Q111}$ striatal neuronal progenitor cell line derived from the Hdh$^{Q7/Q111}$ knock-in mouse model did not reproduce the translational alterations found in the Hdh$^{Q7/Q111}$ KI mouse striatum. In accordance

with our results, Eshraghi et al. (2021) also recently reported that STHdh$^{Q111/Q111}$ cells show a lower global translation rate, which validates the SUnSET assay as an effective and quantitative measurement of the protein synthesis rate in *in vitro* models. The main limitation of mouse model-derived cell lines is that mouse models express artificially extended polyglutamine tracts used to mimic the late-onset of the disease in the short lifespan of the mouse while the majority of patients have CAG repeats between 40 and 50. In addition, transgenic mouse models do not present an accurate gene dosage, resulting in supraphysiological levels of expression. In this context, primary fibroblasts from HD patients presented as a promising model for investigating HD human cell biology due to their clinically significant polyglutamine expansion lengths. Moreover, the fact that they derive from human peripheral tissue could be very useful in defining precise molecular biomarkers of HD progression. However, our results indicate that primary fibroblast cultures do not serve this later goal, since no differences were found between genotypes or disease stages. Interestingly, cluster analysis identified that fibroblasts from patients expressing m*HTT* with less than 42 CAG repeats presented aberrantly high puromycin incorporation.

Another plausible explanation is that, unlike neurons, these models show a high mitotic rate which, in general, is accepted to have a negative impact on global translation (Sivan & Stein, 2008) resulting from altered phosphorylation state of translation initiation factors, mainly eIF2α, during mitosis (Stonyte et al., 2018; Tanenbaum et al., 2015). Thus, we hypothesized that proliferating cell lines were not fit for an *in vitro* model to study translational alterations. This idea was supported by the discovery of increased 4E-BP1 inactivation and puromycin incorporation in striatal neuronal cultures from the R6/1 mice. Overall, since protein synthesis dysregulation seems to be more dependent on the mitotic rate than the genetic background of the cell model, in this Thesis, we used striatal neuronal cultures from the R6/1 mouse model to generate a translation assay for the screen for translation modulators which enabled us to identify an FDA approved drug, the antidepressant sertraline, as potential HD therapeutic option targeting 4E-BP1 activity and cap-dependent translation.

CAP-DEPENDENT TRANSLATION INHIBITION AS A NEW MOLECULAR TARGET OF THE ANTIDEPRESSANT SERTRALINE FOR HD

Since the causal therapeutic techniques targeting the *HTT* gene are still absent, it may be indispensable to adapt existing approved and tested generic drugs that are now on the market to treat the complex HD pattern by targeting dysregulated signaling pathways such as the mTORC1-4E-BP1 axis. With this purpose, in this Thesis, we aimed to determine if the widely prescribed SSRI anti-depressive sertraline was sufficient to normalize translation initiation in an HD molecular context. Sertraline was selected as our drug candidate for its antiproliferative effects through protein synthesis repression and its positive impact on motor integrity and survival already described in other HD animal models. In fact, C. J. Lin et al. (2010) reported an analysis of the polysome profile in cells treated with sertraline which revealed a decrease in polysome content and an increase in 80S ribosomes. In this study, sertraline's impact on translation was proposed to be mediated by a decrease in all the eIF4F complex components levels, nuclear localization of eIF4E through hypophosphorylated 4E-BP1 sequestration, and increased eIF2α phosphorylation (C. J. Lin et al., 2010). Sertraline was also proposed as an autophagy-inducing compound first in a phenotypic screen for autophagy-inducing small molecule FDA-approved drugs from the Johns Hopkins Drug Library (Y. S. Cho et al., 2016). Five years later, the same group focused on sertraline and deciphered the molecular mechanism behind its role as an autophagy inducer. In this paper, Hwang et al. (2021) describe how sertraline acts as a PI3K-AKT and MEK-MAPK-independent mTOR inhibitor through direct binding and inhibition of the mitochondrial VDAC1 (voltage-dependent anion channel 1). Since the latter plays a key role in cell metabolism by transporting ATP through the mitochondrial outer membrane, its inhibition results in ATP levels lowering and increased AMP/ATP ratio, which activates the cellular energy status sensor AMPK. In physiological conditions, AMPK activation inhibits mTOR activity through two different phosphorylation pathways: 1) through TSC activation, which acts as an inhibitor of mTOR or 2) through direct inhibition of RAPTOR, one of the components of the mTORC1. However, the lack of inhibition of the sertraline effect in TSC2 KO cells indicates that AMPK inhibits mTOR activity through the phosphorylation of RAPTOR (C. J. Lin et al., 2010). Moreover, Hwang et al. (2021) concluded their study by showing that sertraline suppresses tauopathy by promoting the autophagic degradation of MAPT (microtubule-associated

protein tau) suggesting its potential as a new drug candidate to treat autophagy-related disorders including neurodegenerative diseases.

However, these studies did not examine the contribution of these translational regulators in hyperphosphorylated 4E-BP1 conditions. For this reason, we used primary neuronal cultures from R6/1 mice as an *in vitro* model to first confirm that sertraline is able to inhibit 4E-BP1 phosphorylation at Thr 37/46 and protein synthesis in mHTT-expressing cells. Interestingly, chronic sertraline treatment increased survival, reduced striatal atrophy and improved motor performance on the rotarod test of R6/2 and N171-82Q HD transgenic mouse models (Duan et al., 2008; Peng et al., 2008). In both cases, sertraline treatment beneficial effects correlated with a normalization of the BDNF levels and enhanced adult neurogenesis, which could be a consequence of the serotoninergic system modulation since the administration of SSRIs has been reported to increase the levels of BDNF (Duan et al., 2004; Duman, 1998; Vaidya et al., 1997, 1999). Along the same line, our work expands the results of the effect of chronic sertraline treatment on the R6/1 mouse motor coordination and learning, accompanied by the normalization of protein synthesis and 4E-BP1 inactivation. Keeping in mind the beneficial effects on motor function induced by protein synthesis inhibition by 4EGI-1, these results suggest that protein synthesis normalization might mediate, at least in part, the beneficial effects on HD phenotype described in several HD animal models. The fact that other SRRIs such as fluoxetine, although increasing adult neurogenesis levels failed to prevent motor dysfunction, strengthens the hypothesis of other sertraline off-targets mediating other molecular mechanisms to exert its effects (Grote et al., 2005). Moreover, in agreement with the aforementioned studies, our results show no effect of sertraline treatment on mHTT aggregation suggesting an independent molecular mechanism of action. However, this possibility could not be ruled out since results obtained on mHTT aggregation in transgenic models expressing the truncated form of HTT cannot be fairly translated to human pathology. Additionally, sertraline arises as a promising candidate for HD clinical trials due to the fact that the serum and brain levels of the antidepressant that were found to be advantageous in these transgenic mice are comparable to those reached by patients (Slaughter et al., 2001).

To confirm the beneficial effects of sertraline treatment in HD patients, we investigated its potential impact when prescribed to a cohort of HD patients as depressive symptomatic therapy, on the longitudinal progression of HD in terms of motor and functional outcomes over a period of seven years. We used the Enroll-HD database, the

largest HD observational study conducted worldwide, to answer this question. This methodology represented a huge advantage of this study since we could work on longitudinal data from a remarkable number of patients recruited at multiple sites worldwide. This is of particular interest since it provides precise and novel knowledge on the long-term outcomes of medications on HD progression.

Remarkably, sertraline treatment appears to have a beneficial effect on delaying the anticipated decline in the functional performance of the HD cohort. Unfortunately, due to the small sample size, we were unable to clearly identify either the positive or negative effects of sertraline on motor function. Interestingly, patients treated with only sertraline or other antidepressants, present a more severely affected functionality and motor scores at baseline. Thus, since it was expected that treated patients would present a faster decline, we can conclude that sertraline exerted a stabilizing effect on functionality scores. However, this effect seems to fade in patients prescribed with sertraline as the only antidepressant which points to a synergistic effect rather than the sum of the effects of the individual drugs. This, however, remains mere speculation since the lack of significance in the effect of sertraline in this group of patients could be explained by the relatively small sample size (n=30).

Observational studies have produced inconsistent results in similar studies due to the heterogeneity of the participants included and the impossibility to isolate the effects of a selected drug in a disease where polypharmacy is very common (Imfeld et al., 2012; Kuan et al., 2017; Moore et al., 2013). However, the strengths of the data presented here consist of the statistical model used, a Linear Mixed Effects Model (LMM) which allowed us to avoid the limitations coming from the baseline differences and potential confounders while focusing on the slope of evolution rather than the mean difference between groups at each time point (Verbeke & Molenberghs, 2000).

Overall, although we were unable to demonstrate an improvement in the pure motor score TMS, our data support our hypothesis regarding a potential neuroprotective effect of sertraline on disease progression in terms of functionality.

CONTRIBUTION OF 4E-BP1 DYSREGULATION TO HD COGNITIVE DEFICITS

The most prominent neuropathological changes in HD consist of cell loss and atrophy of the striatum, the medium spiny neurons being the most severely affected (Vonsattel & DiFiglia, 1998; Waldvogel et al., 2014). This has led the researchers to focus on the striatum although other brain regions are also affected (Spargo et al., 1993). In particular, the hippocampus is strongly associated with non-motor symptoms in both preclinical and clinical HD stages (Paulsen et al., 2008; Ransome et al., 2012). For instance, it is known that mild cognitive impairment can be detected up to 15 years before the motor diagnosis and after this point, hippocampal-dependent deficits like impaired executive function, episodic memory and processing speed are described (Duff et al., 2010; Paulsen et al., 2008). Nevertheless, even though cognitive and behavioral symptoms are the most harmful for patients and their families, clinical trials primarily concentrate on motor symptoms and overall functional ability. Here, we expand the hypothesis of cap-dependent translation dysregulation as a molecular mechanism contributing to HD pathogenesis outside the striatum. Our results show that 4E-BP1 aberrant inactivation was also present in the hippocampus and the motor cortex of the R6/1 mouse model. In particular we confirmed that Pyk2-positive CA1 hippocampal neurons showed increased puromycin incorporation after puromycin infusion through a cannula in the lateral ventricle although we do not discard the presence of translational alterations in other cell types. In the cortex, we also found increased inactivation of 4E-BP1 but no changes in puromycin incorporation at the same age. Since 4E-BP1 was assessed specifically in the motor cortex, this discrepancy could be explained by translation alterations only affecting specific neuronal populations within the cortex like in the case of the striatum where only MSNs are affected (Creus-Muncunill et al., 2019). Moreover, since 4E-BP1's phosphorylation status is technically difficult to assess in post-mortem samples due to the high variability in the post-mortem delay, we cannot prove or rule out the possibility of the presence of translational alterations in the HD human hippocampal region.

Previous results from our group described the translatome profile changes in 15-week-old WT and R6/1 mice whole brains through the application of the PUNCH-P translatome profiling assay (Creus-Muncunill et al., 2019). Since this method is based on the isolation of intact ribosomes by ultracentrifugation followed by an *in vitro* incubation with biotin-puromycin to affinity-label nascent chains it requires large

amounts of starting material. Thus, whole mouse brains must be used, which compromises the spatial resolution of the results (Aviner et al., 2014). Since HD pathology is region-specific, we hypothesized that the HD translatome is likely differently altered throughout the brain (Spargo et al., 1993). For this reason, although the PUNCH-P method *in vitro* labeling strategy confers higher temporal resolution, we used an *ex vivo* puromycin-based labeling assay to identify and compare the purified nascent proteins in the R6/1 mouse model hippocampus.

Interestingly, KEGG pathway analysis revealed that most of the altered proteins are related to metabolic pathways for energy production and nutrient utilization such as glycolysis and pyruvate metabolism. In particular, we identified a selective increase in the synthesis of a subset of proteins related to mitochondrial functions including glycolysis and components of the electron transport chain such as Ndufa5, Mtnd4 and other proteins required for the oxidative phosphorylation system like Timm8a1, Etfdh and Coq9. These results are in line with our previous work in which we described an increase in the translation of proteins related to the oxidative phosphorylation in the R6/1 whole brain through the application of the PUNCH-P method (Creus-Muncunill et al., 2019). In particular, both experiments show an increase in subunits of the complex I of the respiratory chain, such as Ndufa5, which has also been found increased in the R6/1 mice striatum in late disease stages (Herbst & Holloway, 2015). Interestingly, 4E-BP1 inactivity has been shown to promote the translation of components of the respiratory chain which contributes to enhanced OXPHOS and ATP production linking 4E-BP1 activity to mitochondrial respiratory function (Morita et al., 2013, 2015). Furthermore, Morita et al. (2013) also reported that mTORC1 activity, not mTORC2, was responsible for increased glycolysis which could be related to the upregulation of the translation of the glycolytic proteins pyruvate kinase (Pkm) and the pyruvate dehydrogenase complex component Dld we found in the hippocampus of the R6/1 mouse at 16 weeks. Our results are also in consonance with a longitudinal proteomic analysis of brain of R6/2 HD mice at different disease stages in which conclude that protein changes are stage specific and drastic changes occur as early as 2 weeks of age. In this study, KEGG analysis mainly described an up-regulation of the glycolysis/gluconeogenesis pathway that does not seem to show a progressive decline but display transient expression peaks instead. Furthermore, the differences found in the KEGG pathway analysis when comparing protein and mRNA data from the same model evidences that protein expression changes are also highly dependent on

translational alterations (Zabel et al., 2009). Overall, all this data suggests 4E-BP1 dysregulation and 5'cap-dependent translation upregulation could contribute to the early biochemical changes in HD by increasing ATP consumtion and promoting energy producing cellular pathways. Although we observed that CA1 hippocampal neurons showed increased protein synthesis, we can not rule out the possibility that our data may be also reflecting alterations in other cell-types since for instance, astrocytes are also affected by metabolism alterations in HD (Lange et al., 2023)

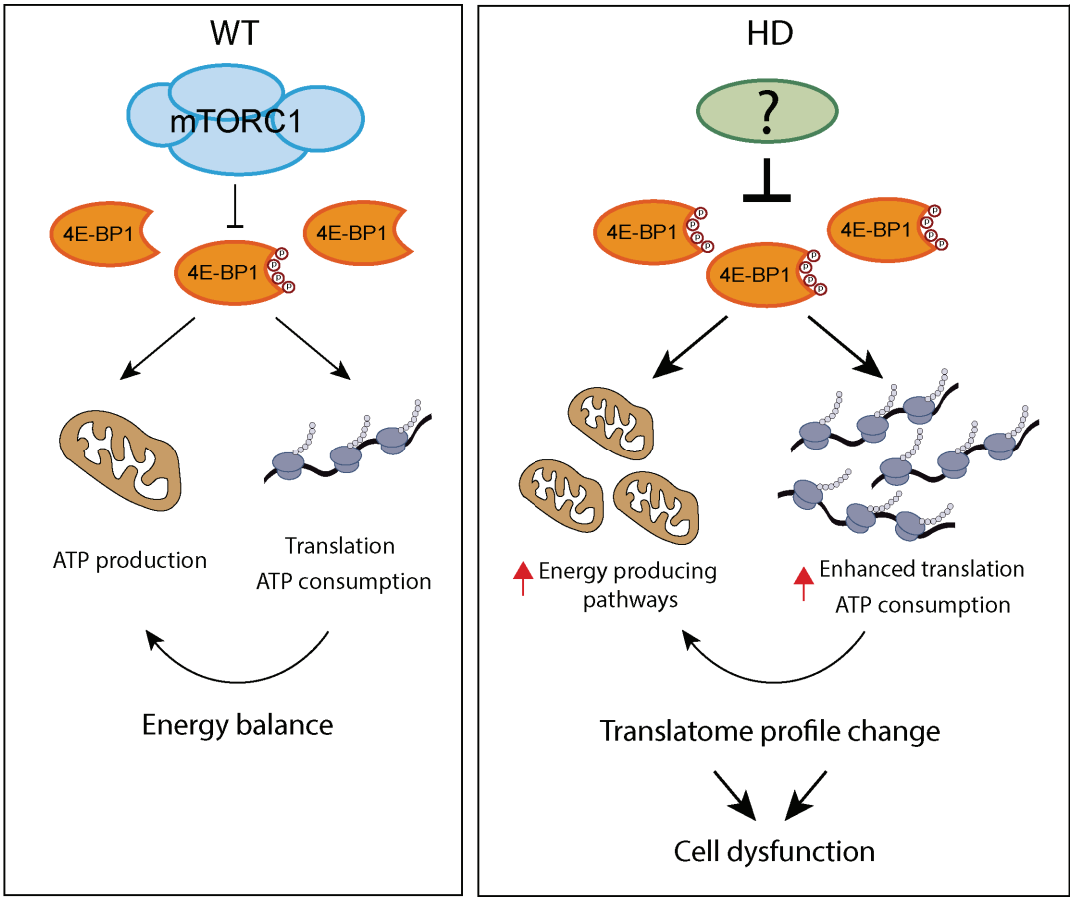


Figure 35. Proposed mechanism for 4E-BP1-dependent cell-dysfunction in HD. Increased 4E-BP1 inactivation and cap-depenent translation contribute to metabolic stress by increasing ATP consumption and promoting energy producing cellular pathways.

Additionally, a remarkable number of synaptic proteins were also altered, mostly downregulated, in the HD hippocampus. Among them, we found a decrease in the translation of several post-synaptic density proteins from the glutamatergic synapse like the Gria1 (GluA1) AMPA receptor subunit and the glutamate receptor-associated scaffold proteins Shank3, Syngap1 and Homer2. Intriguingly, we also found that several presynaptic proteins exhibited higher protein synthesis such as Synapsin-2 (Syn2) and Syntaxin-binding protein 1 (Stxbp1). Our results suggest that dysregulated translation

of proteins involved in synaptic transmission is contributing to the synaptic dysfunction and behavioral abnormalities observed in the R6/1 mouse model. In agreement, the aforementioned longitudinal proteomic analysis of the R6/2 striatum also found alterations in synaptic proteins, in particular they also show upregulated Syn2 protein levels from 2 weeks of age, which indicates that translation dysregulation may be perturbing synaptic activity since early stages (Zabel et al., 2009). Moreover, RPS21, a component of the 40S ribosomal subunit, was found upregulated in the HD samples. Creus-Muncunill et al. (2019) also reported an increase in the translation of ribosomal proteins in the striatum although discrepancies in other proteins can be explained due to different regional properties (striatum vs hippocampus) and methodology employed (PUNCH-P vs puromycin-labelling and pull-down).

Dysregulation also affected components of the unfolded protein response (UPR) and the proteasome which are already known to present changes in early stages in brains from several mouse models and human HD patients (Bennett et al., 2007). We found altered translation levels of two proteins with E3 ubiquitin ligase activity, Nedd4l and Trim32, involved in the ubiquitin-mediated proteolysis. Interestingly, Nedd4l, which, unlike the other proteins listed, appears downregulated in the HD hippocampus is thought to suppress autophagy and mitochondrial metabolism in cellular contexts of mitochondrial stress (D. E. Lee et al., 2020). In the same line, Trap1 up-regulation has been associated with increased UPR in the ER in response to oxidative stress and glucose deprivation (Takemoto et al., 2011). This may reflect the effort to remove protein aggregates and accumulated misfolded proteins from the translational dysregulation here described. However, these results need to be interpreted with caution since puromycin incubation in living cells may induce unwanted effects like cellular stress caused by the accumulation of truncated or misfolded proteins which could be artificially activating the UPR in these experimental conditions (Aviner et al., 2014).

Next, we hypothesized that mimicking the contribution of aberrantly exaggerated cap-dependent translation to the striatal motor dysfunction, increased translation in the HD hippocampus may be contributing to cognitive impairment in the R6/1 mouse model. Our research supported earlier findings showing that R6/1 mice had memory and cognitive flexibility deficits, which are similarly seen in HD patients and provide evidence that cap-dependent translation dysregulation contributes to the hippocampal impairment in a mouse model of HD (Giralt et al., 2012; Pantelis & Andrewes, 1995).

As previously described, early LTP was not induced in standard conditions at the R6/1 mice Schaffer collaterals synapses on CA1 pyramidal neurons, which is the most studied form of LTP underlying cognitive processes such as learning and memory (Bliss & Collingridge, 1993; Bruel-Jungerman et al., 2007; Citri & Malenka, 2008; Giralt et al., 2017). However, the cognitive improvement did not correlate to significant recovery of this synaptic process, although a slight amelioration can be perceived. However, further research should be performed since although an improvement in hippocampal-dependent tasks is usually attributed to early LTP recovery, late LTP has been described to be more dependent on translational changes and it has been related to the activation of 4E-BP1 (Ran et al., 2009).

Normalization of bulk protein synthesis in hippocampal neurons was validated by the application of the *ex vivo* puromycin-based labeling assay previously used in this Thesis to assess translatome changes in the R6/1 compared to WT mice. Interestingly, we found the recovery of several proteins associated with response to metabolic and oxidative stress. Among them, we identified some mitochondrial proteins such as Ndufa5, a subunit of the enzyme NADH dehydrogenase (ubiquinone), the largest of the respiratory complexes and the mitochondrial UCP4 (Slc25a27) which has been reported to mediate an adaptive shift in energy metabolism and increase the resistance of neurons to metabolic and oxidative stress (D. Liu et al., 2006). We also found a recovery of the vacuolar H+-ATPase (Atp6v1e1) which has been also associated with AD pathogenesis by affecting the synaptic vesicle cycle, the phagosome, and oxidative phosphorylation (Zhou et al., 2021). These data suggest that normalization of protein synthesis through 4EGI-1 treatment prompted metabolic and oxidative stress relief.

Remarkably, we found an increased hippocampal translation of the RNA binding motif protein 3 (RBM3) in the R6/1 mice, a stress-response mRNA binding protein known to enhance global protein translation at both physiological and mild hypothermic temperatures through phosphorylation of translation initiation factors and active polysome formation. Strikingly, RBM3 overexpression has been reported to greatly enhance the phosphorylation of eIF4E and 4E-BPs arising as a candidate for 4E-BP1 activity dysregulation in HD (Dresios et al., 2005; Smart et al., 2007). This hyphotesis was strengthened by the recovery of the RBM3 translation levels by improvement of the protein homeostasis through 4EGI-1 administration.

METFORMIN INHIBITS CAP-DEPENDENT TRANSLATION IN MHTT EXPRESSING CELLS

Emerging evidence suggests that dimethylbi-guanide (metformin), a first-line treatment for type 2 diabetes mellitus, may have neuroprotective effects in a variety of brain disorders including HD. For instance, neuropsychiatric and motor symptoms amelioration and prevention of BDNF decline and mHTT aggregation by chronic metformin treatment was reported in the zQ175 mouse model of HD (Sanchis et al., 2019). Metformin use was also linked to better outcomes on cognitive tests and a favourable trend toward improved motor function was identified in a statistical analysis of a sample population of patients participating in the Enroll-HD database (Hervás et al., 2017).

Metformin's mechanism of action is well-studied. It inhibits the complex 1 of the ETC resulting in an elevation of the AMP levels that activates the cellular energy sensor AMPK by phosphorylation of AMPK α-subunit at Thr-172 (El-Mir et al., 2000; Owen et al., 2000). AMPK antagonizes mTOR activity through two signaling pathways. First, it activates tuberous sclerosis complex 2 (TSC2), which inhibits mTORC1 activation through the activity of Ras homolog enriched in brain (Rheb) (Howell et al., 2017). Secondly, it directly phosphorylates the scaffolding mTORC1 protein RAPTOR (Gwinn et al., 2008). Furthermore, metformin has been proven to decrease translation in a 4E-BP–dependent manner in MCF-7 cells, which as a breast cancer cell line present hyperactivated mTORC1 (Holz & Blenis, 2005; Larsson et al., 2012). Intriguingly, Larsson et al. (2012) also describe metformin's effect on the cell translatome which includes the suppression of mRNAs encoding mitochondrial proteins. For this reason, we hypothesized that metformin's neuroprotective effects in HD models could be related to the antagonizing effect of metformin on the increased cap-dependent translation and up-regulation of a subset of mitochondrial-related proteins. Therefore, we tested metformin's effect on protein synthesis in different HD models including neuronal primary striatal cultures and striatal and hippocampal slices of the R6/1 mice. The dose used (10 mM) was reported to effectively decrease 4E-BP1 phosphorylation levels in MCF-7 cells (Larsson et al., 2012) and increase the AMP/ATP ratio activating AMPK (El-Mir et al., 2000; Owen et al., 2000). However, in primary neuronal cultures, we detected that this dosage was causing cell death. As a result, we decreased the concentration of metformin to 1 mM, a dosage that has previously been used in primary cultures studies

(Utami et al., 2020). The ability of each cell type to actively introduce metformin through its transporters, the organic cation transporters, which will vary based on their expression levels in each cell type, is most likely the cause of these variations in drug concentration (M. K. Choi & Song, 2008; Creus-Muncunill et al., 2019). As expected, metformin decreased protein synthesis levels as well as phosphorylation levels of 4E-BP1 in primary striatal neuron cultures and hippocampal slices from the R6/1 mice. Unfortunately, we failed to reproduce the results in the striatum slices, yet a decreasing trend was detected.

Although the most studied molecular mechanism for metformin's effects is the activation of AMPK (Howell et al., 2017; Mascaraque et al., 2020; Shaw et al., 2005, larsson), other groups have proposed that it is also able to inhibit protein synthesis by decreasing Raf–MEK–ERK signaling pathway resulting in a reduction in eIF4E phosphorylation (Gantois et al., 2017). Moreover, metformin has also been proven to induce downregulation of the RBM3, an RNA-binding protein that we found up-regulated in the hippocampus of the R6/1 mice which is known to enhance global protein translation through phosphorylation of eIF4E and 4E-BPs (Laustriat et al., 2015). This is especially interesting since data from *in vitro* and *in vivo* models along with the discovery of over-activated forms of AMPK in the brain tissue of HD patients suggest that activating AMPK during the late stages of HD may have detrimental effects so AMPK may be best considered as a target to treat HD during the early stages of the disease (Chou et al., 2005; Ju et al., 2011).

Altogether, our results show for the first time that metformin is able to inhibit 4E-BP1 hyperphosphorylation in two HD models and open a new research line in search of the exact molecular pathway through which metformin is able to reduce cap-dependent translation.

Summarizing, in this doctoral Thesis, we provided further evidence for the contribution of 4E-BP1 activity dysregulation and exaggerated cap-dependent translation to the HD neuronal dysfunction and phenotype. Although which particular pathogenic mechanism causes the inactivation of 4E-BP1 in the HD striatum is yet unknown, our data strengthen the hypothesis that it may constitute a compensatory mechanism, since overexpression of constitutively active form 4E-BP1 prompted an unexpected increase in 4E-BP1 phosphorylation and puromycin incorporation in the striatum of the R6/1 mice. Moreover, this hypothesis is in line with a recent study showing ribosome stalling induced by mHTT expression in STHdh cells (Eshraghi et al., 2021), although in contrast, in this model, translation results inhibited, maybe due to the proliferating state of these cells as opposed to the neuronal cell cycle suppression. Interestingly, it has been recently shown that treatment with 4EGI-1 attenuates ribosome collisions in cells expressing mHTT (Aviner et al., 2022), which is in line with our results showing that treatment with 4EGI-1 prevents R6/1 mice motor deficits and normalizes *de novo* protein synthesis and ribosomal content in the striatum (Creus-Muncunill et al., 2019) .

On the other hand, we found that striatal neuronal cultures from the R6/1 mouse model replicate the translational alterations found in the R6/1 striatum which allowed us to identify the FDA-approved antidepressant sertraline as a potential HD therapeutic option by targeting 4E-BP1 activity and cap-dependent translation. Furthermore, we show that chronic sertraline treatment normalized protein synthesis in the striatum of the R6/1 mice and confirmed that it was able to improve the motor phenotype in this model, as described by other studies in other HD mouse models (Duan et al., 2008; Peng et al., 2008). Also, we took advantage of the Enroll-HD database to perform a longitudinal study on the effect of sertraline treatment on the progression of motor symptoms and functionality decline in HD patients in which we identified a potential neuroprotective effect of sertraline on disease progression in terms of functionality.

Until now, our research was only focused on the study of translation control in the striatum. In this work, we expanded our research to other affected areas of the HD brain, with a specific focus on the hippocampus. Interestingly, our results suggest that protein synthesis dysregulation also contributes to hippocampal-dependent cognitive deficits probably due to energy deficits and altered translation of selective transcripts including a set of mitochondrial-related proteins.

Finally, we provide evidence that, among its different mechanisms of action, metformin's beneficial effects on HD models could also be attributed to the modulation of 4E-BP1's activity and translation although the upstream mechanism still requires further investigation.

CONCLUSIONS

1. Catalytically active 4E-BP1 striatal overexpression exacerbates protein synthesis and induces motor alterations in WT mice.

2. Protein synthesis is aberrantly increased in primary fibroblast cultures from HD patients expressing less than 42 CAG repeats.

3. Primary striatal neuronal cultures from R6/1 mice recapitulate translational alterations present in the R6/1 mouse striatum and emerge as an *in vitro* model for the primary screening of translation modulators in an HD molecular context.

4. Sertraline treatment improves motor function and normalizes 4E-BP1 activity and cap-dependent translation in the R6/1 mice striatum.

5. Sertraline treatment slows down the expectable functional performance decline in HD patients.

6. Translation is increased in the hippocampus of the R6/1 mouse model affecting proteins from selective pathways involved in energy production and nutrient utilization associated to 4E-BP1's increased phosphorylation.

7. Normalization of hippocampal cap-dependent translation through 4EGI-1 treatment improves cognitive function and slightly ameliorates hippocampal LTP deficit in R6/1 mice.

8. Normalization of hippocampal translation prompted the recovery of several proteins associated with response to metabolic and oxidative stress in R6/1 mice.

9. Metformin treatment downregulates protein synthesis and 4E-BP1 inactivation in *in vitro* and *ex vivo* models of HD.

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