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Uncovering new therapeutic strategies for motor and cognitive deficits in Huntington's Disease

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Dissertation submitted by:

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Programa de Doctorat en Biomedicina per la Universitat de Barcelona

RESUM

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La malaltia de Huntington (MH) és un trastorn neurodegeneratiu de tipus hereditari caracteritzat per símptomes motors, cognitius i psiquiàtrics, descrit en detall per primera vegada per George Huntington al 1872 (Huntington, 2003; Ross & Tabrizi, 2011). La malaltia té un inici tardà i és deguda a una expansió aberrant de la repetició trinucleotídica CAG en el gen *HTT* descoberta l'any 1993, que provoca una extensió de poliglutamines a la proteïna Huntingtina (MacDonald et al., 1993). El nombre de repeticions CAG està inversament relacionat amb l'edat d'inici de la malaltia (M. Duyao et al., 1993; Rubinsztein et al., 1996). Les persones amb 40 o més repeticions mostren plena penetrància, mentre que aquelles amb 36-39 repeticions mostren una penetrància reduïda (Walker, 2007). D'altra banda, la MH juvenil es produeix en persones amb 50 o més repeticions (Gordon, 2013).

La MH és heterogènia, amb baixes taxes de prevalença en poblacions no-europees (McColgan & Tabrizi, 2018), i les taxes més altes en poblacions específiques amb origen en un nombre reduït de predecessors, com ara Tasmània i l'àrea al voltant del llac de Maracaibo a Veneçuela (Okun & Thommi, 2004). Els símptomes motors inclouen moviments coreics i discinètics, distonia i rigidesa, mentre que els símptomes cognitius i psiquiàtrics apareixen primer i empitjoren gradualment fins que coexisteixen amb els motors. La malaltia presenta una etapa pre-simptomàtica, una fase prodròmica i una fase manifesta, que es diagnostica per l'aparició dels trastorns motors (G. P. Bates et al., 2015; Walker, 2007). Al final d'aquest llarg procés, 15-20 anys després de l'edat d'inici, la principal causa de mort és la pneumònia per aspiració, com a complicació de la disfàgia, seguida del suïcidi (Heemskerk & Roos, 2012). Neuropatològicament, la MH es caracteritza per l'atròfia bilateral dels nuclis caudat i putamen, que condueix a un augment dels ventricles laterals (J. P. Vonsattel et al., 1985). No obstant això, la malaltia també afecta altres regions cerebrals, incloent el còrtex cerebral, l'hipocamp, el tàlem i el cerebel entre d'altres, apuntant a una alteració de les xarxes cerebrals a gran escala que ajuda a comprendre les diverses presentacions clíniques de la malaltia, relacionades amb les alteracions motores, cognitives i psiquiàtriques (Glikmann-Johnston, Fink, et al., 2019; Nana et al., 2014; J. P. G. Vonsattel, 2008). Tot i que la identificació de la mutació responsable de la MH es va produir fa 30 anys, encara no està clar per què l'estriat i concretament les neurones espinoses de mida mitjana

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(MSN) són selectivament més vulnerables que d'altres malgrat l'àmplia expressió de la Huntingtina en l'organisme. Gràcies a l'estudi en models de ratolí s'ha posat de manifest que la disfunció neuronal precedeix la mort neuronal (Mangiarini et al., 1996), suggerint que abordar les vies moleculars afectades durant les primeres fases de la malaltia pot ajudar a prevenir frenar la progressió d'aquesta. Donat que a dia d'avui encara no existeixen tractaments específics que frenin la progressió de la malaltia, el present treball es centra en l'estudi de dos mecanismes moleculars i el seu potencial terapèutic: l'estrès del reticle endoplasmàtic (ER) i les alteracions en el secretoma astrocític de la MH.

En primer lloc, l'estrès de reticle és una característica patològica comuna de diverses malalties neurodegeneratives (Hetz & Saxena, 2017), que provoca l'activació de la cascada de senyalització UPR per tal d'assegurar una correcta proteostasis cel·lular (Hetz & Mollereau, 2014; Oakes & Papa, 2015). En el cas de la MH, existeix un augment crònic dels marcadors UPR en models cel·lulars i animals de la malaltia així com en mostres humanes de pacients (Shacham et al., 2019). No obstant, les branques precises de la UPR que es veuen afectades depenent de la regió cerebral i el possible vincle amb la cognició i memòria romanen inexplorats en el context de la MH. De fet, diversos estudis han assenyalat la importància de l'estrès de reticle en la funció sinàptica i la formació de memòria tant en contextos no patològics (N. Sen, 2019) així com en el cas de l'Alzheimer (Lin et al., 2018). Per tant, en aquest primer projecte, hem definit la contribució de l'eix GRP78/PERK, un transductor important de la senyalització d'estrès de reticle, a la neuropatologia estriatal i hipocampal així com a la disfunció de la memòria en la malaltia de Huntington. Per fer-ho, primerament vam demostrar l'activació selectiva d'estrès de reticle a l'hipocamp d'animals R6/1 en etapes pre-simptomàtiques de la malaltia, amb un augment de GRP78 i CHOP, considerats marcadors clàssics de la via. Un cop confirmat i amb l'objectiu de poder estudiar el paper de GRP78 a la neuropatologia de la MH i la seva possible contribució als defectes cognitius, vam generar un model doble mutant creuant animals huntingtonians R6/1 amb animals heterozigots per $GRP78^{+/-}$. Vam avaluar els efectes d'aquesta reducció genètica amb la realització de proves de comportament cognitiu i motor i amb l'avaluació bioquímica i molecular dels teixits cerebrals dels animals un cop finalitzats

aquets. Així, els nostres resultats han demostrat per primer cop que la reducció genètica de GRP78 és capaç de revertir el deteriorament de diverses funcions cognitives en els ratolins R6/1, com és la memòria espacial i de reconeixement a llarg termini així com els defectes de coordinació motora. De manera similar, altres recerques també han demostrat com la reducció dels nivells de GRP78 per tractament amb curcumina millora les deficiències de la memòria espacial en ratolins transgènics ApoE4 utilitzats com a model d'Alzheimer (Kou et al., 2021). En el nostre cas, a nivell neuropatològic, aquestes millores cognitives i motores han anat associades a un augment de la densitat d'espines dendrítiques tant en MSN de l'estriat com en neurones piramidals de la regió CA1 de l'hipocamp. A més, la reducció genètica de GRP78 s'ha acompanyat també d'una reducció en el nombre d'agregats de Huntingtina en ambdues regions.

Amb l'objectiu de poder definir quina via de la UPR podia estar implicada en aquestes millores i l'evidència que l'activació sostinguda de PERK afecta la funció cognitiva (M. Ohno, 2018; Taalab et al., 2018), vam analitzar els nivells dels 3 sensors de la via UPR en els ratolins R6/1. Els resultats indiquen l'activació selectiva de PERK sense canvis significatius en IRE1α o en els nivells d'ATF6. Per tant, ens vam centrar en aquesta branca com a possible mecanisme subjacent a la disfunció cognitiva depenent de l'hipocamp en la MH. Per tal d'estudiar-ho, vam tractar intraventricularment de manera crònica a animals R6/1 amb un inhibidor de PERK conegut amb el nom de GSK2606414 (Axten et al., 2012) a partir de les 12 setmanes, just quan comencen a manifestar-se els símptomes cognitius. Els nostres resultats mostren com gràcies a la inhibició farmacològica de PERK, es poden prevenir els dèficits de memòria espacial i de reconeixement a llarg termini dels animals R6/1 fomentant la millora en la densitat i formació d'espines dendrítiques així com modulant positivament els nivells de gens de resposta immediata (IEG) tals com Arc i c-Fos. És per això, que les dades recopilades en aquest primer treball identifiquen un nou mecanisme involucrat en els dèficits cognitius de la MH basat en la desregulació de l'eix GRP78/PERK que podria utilitzar-se com a nova oportunitat terapèutica en el tractament de la simptomatologia cognitiva de la malaltia.

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La segona part d'aquesta tesis es centra en la comunicació entre neurones i astròcits, que implica l'alliberament de diversos factors que poden afectar ambdós tipus cel·lulars i regular la funció neuronal (Robertson, 2018; Schiera et al., 2020). En contextos patològics com en la MH, hipotetitzem que les alteracions en el secretoma astrocític podrien conduir a vulnerabilitat i disfunció neuronal per una deficient secreció de BDNF, així com per una resposta inflamatòria astrocítica anormal a causa de l'expressió aberrant de la proteïna ARMS/Kidins220.

Malgrat que hi ha pocs estudis sobre ARMS, la proteïna es va descobrir a l'any 2000 (Iglesias et al., 2000) i es coneix que es troba majoritàriament expressada al sistema nerviós modulant processos neuronals de supervivència, diferenciació i plasticitat sinàptica (Neubrand et al., 2012). Estructuralment, ARMS es caracteritza per tenir una gran quantitat de dominis amb capacitat per interaccionar amb altres proteïnes, cosa que la converteix en una perfecta plataforma de senyalització capaç d'associar-se amb molts receptors. Estudis recents suggereixen que les alteracions en ARMS estan associades a diferents patologies tant en diverses formes de càncer (Liao et al., 2007, 2011) com en malalties neurològiques i neuropsiquiàtriques com ara l'Alzheimer (López-Menéndez et al., 2009), el Parkinson (Simunovic et al., 2009), l'autisme (S. W. Kong et al., 2012) o l'esquizofrènia (Kranz et al., 2015). En el context de la malaltia de Huntington, l'any 2018 el nostre grup va definir per primera vegada nivells elevats d'ARMS en models animals de la malaltia així com en mostres humanes i com la normalització d'aquests es traduïa a un augment de la secreció de BDNF (López-Benito et al., 2018). Així, la recerca senyala ARMS com una proteïna crucial en la diferenciació neuronal, la supervivència i la plasticitat, convertint-se en un efector clau de les vies neurotròfiques. No obstant, la funció d'ARMS en les cèl·lules glials no està gaire explorada. Estudis recents han posat de manifest el paper d'ARMS astrocitari en la regulació de les dinàmiques de Ca^{2+} glial, les vies de supervivència/mort i la comunicació astròcit-neurona (Jaudon et al., 2020, 2021), ressaltant ARMS en el creixent nombre de malalties associades a disfuncions astrocítiques. Per tant, en aquest segon projecte hem definit el rol d'ARMS específicament en les alteracions del secretoma astrocític de la MH i avaluat el seu potencial terapèutic com a nova estratègia en el tractament dels dèficits motors observats a la malaltia. Per fer-ho,

primerament vam demostrar l'augment aberrant dels nivells d'ARMS en astròcits huntingtonians estriatals. Donat aquest increment, el següent objectiu va ser determinar si la normalització de l'expressió d'ARMS en astròcits podia afectar els dèficits motors de la malaltia. Mitjançant injeccions estereotàctiques d'AAV2/5-GFAP-shARMS-GFP al nucli estriat de ratolins WT i R6/1, vam demostrar que la supressió d'ARMS en astròcits millora moderadament els dèficits d'aprenentatge motor, coordinació i equilibri en animals R6/1, oferint una nova i prometedora diana terapèutica en el tractament de la MH. Ara bé, per tal d'investigar els mecanismes subjacents de l'impacte positiu de modular ARMS en astròcits, ens vam centrar en la seva possible implicació en la alliberament de BDNF, citocines i quimiocines per part d'aquests. Donada l'evidència científica que relaciona els nivells de BDNF amb efectes protectors de millora neuropatològica i de comportament en models animals de MH (Giralt, Carretán, et al., 2011; Zuccato et al., 2005), els nostres resultats *in vitro* són importants ja que indiquen per primera vegada que l'augment aberrant dels nivells d'ARMS astrocitaris impedeixen la correcta secreció de BDNF en astròcits R6/1. A més, els astròcits activats també poden secretar citocines i quimiocines amb efectes neuroprotectors o neurotòxics, contribuint així a la patogènesi de la MH (Hsiao et al., 2013; Linnerbauer et al., 2020; Rocha et al., 2016; Stephenson et al., 2018). En el nostre cas, la modulació d'ARMS altera la secreció de diverses citocines i quimiocines destacant sobretot la disminució de TNFα i l'augment de CXCL12. En conjunt, els nivells aberrant d'ARMS astrocitari afecten negativament la secreció de BDNF i contribueixen a un perfil inflamatori astrocític atípic que condueix a vulnerabilitat neuronal modificant la comunicació neurona-astròcit i contribuint als dèficits motors de la MH.

En conclusió, els resultats presentats en aquesta tesi han proporcionat un coneixement detallat de la contribució de les proteïnes GRP78, PERK i ARMS en la fisiopatologia de la MH convertint-se en prometedores dianes terapèutiques per tractar la simptomatologia cognitiva i motora. De la mateixa manera, aquestes investigacions posen de manifest la necessitat de comprendre els mecanismes moleculars i cel·lulars de la malaltia, no només des d'una perspectiva neuronal, sinó també considerant el paper dels astròcits. Els nostres resultats posen de relleu la importància de la

comunicació entre neurones i astròcits i suggereixen que una combinació de diferents dianes terapèutiques podria ser necessària per tractar eficaçment la MH.

ABBREVIATIONS

3-NP	3-nitropropionic acid
AAV	Adeno-associated virus
ACM	Astrocyte conditioned medium
AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ANOVA	Analysis of variance
AP	Antero-posterior axis
APP	Amyloid-beta precursor protein
Arc	Activity-regulated cytoskeleton-associated protein
ARMS	Ankyrin-Repeat Rich Membrane Spanning/kinase D-interacting substrate of 220 kDa
ARTP	Accelerating Rotarod Training Procedure
ASK1	Apoptotic signal-regulating kinase 1
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Aβ	Amyloid-beta
BAC	Bacterial artificial chromosome
BDNF	Brain-derived neurotrophic factor
CA1	Cornu ammonis 1
Ca^{2+}	Calcium
CAG	Cytosine, Adenine, Guanine
CBP	CREB Binding Protein
cDNA	Complementary DNA

ABBREVIATIONS

CHOP	C/EBP homologous protein
CN	Caudate nucleus
CNS	Central nervous system
CREB	cAMP-response element-binding protein
CXCL12	Chemokine (C-X-C motif) ligand 12 (Stromal cell-derived factor 1)
CXCR4	C-X-C chemokine receptor type 4
D1R	Dopamine D1-like receptor
D2R	Dopamine D2-like receptor
DARPP-32	Dopamine- cAMP-regulated phosphoprotein of molecular weight 32 kDa
DG	Dentate gyrus
DIV	Days in vitro
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EAAT2	Excitatory amino acid transporter 2
eIF2α	Eukaryotic translation initiation factor 2 α
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
FDA	U.S. Food and Drug Administration
FTD	Frontotemporal Dementia
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate/aspartate transporter

GLT-1	Glutamate transporter-1
GPe	External globus pallidus
GPi	Internal globus pallidus
GRP78	Glucose-regulated protein of 78 kDa
HD	Huntington's Disease
Hdh	Huntington's disease homolog
HIV	Human immunodeficiency syndrome
Htt	Huntingtin
IEG	Immediate early gene
INF-Y	Interferon-gamma
IP3R	Inositol (1,4,5)-triphosphate receptor
IRE1α	inositol-requiring protein 1α
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KI	Knock-in
KO	Knock-out
MAMs	Mitochondria-associated membranes
MAP2	Microtubule-associated protein 2
mHtt	Mutant huntingtin
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
MSN	Medium spiny neuron
NF-κB	Nuclear factor NF-kappa-B

ABBREVIATIONS

NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NOLT	Novel Object Location Test
NORT	Novel Object Recognition Test
NT	Neurotransmitter
P	Putamen
$P75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's Disease
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
PFA	Paraformaldehyde
PMD	Protein misfolding disorder
PolyQ	Polyglutamine
PrD	Prion Disease
PSD-95	Post Synaptic Density 95ynaptic
PSP	Progressive Supranuclear Palsy
PTP	Permeability transition pore
RIDD	Regulated IRE1-dependent decay
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
Rpm	Rotations per minute

RT	Room Temperature
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
S1P	Sphingosine-1-phosphate
S2P	Sphingosine-2-phosphate
SEM	Standard Error of the Mean
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TBS	Tris-buffered Saline
TBS-T	Tris-buffered saline-Tween 20
TGF-β	Transforming growth factor beta
TIMP-1	Metalloproteinase inhibitor 1
TNFα	Tumor necrosis factor α
TrkB	Tropomyosin receptor kinase
TUDCA	Tauroursodeoxycholic acid
UPR	Unfolded protein response
WT	Wild-type
XBP1	X-box binding protein 1
YAC	Yeast artificial chromosome

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INTRODUCTION

1. HUNTINGTON'S DISEASE

1.1. A little bit of history

It was in 1872 when George Huntington described a hereditary type of chorea that ran with mental illness in the *Medical and Surgical Reporter* of Philadelphia (Huntington, 2003). It was certainly not the earliest medical account of it, but it was the most accurate. The "dancing propensities of those affected" gave an alternative name to the disorder, Huntington's chorea (from the ancient Greek *choreia*, meaning dance) ultimately referred to as Huntington's Disease (HD). Indeed, Huntington defined HD's cardinal particularities, including its hereditary nature, a tendency to insanity and suicide, and its late adulthood onset.

1.2. Epidemiology

Worldwide the incidence of HD is heterogeneous even among communities from the same region. Prevalence rates are much lower in populations of non-European ancestry like Africa and Asia ranging from 0.11 to 0.72 cases per 100,000 whereas in Western Europe, North America, and Australia go from 4 to 12 cases every 100,000 (McColgan & Tabrizi, 2018; Rawlins et al., 2016). However, the highest prevalence on earth is found in specific populations that originated from a reduced number of predecessors, such as Tasmania and the area around Lake Maracaibo in Venezuela where the prevalence is about 700 cases per 100,000 (Okun & Thommi, 2004).

1.3. Etiology

Nancy Wexler and James Gusella selected Lake Maracaibo as their research site for HD in 1979. It was there that, four years later in 1983, they pinpointed the genetic locus of the disease (Gusella et al., 1983). They discovered that the cause of HD was located on the short arm of chromosome 4, specifically in region 4p16.3. However, it took another decade before the exact genetic cause of HD was identified. In 1993, MacDonald et al. revealed that an abnormal expansion of the trinucleotide repeat cysteine, adenosine,

and guanine $(CAG)_n$ in the exon 1 of the *Huntingtin* gene (*IT15*) was responsible for the disease and affected the age of onset (MacDonald et al., 1993) (Figure 1). The gene spans 180 kb containing 67 exons (Ambrose et al., 1994) and the CAG expansion codifies for a polyglutamine (polyQ) stretch in the Huntingtin protein altering its biological functions when it exceeds 36 repeats. That year, the *Htt* mouse homolog was mapped to the murine chromosome 5 (*Hdh*) opening new possibilities for HD research (Barnes et al., 1994; Nasir et al., 1994).

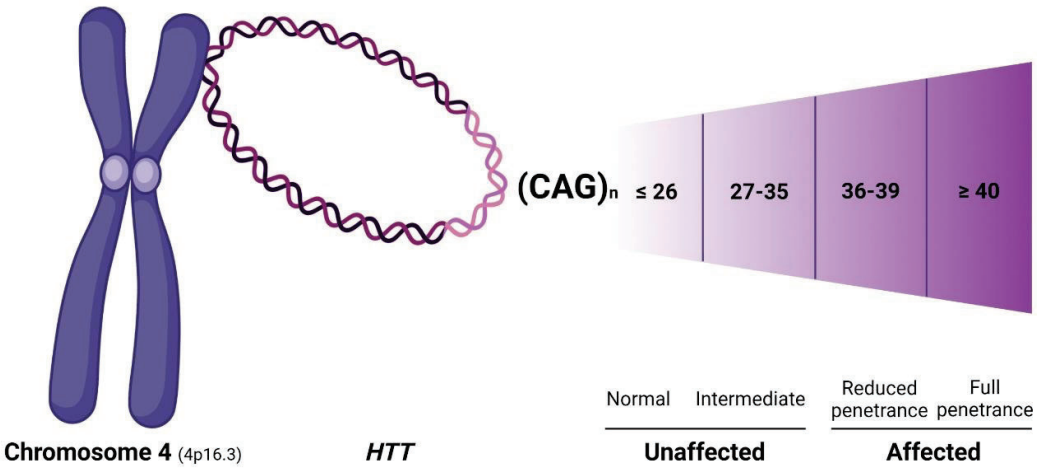


Figure 1. Huntington's disease mutation. Localization of the *HTT* gene at human chromosome 4. Within its exon 1, a CAG repeat exists that is unstable. The number of CAG repeats in an individual's *Huntingtin* gene can determine their classification as either unaffected or affected. Specifically, 36 repeats or more lead to the manifestation of the disease. However, affected individuals may exhibit varying penetrance levels based on the expansion length. Some may experience reduced penetrance, wherein clinical signs are absent or mild, while others may experience full penetrance, resulting in the inevitable manifestation of Huntington's disease symptoms.

In normal conditions, non-HD subjects present less than 36 CAG repeats while full penetrance of the disease is found in individuals carrying 40 repeats or more (Kremer et al., 1994; Nance, 1998). Those with 36-39 repeats show reduced penetrance meaning a delayed onset of symptoms (Tabrizi et al., 2020; Walker, 2007). Interestingly, the age of onset is inversely correlated with the number of CAG repeats (Andrew et al., 1993; M. Duyao et al., 1993; Rubinsztein et al., 1996). Thus, individuals with 50 CAG repeats or more will develop the most aggressive form of the disease called juvenile HD which usually starts at the age of 20 and represents 5% of all cases (Cronin et al., 2019; Gordon, 2013). Finally, although HD is an autosomal dominant disorder,

intermediate alleles (IA: 27 to 35 CAG repeats) exist and although they were thought to be non-pathogenic, these individuals might experience signs of chorea and cognitive impairment (Squitieri & Jankovic, 2012). Besides, they may pass on the mutation to their offspring due to the gametic meiotic instability of the CAG track (Kelly et al., 1999) leading to longer CAG repeats each generation, which is termed genetic anticipation. This is more pronounced in the male germline as CAG instability is higher during spermatogenesis (Telenius et al., 1994; Wheeler et al., 2007). Therefore, *de novo* HD cases are rare and thought to be a consequence of intergenerational instability of an intermediate allele (M. Duyao et al., 1993; Goldberg et al., 1993).

1.4. Clinical aspects

HD is classically considered a motor disorder, yet patients exhibit a triad of motor, cognitive, and psychiatric symptoms that take place at different times and can vary in degree during the premanifest and manifest stages of the disease (Ross & Tabrizi, 2011) (Figure 2). These two stages are distinguished by a positive genetic test and the onset of motor disturbances, which is considered the clinical onset of the disease (McColgan & Tabrizi, 2018). During the pre-symptomatic stage of the disease, affected individuals do not present symptomatology, while in the prodromal phase, subtle motor, cognitive, and psychiatric symptoms appear and can be present many years before the clinical onset of the disease (G. P. Bates et al., 2015; Walker, 2007). Regarding motor disturbances, adult-onset HD is first characterized by choreic and dyskinetic movements affecting the limbs, trunk, and face that eventually morph into bradykinesia, impairing voluntary movements together with incoordination, dystonia, and rigidity (Novak & Tabrizi, 2010; Young et al., 1986). Secondly, cognitive symptoms take place up to 20 years before the onset of motor dysfunction and gradually worsen until coexisting with motor symptoms (Paulsen et al., 2008). These are related to executive functions including alterations in attention and emotion recognition, working and spatial memory impairment, and deficits in language comprehension and verbal fluency which gradually give rise to subcortical dementia (Begeti et al., 2016; Giralt, Saavedra, et al., 2012a; Paulsen et al., 2017). Finally, psychiatric symptoms are very

frequent and often cause the most distress to HD carriers. The most common symptom is major depression together with apathy and anxiety (Duff et al., 2007; Julien et al., 2007; Paulsen, 2005). Patients can also develop obsessive-compulsive behaviors, irritability, and suicidal thoughts (Paulsen et al., 2001; van Duijn et al., 2014) being suicide rates higher in HD patients compared to the general population (Wetzel et al., 2011). HD patients can also exhibit other lesser-known clinical manifestations including changes in sexual behavior and circadian rhythms, and even non-brain related symptoms such as unintended weight loss, heat, and cold intolerance, sialorrhea, and urinary difficulties (Walker, 2007). At the end of this difficult journey 15-20 years after the age of onset, the main cause of death in HD patients is aspiration pneumonia, as a complication of dysphagia, followed by suicide (Heemskerk & Roos, 2012; Sørensen & Fenger, 1992).

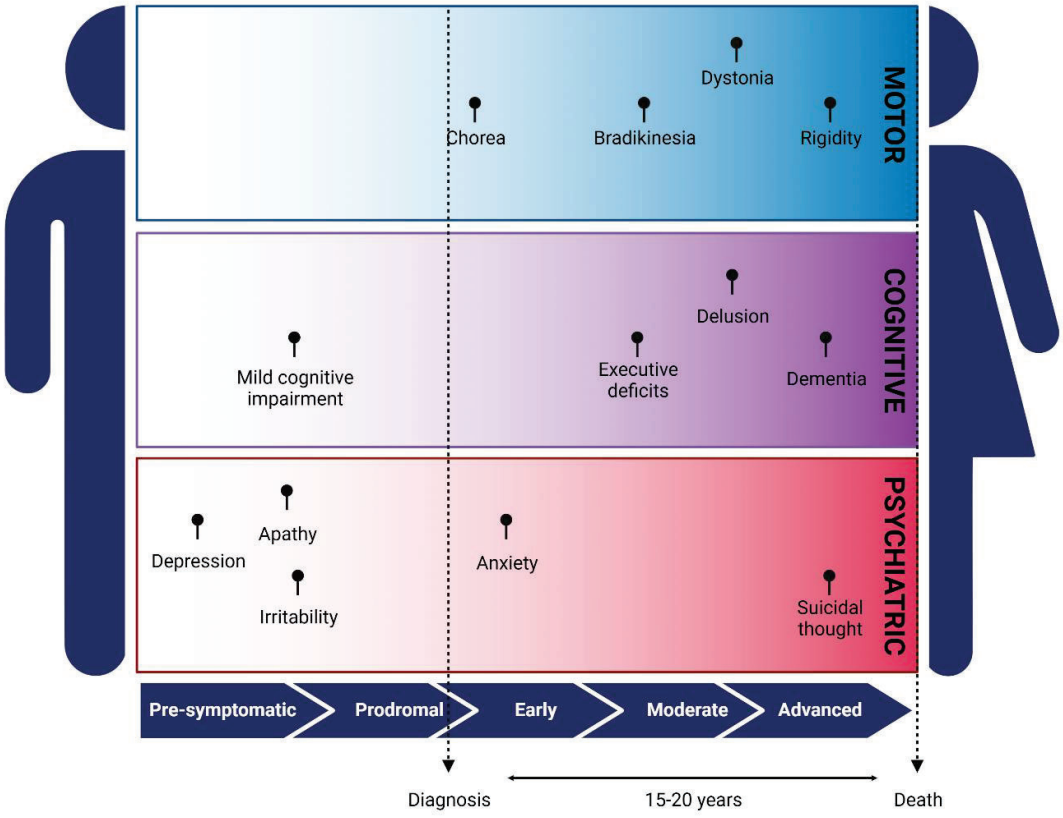


Figure 2. Clinical progression of HD symptomatology. A schematic representation is presented to illustrate the principal changes in motor, cognitive, and psychiatric aspects that occur as Huntington's disease progresses in patients. As time passes, functional capabilities decline, and motor deficiencies become increasingly severe.

1.5. Huntingtin protein

The product of the *HTT* gene is a large protein of around 350 kDa containing 3,144 amino acids with an expandable polyglutamine (polyQ) stretch located at the N-terminal fragment. HTT is ubiquitous and it is found differently expressed throughout the body with the highest levels in the brain and testis (DiFiglia et al., 1995; Trottier et al., 1995). Within the brain, HTT is most expressed in the striate nucleus, cerebellum, cortex, and hippocampus (Borrell-Pagès et al., 2006).

Huntingtin's conformation is dynamic depending on its subcellular location within the cell (Kegel et al., 2005). Originally, it was thought to be a predominantly cytoplasmic protein associated with vesicles (DiFiglia et al., 1995), but later studies soon proved its presence in the nucleus (Kegel et al., 2002), plasma membrane (Kegel et al., 2005), microtubules (Hoffner et al., 2002), the Golgi complex and ER (Rockabrand et al., 2007) and mitochondria (Borrell-Pagès et al., 2006). According to this broad subcellular distribution and given that HTT is a multi-domain protein, it can interact with many partners participating in numerous cellular functions (Tourette et al., 2014). Most of the interactors are related to cellular dynamics, metabolism, protein turnover, and gene expression. Therefore, it is not shocking that HTT plays a key role in vesicle trafficking (Caviston et al., 2007), endocytosis, cell division (Elias et al., 2014), autophagy (Martin et al., 2014), and transcription (Valor, 2015) reviewed in (Saudou & Humbert, 2016). All of these functions primarily influence embryonic development, tissue maintenance, and cell morphology and survival.

As a result of the expanded PolyQ stretch in the N-terminal fragment, mutant HTT (mHTT) folds abnormally resulting in soluble monomers that combine to form oligomers, gradually increasing in size, and eventually forming large inclusions or aggregates that accumulate in the nucleus and cytoplasm (J. K. Cooper et al., 1998; Hoffher et al., 2005) (Figure 3). Whether their role is pathogenic or protective remains under debate. Inclusions are thought to be vital in the pathogenesis of HD, hindering normal HTT function (Becher et al., 1998), however, more recent studies suggest that oligomeric mHTT is a more toxic form and that accumulation into aggregates could be a protective mechanism (Arrasate et al., 2004; Herrera et al., 2011).

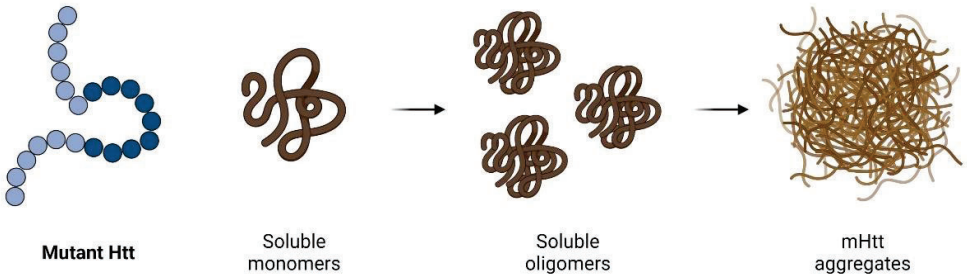


Figure 3. Generation of mutant Htt aggregates. The expansion of CAG causes misfolding of mutant Htt (mHtt), leading to the formation of soluble monomers that combine to form oligomers, eventually resulting in insoluble aggregates. The impact of mHtt aggregates is a topic of debate since they have been shown to have both pathologic and protective effects.

Given the crucial roles of HTT within the cell, it has been suggested that a loss of HTT function contributes to HD pathology. Adult mice with *Htt* expression knocked out (KO) using a Cre/LoxP system exhibit progressive neurodegeneration, lending support to this hypothesis (Dragatsis et al., 2000). Additionally, it has been demonstrated in recent years that loss of WT *Htt* expression in HD cellular and mouse models recapitulates the behavioral changes and striatal synaptic defects seen in HD, which can be corrected when WT *Htt* is re-expressed (Burrus et al., 2020; McAdam et al., 2020; Migazzi et al., 2021). However, because complete ablation of *Htt* in mice results in embryonic lethality at E7.5 (M. P. Duyao et al., 1995; Zeitlin et al., 1995), the neurotoxic effects of HTT aggregates (Paine, 2015) and its dominant inheritance, the consensus is that HD is mainly caused by a toxic gain-of-function mutation (G. P. Bates et al., 2015; Tabrizi et al., 2020).

1.6. Neuropathology

The most remarkable neuropathological feature of HD is the bilateral atrophy of the striatum (caudate and putamen nuclei) accompanied by neuronal loss and astrocytic gliosis leading to an enlargement of the lateral ventricles (J. P. Vonsattel et al., 1985). Striatal atrophy develops well before the onset of symptoms, with a 50% reduction in striatal volume at the time of diagnosis (Aylward et al., 1994, 2004). Striatal degeneration follows an ordered progression starting caudally and progressing from a dorsomedial to a ventrolateral direction (Reiner et al., 2011; Roos et al., 1985). Because of this, neuropathologist Jean Paul Vonsattel created a universally accepted grading

system ranging from 0 to 4 that ranks severity based on macroscopic and microscopic criteria (Figure 4). Grade 0 includes those brains with no significant macroscopic alterations although there is a loss of 30-40% of the caudate neurons. In grade 1, the caudate shows 50% of neuronal loss together with moderate astrogliosis. In grades 2 and 3, atrophy, neuronal loss, and astrogliosis are increasingly more pronounced until grade 4 when the striatum presents up to 95% of neuronal loss and the ventricular surface of the caudate nucleus has transformed from convex to concave (Reiner et al., 2011; J. P. Vonsattel et al., 1985a; J. P. Vonsattel & DiFiglia, 1998; J. P. G. Vonsattel, 2008).

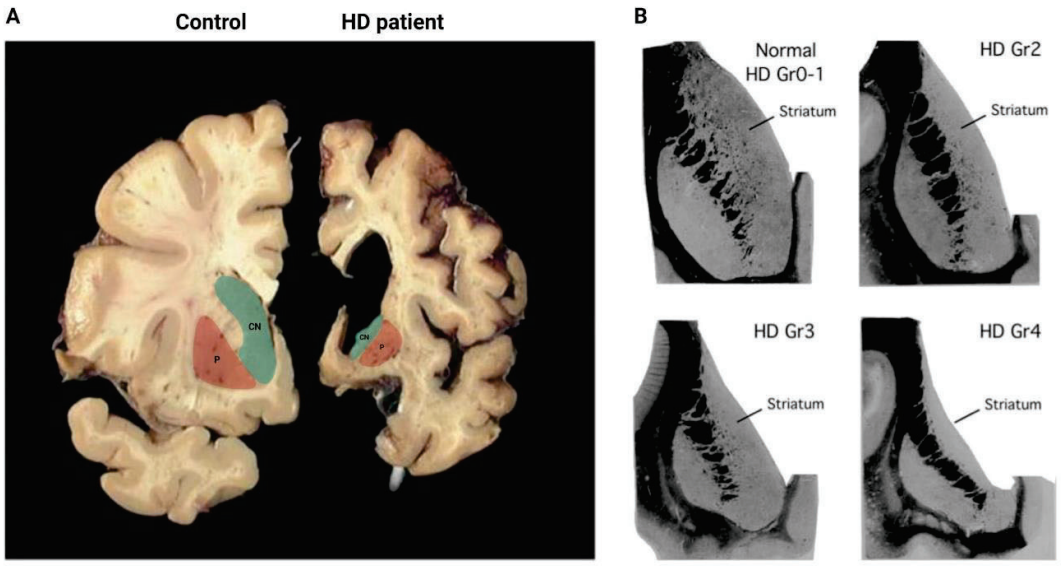


Figure 4. Macroscopic HD neuropathology. (A) Coronal brain sections of a human control case (left) and a Grade 3/4 HD patient (right) are presented. The caudate nucleus (CN) and putamen (P) show significant atrophy as indicated. Cortical atrophy and lateral ventricle enlargement are also observable. (B) Schematic diagrams depicting the Vonsattel grading system for striatal neurodegeneration are shown, where the caudate nucleus surface alteration can be appreciated. See the text for more information. Modified from (Reiner et al., 2011).

Besides the striatum, HD also affects other brain regions, including the cerebral cortex (layers III, V, and VI), hippocampus, thalamus, globus pallidus, subthalamic nucleus, substantia nigra, white matter, and cerebellum (Nana et al., 2014; J. P. G. Vonsattel, 2008; Walker, 2007). The involvement of so many brain structures helps to better understand the many different clinical presentations of the disease and correlates with the motor, cognitive and psychiatric alterations (McColgan & Tabrizi, 2018; Mehrabi et

al., 2016). In this Thesis, we will focus on the striatum and hippocampus; two of the most important brain structures that contribute to HD-related behavioral impairments such as motor coordination and learning, and cognition.

1.6.1. Striatal pathology

GABAergic medium-sized spiny neurons (MSN) account for 90-95% of the total neurons within the striatum (Dubé et al., 1988; Gerfen, 1988) and are the main cell type that degenerates in HD affecting the connectivity and functionality of the basal ganglia, the hub of movement control (reviewed in (Cepeda et al., 2007; Morigaki & Goto, 2017)). Basal ganglia include the striatum, the internal and external *globus pallidus* (GPi and GPe), the subthalamic nucleus (STN), and *substantia nigra pars compacta* (SNc) and *reticulata* (SNr). In this complex circuit, MSNs can be divided into two subpopulations depending on the brain areas they project and the neuropeptides they express, forming two important inhibitory pathways: the direct and the indirect pathway (Kawaguchi et al., 1990). MSNs from the indirect pathway express the dopamine D2 receptor and project to the *globus pallidus pars externa* (Aizman et al., 2000; Steiner & Gerfen, 1999; Surmeier et al., 2007). In turn, MSNs from the direct pathway express the dopamine D1 receptor and project to the *globus pallidus pars interna* and *substantia nigra reticulata* (Gerfen et al., 1990). Eventually, the coordination of these two pathways is essential to maintain a correct balance of cortical excitation to generate voluntary movement. While the direct pathway activates the cortex, the indirect pathway has the complete opposite effect. In HD, MSNs from the indirect pathway are the first to degenerate which leads to a disinhibition of the thalamus and, consequently an overactivation of the cortex that explains the hyperkinetic symptomatology that HD patients experience during the first stages of the disease (Reiner et al., 1988; Richfield et al., 1995). With disease progression, MSNs from the direct pathway also degenerate, resulting in an over-inhibition of the thalamus and cortex, which explains the hypokinetic movements (Y. P. Deng et al., 2004).

1.6.2. Hippocampal pathology

The hippocampus is a vital structure of the limbic system that is considered essential for acquiring and consolidating various forms of memory and spatial navigation and participates in regulating emotions, anxiety, stress, or depression (Bartsch & Wulff, 2015; Manns et al., 2003). This region has a remarkable ability for structural reorganization, altering the dendritic branching and number of spines of preexisting neural connections and establishing new ones through neurogenesis to support essential functions such as learning and memory, and regulation of anxiety and stress (Leuner & Gould, 2010). The hippocampus typically consists of two main regions: the Cornu Ammonis (CA), which is further divided into various subregions and is packed with pyramidal neurons, and the dentate gyrus (DG) which contains mostly granular neurons (W. Deng et al., 2010; Freund & Buzsáki, 1996; Klausberger, 2009). Although gross hippocampal volumes do not differ significantly between HD and control groups like striatal volumes do (Glikmann-Johnston et al., 2021), evidence points towards a large-scale brain network alteration that leads to HD hippocampal-dependent memory deficits (Glikmann-Johnston, Carmichael, et al., 2019; Glikmann-Johnston et al., 2021; Glikmann-Johnston, Fink, et al., 2019). Importantly, several studies have found that HD patients exhibit alterations in learning and memory tasks including recognition and spatial working memory (Glikmann-Johnston, Fink, et al., 2019; Paulsen et al., 2013) that correlate with estimated years to diagnosis, indicating that hippocampal dysfunction is an important and early contributor to HD cognitive decline (Begeti et al., 2016) and may act as a marker of HD cognitive deterioration (Harris et al., 2019). Indeed, some studies have provided evidence of a 35% neuronal loss specifically in the CA1 region of HD brains already showing massive atrophy in the striatum (Spargo et al., 1993) supporting the idea that beyond the atrophy of the striatum and cortex, a more general neurodegenerative process that involves the hippocampus could contribute to HD memory impairment.

Accordingly, evidence of early hippocampal affectation also comes from studies conducted on genetic mice models. Impaired hippocampal-dependent functions like navigation and spatial and recognition memories are compromised in most HD models before the onset of motor symptoms (Alvarez-Periel et al., 2018; Brito, Giralt, Enriquez-

Barreto, Puigdellívol, Suelves, Zamora-moratalla, et al., 2014; Carter et al., 1999; Giralt, Puigdellívol, et al., 2012; Giralt, Saavedra, et al., 2011, 2012a; Miguez et al., 2015; Pupak et al., 2022). Besides, these are accompanied by the presence of structural and functional synaptic changes as well as biochemical and transcriptional abnormalities preceding neuronal loss.

Overall, although the striatum is the most affected brain region in HD, the neuropathology extends well beyond to other areas, such as the hippocampus, cortex, and cerebellum. The involvement of these structures is crucial for understanding the diverse symptomatology of HD. In this Thesis, we will explore cellular mechanisms underlying both striatal and hippocampal pathology. While the former may correlate with motor symptoms, the latter is also particularly significant as cognitive deterioration appears before the onset of motor symptoms and causes the most distress to HD patients.

1.7. Huntington's disease models

The study of human *postmortem* samples is key to defining HD neuropathology but gives little insight into the cellular and molecular mechanisms responsible for neuronal dysfunction and degeneration. Before the discovery of the causative HD mutation in 1993 (MacDonald et al., 1993), HD studies were performed in murine models based on chemically induced lesions in the striatum using excitotoxic glutamate analogs such as kainic (Coyle & Schwarcz, 1976) or quinolinic acid (Beal et al., 1986) or the mitochondrial toxin 3-nitropropionic acid (3-NP) (Borlongan et al., 1995). The disadvantage of these neurotoxin-based models is that they neither replicate the slow progression of the disease nor its main pathological feature, the presence of mHtt aggregates. Therefore, genetically engineered animal models of HD have had a tremendous role in increasing our understanding of HD pathogenesis and have granted us the opportunity to test therapeutic compounds. Over the years, the disease has been modeled in several species including mice (Mangiarini et al., 1996), rats (von Hörsten et al., 2003), worms (Faber et al., 1999), flies (Jackson et al., 1998), fish (Best & Alderton, 2008), minipigs (Yan et al., 2018), sheep (Jacobsen et al., 2010), and non-

human primates (S. H. Yang et al., 2008) yet among all of them, rodents are the most commonly used species. Based on the generation strategies, these rodent models can be divided into 3 different categories: the fragmented transgenic models, the full-length models, and the knock-in models.

1.7.1. Fragmented transgenic models

N-terminal transgenic mice express the 5' N-terminal fragment of the human m*HTT* gene including the exon 1 with a specific number of CAG repeats randomly inserted into the mouse genome. These models include the R6 and the N171-82Q lines and have proved that the expression of exon 1 is enough to trigger behavioral and neuropathological alterations. R6 mice were the first transgenic HD mouse models ever generated and have the most aggressive phenotype with the fastest disease course (Mangiarini et al., 1996). Depending on the number of CAG repeats we can distinguish between the R6/1 and the R6/2 lines that express 116 and 144 CAG repeats respectively. Besides, R6/1 mice only express one copy of the transgene whereas the R6/2 express three (Mangiarini et al., 1996) which relates to a significant lifespan decrease. Both R6/1 and R6/2 models show an early onset of symptoms characterized by the appearance of cognitive dysfunction before motor impairment. Behavioral phenotype is accompanied by neuronal atrophy, enlargement of the lateral ventricles, and progressive increase in mHtt aggregation (reviewed in (Puigdellívol et al., 2016; Switonski et al., 2012)). However, due to differences in CAG repeat length, R6/1 and R6/2 mice show different temporal patterns of HD pathology. The main weakness of these genetic mouse models is that do not exhibit neuronal death, atrophy is more related to neuronal dysfunction, and their fast disease progression makes them unfit to study the earliest HD symptoms (Francelle et al., 2014).

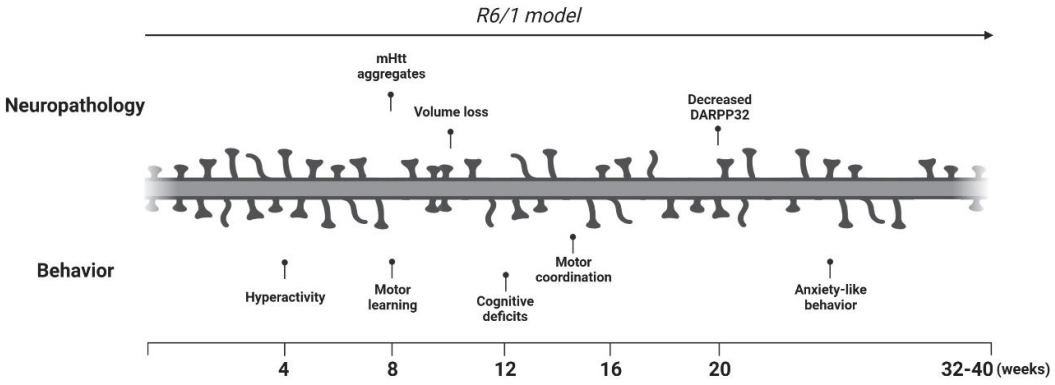


Figure 5. Timeline of the behavioral and neuropathological features of the R6/1 mouse model used in this thesis. An illustration is provided to depict the neuropathologic changes accompanied by motor, psychiatric, and cognitive alterations exhibited by the R6/1 mouse model over the course of weeks. See the main text for references.

N171-82Q mice express the N-terminal fragment of the human HTT protein with 171 amino acids and 82 glutamines under the regulation of the mouse prion promoter (*Prp*) limiting the expression to neurons (Schilling, 1999). These mice develop neuropathological and behavioral phenotypes like those in R6 mice, along with some cell death in the cortex and striatum. In contrast, since they have fewer CAG repeats than R6, the onset of symptoms is later (Yu et al., 2003).

1.7.2. Full-length transgenic models

Full-length models carry the full-length human *HTT* gene containing expanded CAG triplets and all introns, exons, and regulatory elements in either a yeast (YAC) or a bacterial artificial chromosome (BAC). Therefore, mHtt shows a more accurate tissue and temporal gene expression than the fragmented transgenic models. These mice have a longer disease course and disease phenotypes appear gradually over months with a relatively normal survival rate. There are several YAC lines depending on the CAG length (48, 72, and 128) but the best-studied YAC-derived mouse model is YAC128, which expresses the complete m*HTT* gene with 128 CAG repeats (Hodgson et al., 1999; Slow et al., 2003). On the other hand, the BACHD mouse model expresses the full-length human m*HTT* gene with 97 CAG triplets (Gray et al., 2008). In addition to the motor and cognitive impairment, both lines show a selective mild neuronal loss in the

striatum and cortex, making it an attractive model to study mechanisms involved in cell death (Gray et al., 2008; Slow et al., 2003). However, since YAC mice have increased body weight, the main disadvantage is the inability to study HD-related metabolic alterations (van Raamsdonk et al., 2007).

1.7.3. Knock-in models

In transgenic mice, the gene of interest is randomly inserted into the genome which can affect the activity of other genes. In addition, overexpression of the transgene can lead to artefactual results. To overcome these disadvantages, knock-in HD mice (Hdh^{Q}) were generated by inserting the human *HTT* exon 1 with CAG lengths ranging from 50 to 200 into the endogenous mouse *Htt* gene (Wheeler et al., 1999; White et al., 1997). These models can be homozygous or heterozygous with a wild-type *Htt* allele and a CAG-expanded allele. KI models are generally considered to be the most faithful to human disease because mutated *Htt* is placed in the appropriate context within the mouse genome and expression is controlled by the endogenous *Htt* promoter (Menalled & Brunner, 2014; Pouladi et al., 2013). Compared to truncated HD models, these mice exhibit a slower disease progression and are particularly suitable for studying the early stages of HD. Our research group has demonstrated that $Hdh^{Q7/Q111}$ mice display depressive-like behavior at 2 months (Brito et al., 2019), impaired long-term memory at 6 months, and motor symptoms at 8 months old (Brito et al., 2019; Brito, Giralt, Enriquez-Barreto, Puigdellívol, Suelves, Zamora-moratalla, et al., 2014; Puigdellívol et al., 2015). However, neuropathology in knock-in HD mice is typically observed at an advanced age, with striatal intranuclear inclusions at 6 months and hippocampal inclusions with reduced spine density at 8 months of age (Brito, Giralt, Enriquez-Barreto, Puigdellívol, Suelves, Zamora-moratalla, et al., 2014; Menalled et al., 2009; Puigdellívol et al., 2015; Suelves et al., 2019).

2. FROM MOLECULAR PATHOGENIC MECHANISMS TO POTENTIAL THERAPEUTIC STRATEGIES IN HD.

2023 marks 30 years since the discovery of the mutation responsible for HD (MacDonald et al., 1993). However, why the striatum, and MSNs in particular, are selectively vulnerable despite widespread expression of HTT in the brain and body and its involvement in multiple cellular pathways remains unclear (Ross & Tabrizi, 2011). Studying HD mouse models has helped us to understand some of the cellular and molecular mechanisms affected by mHTT and that neuronal dysfunction precedes neuronal death. This is significant because by addressing the pathways affected in the early stages of the disease, it would be possible to prevent or slow down disease progression. Some of these pathways are cell-autonomous processes and others involve interactions with other cell types. For instance, it has already been described the disruption of cortical delivery of the neurotrophic factor BDNF to the striatum (Baquet et al., 2004; Gauthier et al., 2004), excitotoxicity due to increased glutamate release from cortical afferents (Fan and Raymond, 2007), decreased glutamate uptake by astroglia (Estrada-Sánchez & Rebec, 2012; B. R. Miller et al., 2012), mitochondrial dysfunction (Cherubini et al., 2015, 2020), altered synaptic transmission, impairment of vesicle transport, transcriptional dysfunction, changes in calcium homeostasis, endoplasmic reticulum stress, autophagy deficits, and proteasomal impairments (G. P. Bates et al., 2015; Ross & Tabrizi, 2011; Zuccato et al., 2010). However, the impact of each proposed molecular mechanism may vary depending on the brain region, leading to a distinct set of signs and clinical manifestations in patients with HD. This Thesis is centered around the hippocampus and striatum and aims to identify underlying mechanisms that can explain the vulnerability of these areas in HD. Our goal is to test potential new therapeutic strategies that can alleviate both motor and cognitive symptoms of HD. Specifically, we have focused on two molecular mechanisms: ER stress and alterations in the astrocytic HD secretome. The first part of the Thesis investigates ER stress in hippocampal neurons and its potential novel link to HD cognitive deficits. In the second part, we study the role of the protein ARMS in the

astrocytic-secretome alterations in the striatum and its possible novel contribution to HD motor deficits. By doing so, we hope to emphasize the significance of identifying multiple targets to effectively treat HD and move beyond the current *neurocentric* perspective that dominates HD research.

2.1. CONTRIBUTION OF THE ER STRESS TO HD PATHOLOGY

2.1.1. Misfolding and protein aggregation

Neurodegenerative diseases are progressive conditions characterized by the loss of function of neurons and glial cells in specific areas of the nervous system. They include Alzheimer's, Parkinson's, ALS, Huntington's, prion disorders, retinitis pigmentosa, and some myelin disorders, each with unique pathophysiological and clinical characteristics. However, they share a common archetypical feature: abnormal accumulation of misfolded proteins (Aguzzi & O'Connor, 2010; Bertram & Tanzi, 2005; Soto, 2003). Therefore, these diseases are also called proteinopathies or protein misfolding disorders (PMDs) as the presence of a specific misfolded protein causes the disease (Taylor et al., 2002). Normally, endoplasmic reticulum (ER) and cytosolic chaperones help newly synthesized proteins to fold properly, while misfolded proteins can be degraded through the proteasome, lysosome, or autophagy pathways, maintaining protein homeostasis (proteostasis) (Balch et al., 2008; Kaushik & Cuervo, 2015). However, in PMDs, this process becomes defective due to the accumulation of misfolded proteins, leading to ER stress, that triggers the unfolded protein response (UPR) to restore proteostasis (Hetz & Mollereau, 2014; Oakes & Papa, 2015; M. Wang & Kaufman, 2016).

2.1.2. ER stress and the UPR response

The ER is crucial for maintaining the proteostasis network by controlling the folding of newly synthesized secretory proteins (Matus et al., 2008; Roussel et al., 2013; M. Wang & Kaufman, 2016). After undergoing quality control, properly folded proteins are transported to their final destinations, such as the ER itself, the Golgi apparatus, lysosomes, the endosomal system, the plasma membrane, or the extracellular space, with the assistance of chaperones, enzymes, foldases, and cofactors involved in post-translational modifications (Braakman & Bulleid, 2011). Any disturbances to this process can result in ER stress, which activates the UPR. The UPR regulates RNA stability, protein synthesis, and the transcription of genes involved in various aspects of the secretory pathway, including folding, degradation, vesicular trafficking, autophagy, redox control, amino acid metabolism, and lipid synthesis (Bernales et al., 2006; Chow et al., 2015; Hetz et al., 2015). The UPR is a signal transduction pathway consisting of stress sensors at the ER membrane and downstream transcription factors that can either mitigate stress or induce proapoptotic programs depending on the ER's protein folding status. The three main transmembrane proteins that initiate the UPR are inositol-requiring protein 1α (IRE1α) (Tirasophon et al., 1998), activating transcription factor 6 (ATF6) (Haze et al., 1999), and protein kinase RNA-like ER kinase (PERK) (Harding et al., 1999; Walter & Ron, 2011).

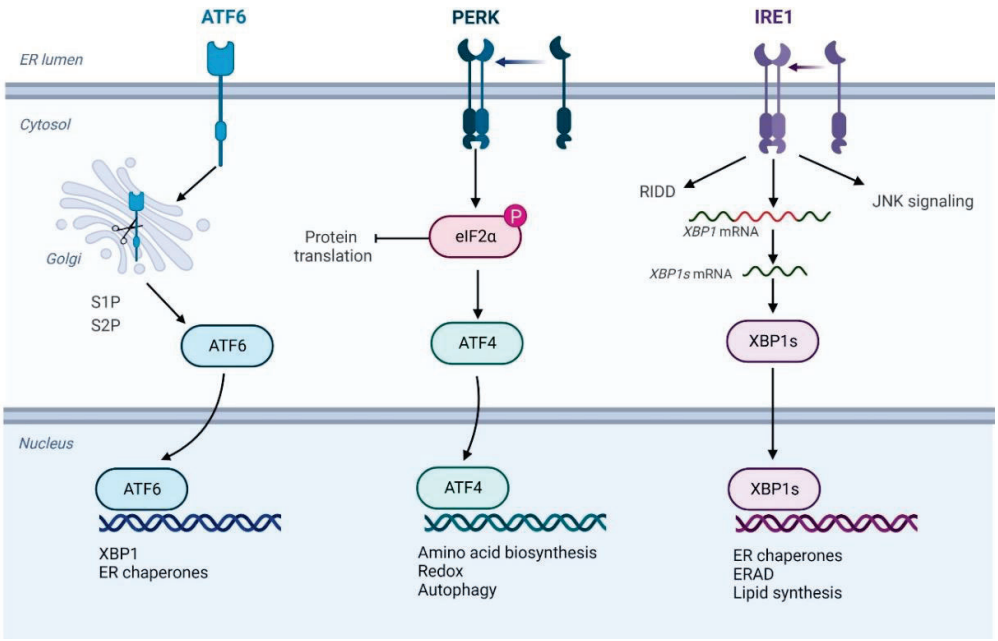


Figure 6. The three arms of the UPR. All three ER stress sensors (PERK, IRE1α, and ATF6) are initially activated in the ER membrane, leading to signaling events that restore protein-folding homeostasis and promote cell survival. PERK phosphorylates eIF2α, which globally shuts down translation while inducing the expression of ATF4, a transcription factor that upregulates select genes involved in restoring proteostasis and CHOP. IRE1α regulates gene expression at the transcriptional and post-transcriptional levels via its RNase activity, which splices XBP1 mRNA or degrades RNAs (RIDD activity). ATF6 is transported from the ER to the Golgi complex, where it is cleaved by S1P and S2P proteases, releasing its cytosolic domain, which acts as a potent transcription factor. Together, UPR transcription factors regulate distinct subsets of target genes involved in restoring ER homeostasis or inducing apoptosis, determining cell fate.

Inositol-requiring protein 1

The IRE1α and XBP1 pathway initiate the more conserved adaptive response of the UPR. When activated, IRE1α dimerizes and autotransphosphorylates, acting as a Serine/Threonine protein kinase and endoribonuclease, splicing the mRNA for XBP1 to produce the stable protein XBP1s, which travels to the nucleus and regulates the expression of UPR-related genes (Calfon et al., 2002; K. Lee et al., 2002; Yoshida et al., 2001). XBP1s has been linked to cell survival by inducing the expression of genes involved in protein folding and quality control, and by activating the ER-associated protein degradation (ERAD). IRE1α also regulates the expression of multiple mRNA and microRNA targets, which can have various effects including stress mitigation, inflammation, and apoptosis (Maurel et al., 2014). IRE1α also has the ability to interact with other stress pathways, including MAP kinase, autophagy, and Nuclear factor κB-mediated inflammation (Hetz & Glimcher, 2009).

Activating transcription factor 6

The ATF6 protein is made up of two isoforms, α and β, that are widely present in the ER. When there is stress in the ER, ATF6 is transported from the ER to the Golgi, where it is processed by two proteases called S1P and S2P creating a fragment called ATF6f that travels to the nucleus and functions as a transcription factor, activating the expression of XBP1, and genes related to ERAD and protein folding (X. Chen et al., 2002; Haze et al., 1999; Yamamoto et al., 2007). Together with XBP1s, ATF6f helps regulate gene expression during stress (A.-H. Lee et al., 2003; Yoshida et al., 2001).

Protein kinase RNA-like ER kinase

Activation of PERK results in the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), reducing protein synthesis and decreasing the accumulation of unfolded proteins in the ER (Harding et al., 2000; Ron & Walter, 2007; Scheuner et al., 2001; Schröder & Kaufman, 2005). This results in the selective translation of the transcription factor ATF4, which is necessary for the increase in gene expression of proteins involved in redox regulation, amino acid metabolism, autophagy, and protein folding and synthesis (Cullinan & Diehl, 2004; Harding et al., 2003; Rutkowski et al., 2006; Ye & Koumenis, 2009). In cells with irreversible damage, ATF4 can also trigger cell death pathways through the induction of the transcription factor DDIT3 commonly known as CHOP, reactive oxygen species, and members of the BCL2 apoptosis regulator family. Based in these functions, PERK is being explored as a potential target for the treatment of neurodegenerative diseases including HD, AD, TBI and others (Shacham et al., 2021).

2.1.3. Chronic ER stress and apoptosis

The UPR activation has both adaptive and apoptotic impacts on the cell's fate. In response to an imbalance in ER homeostasis, the first step is to reduce the influx of newly synthesized proteins into the ER through global translation reduction. This is achieved through eIF2α phosphorylation by PERK and mRNA degradation by regulated IRE1-dependent decay of mRNA (RIDD) (Reid et al., 2014). The ER also increases the removal of misfolded proteins, which can either be achieved by ERAD and autophagy or by the lysosomal degradation of GPI-anchored proteins at the plasma membrane (Ogata et al., 2006; Satpute-Krishnan et al., 2014; Senft & Ronai, 2015). Additionally, the ER activates amino acid metabolism and antioxidant responses, along with improving folding and quality control mechanisms to re-establish homeostasis (Schröder & Kaufman, 2005). If the UPR fails to restore ER proteostasis, it switches to a terminal UPR and activates a program of pro-apoptotic signals that lead to cell death (Hetz & Papa, 2018; Riccardi et al., 2015) (Figure 7).

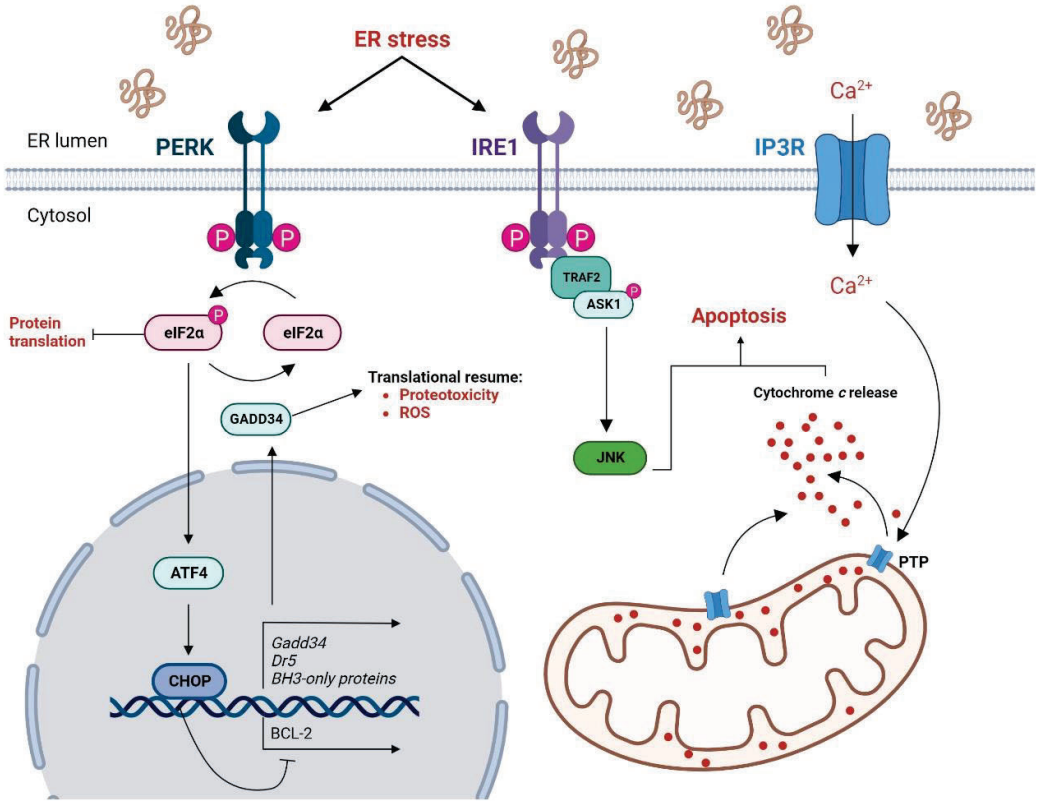


Figure 7. Sustained ER stress and induction of apoptosis. In cases of irreversible ER stress, the UPR actively promotes proteotoxicity, inflammatory responses, and apoptosis through multiple signaling outputs from both PERK and IRE1α pathways. Downstream of PERK, ATF4 targets also include genes involved in the control of cell death, such as DR5 or BH3-only proteins. IRE1α, on the other hand, signals through the JNK pathway. Additionally, ER calcium release plays a role in ER stress-mediated cell death, potentially involving IP3 receptors. Overloading of calcium leads to the activation of the permeability transition pore (PTP), triggering the release of cytochrome c and downstream caspase activation.

One of these signals is the sustained PERK activation that leads to a prolonged block in translation that is not compatible with cell survival. Under chronic ER stress conditions, the continuous PERK signaling increases the expression of the CHOP transcription factor (also known as GADD153) (Tabas & Ron, 2011; Urra et al., 2013), which increases the chances of cell death by suppressing the anti-apoptotic BCL-2 protein (McCullough et al., 2001). CHOP works together with ATF4 to activate the transcription of pro-death targets, causing an increase in ROS production and depletion of ATP (Marciniak et al., 2004). They also activate targets that enhance protein synthesis in cells already overwhelmed by unfolded proteins in the ER (Han et al., 2013; Marciniak et al., 2004). IRE1α can also trigger cell death by assembling an activation platform for apoptosis

signal-regulating kinase 1 (ASK1) and its downstream target c-Jun NH2-terminal kinase (JNK) (Nishitoh et al., 2002; Urano et al., 2000). Moreover, chronic ER stress can also trigger the mitochondrial apoptotic pathway governed by pro- and anti-apoptotic BCL-2 family proteins that regulate the permeability of the outer mitochondrial membrane and the release of cytochrome c leading to apoptosis (Danial & Korsmeyer, 2004; D. Ren et al., 2010; Tait & Green, 2010). Altogether, the relationship between ER stress and apoptosis is complex. The UPR sensors can either support cell survival and fix cell stress or inhibit adaptive responses and trigger apoptosis. The outcome depends on the duration of UPR activation and the cell's ability to effectively manage ER stress.

2.1.4. What causes ER stress in HD

Although the exact causes of ER homeostasis disturbances in HD remain unclear, it is known that, unlike other neurological disorders, mHTT does not accumulate in the ER lumen but instead interacts with the cytosolic surface of organelle membranes, suggesting an indirect impact on the ER (Matus et al., 2008; R. Vidal et al., 2011). A research study in 2008 shed some light on the possible explanation for the occurrence of ER stress in HD. The researchers expressed mHtt in yeast and PC12 cells, which resulted in a rapid defect in ER-associated protein degradation due to the entrapment of essential ERAD proteins such as Npl4, Ufd1, and p97 into mHtt aggregates, thus triggering ER stress (Duennwald & Lindquist, 2008). Moreover, the expression of these ERAD components improved mHtt pathogenesis and significantly reduced ER stress as evidenced by the levels of GRP78 and CHOP. mHtt can also trigger ER stress by interfering with the function of gp78, an ER membrane-anchored ubiquitin ligase that plays a role in ERAD (H. Yang et al., 2010). Additionally, although still in controversy, some research suggests that mHtt could be further hindering the degradation of ERAD substrates by inhibiting the proteasome (Davies et al., 2007; P. H. Ren et al., 2009). Thus, ER stress in HD is a complex phenomenon that can be caused by various factors. Besides the impairment of ERAD as previously discussed, disturbances in vesicular trafficking between the ER and Golgi and alterations in autophagy are also possible contributors. To maintain proper cellular function, the trafficking of vesicles between the ER and Golgi is a crucial checkpoint in the secretory pathway. However, disruptions

at any stage can cause a buildup of cargo vesicles, which can affect ER function. Experimental studies have shown that mHtt expression can perturb this ER/Golgi trafficking, leading to ER stress (del Toro et al., 2006), similar to what is observed in PD models with mutant α-synuclein (A. A. Cooper et al., 2006). Autophagy plays a crucial role in breaking down cytoplasmic components, including damaged organelles, toxic protein aggregates, and intracellular pathogens, and helps cells survive against ER stress (Levine & Kroemer, 2008; Mizushima et al., 2008). Changes in autophagy can result in the buildup of misfolded proteins and ER stress, as seen in recent studies where its inhibition can lead to neurodegeneration (Fleming et al., 2022; Menzies et al., 2017). Research suggests that mHtt inclusions impair the activity of the autophagy regulator Beclin-1 and lead to altered protein homeostasis and ER stress (Cortes & la Spada, 2014; Martin et al., 2015). Overall, although the exact mechanisms are still unclear, it is evident that mHTT disrupts critical cellular pathways such as ERAD, the secretory pathway, and autophagy, ultimately leading to ER stress.

2.1.5. ER stress in HD models and patients

The ER stress response in HD is believed to be due to the presence of soluble oligomers of mHtt (Leitman et al., 2013), which are more toxic than the aggregates (Arrasate et al., 2004; Herrera et al., 2011; Lajoie & Snapp, 2010). This leads to the upregulation of markers of the UPR such as P-PERK, P-eIF2α, CHOP, GADD34, GRP78, ATF6, Herp, and XBP1s in both cellular and animal models of HD, as well as in *postmortem* samples from HD patients (Carnemolla et al., 2009; Leitman et al., 2013, 2014; H. Luo et al., 2018; Reijonen et al., 2008; Shacham et al., 2019; R. Vidal et al., 2011). Moreover, studies have found that other cellular factors, such as the ubiquitin-specific protease-14 and ATF5, which also play important roles in reducing ER stress, are sequestered and depleted during mHtt aggregation (Hernández et al., 2017; Hyrskyluoto et al., 2014). In 2009, a study was conducted to evaluate the presence of ER stress in HD by analyzing the levels of two critical ER stress-related proteins - GRP78, the main chaperone that acts as an ER stress sensor (Casas, 2017; M. Wang et al., 2009), and CHOP, a key pro-apoptotic player that upregulates under chronic ER stress conditions (Cai et al., 2015; Tabas & Ron, 2011; Urra et al., 2013). The study found that even at pre-symptomatic

disease stages in KI mice and in human HD brains, the levels of GRP78 and CHOP were elevated (Carnemolla et al., 2009), indicating the presence of an ER stress response in HD. In the case of R6/2 mice, inhibiting ASK1 reduced ER stress and nuclear mHtt aggregates (Cho et al., 2009). Moreover, research has also shown that ER stress increases mHtt aggregation through the upregulation of SCAMP5 which is significantly increased in the striatum of HD patients and induced in cultured striatal neurons by either ER stress or mHtt expression (Noh et al., 2009). In addition to the aforementioned research, studies have also investigated the modulation of the three main UPR sensors and downstream targets to better understand ER stress in HD. For instance, Vidal et al. in 2012 found that reducing XBP1 expression in HD transgenic mice reduced neural loss, improved motor skills, and enhanced autophagy and mHtt clearance. The authors suggested that XBP1 suppression allowed for higher expression of FoxO1, which may regulate autophagy, making it a key factor in the altered proteostasis seen in HD (R. L. Vidal et al., 2012). Research also shows that acute IRE1 inhibition can have opposing effects, preventing ER stress-induced apoptosis (Upton et al., 2012) or increasing it (M. Lu et al., 2014). However, in the context of HD, the downregulation of IRE1 rescued the HD phenotype in a fly model of HD (H. Lee et al., 2012). Notably, PERK is also important for modulating and improving cellular adaptation to ER stress, making it a potential target for neurodegenerative diseases. However, the effects of blocking PERK have been so far mixed, as it can either slow neurodegeneration or worsen symptoms in different disease contexts (Shacham et al., 2021). Thus, further investigation is necessary to understand the intricate UPR pathways and the potential benefits of their modulation in HD and other diseases. As a result, studies that explore the therapeutic impact of compounds that alleviate ER stress are crucial and could provide valuable insights into their efficacy in HD therapeutics. For instance, treatment with chemical chaperones 4-PBA and TUDCA reduced ER stress in type 2 diabetes and HD mouse models by improving the overall ER function and decreasing ER stress-induced apoptosis (Keene et al., 2002a; Özcan et al., 2006). Furthermore, a novel peptide has been developed that blocks the interaction between mHtt and p97/VCP, a key factor in the development of ER stress in HD as p97/VCP is an essential ERAD factor that is sequestered into mHtt aggregates and depleted (Duennwald & Lindquist, 2008; Guo et al., 2016). Besides, Sigma-1 Receptor

agonists have shown neuroprotective effects in HD models alleviating ER stress by upregulating antioxidant activity, normalizing calcium homeostasis, and decreasing ROS levels (Hyrskyluoto et al., 2013).

Overall, the research focus so far has been centered on understanding the role of ER stress in striatal vulnerability, as it is the most affected brain region in HD. Besides, evidence suggests that striatal neurons are highly susceptible to ER stress due to the low activity of PERK-mediated eIF2α phosphorylation in this area. Although the increase in eIF2α phosphorylation in the presence of mHtt was initially thought to be harmful, it was later determined to be a cellular attempt to restore balance (Leitman et al., 2014). Nevertheless, the elevated levels of P-eIF2α have been linked to memory and long-term potentiation (Costa-Mattioli et al., 2005, 2007; Sidrauski et al., 2013), implying a potential connection between ER stress and the cognitive impairment seen in HD. For this reason, this Thesis aims to study the role of ER stress in HD, how the different UPR branches are affected depending on the brain region, its impact on the hippocampus, and the possible link with cognition.

2.1.6. ER stress and cognition

According to recent studies, the PERK and IRE1α-XBP1 signaling pathways are involved in neurodegenerative diseases in more ways than just reducing chronic ER stress. They can also impact synaptic function through new mechanisms by regulating the expression of important synaptic proteins. These findings have important implications for understanding the connection between UPR signaling, synaptic formation, neuronal plasticity, and cognitive and memory function in neurodegenerative disorders. The impact of PERK on synaptic plasticity and hippocampal-dependent memory was first explored using a PERK conditional KO mouse model, where PERK was deleted specifically in excitatory neurons, and mice exhibited impaired behavioral flexibility (Trinh et al., 2012, 2014). On the contrary, conditional PERK knockout in a mouse model of AD improved memory and synaptic plasticity (Ma et al., 2013). Similarly, knocking down PERK in the cortex of adult rats also led to enhanced memory and improved behavioral flexibility (Ounallah-Saad et al., 2014), identifying PERK as a novel

regulator of cognitive functions. In addition, PERK has also been implicated in age-dependent brain diseases. Studies have shown that PERK inhibitors such as GSK2606414 and GSK2656157 are protective against several neurodegenerative diseases, including frontotemporal dementia (FTD) (Radford et al., 2015), prion disease (Mori et al., 2013), PD (Mercado et al., 2018), traumatic brain injury (TBI) (T. Sen et al., 2017), and AD (W. Yang et al., 2016). Moreover, Echinacoside has also been reported to be a PERK inhibitor and exhibits neuroprotective effects in a mouse model of PD (Zhao et al., 2010) and in AD, reducing the accumulation of Aβ protein in AD mice (W. Chen et al., 2018; Dai et al., 2020). However, PERK inhibition has also resulted in secondary side effects, including pancreatic toxicity (Atkins et al., 2013; Harding et al., 2012). Therefore, the most recent approaches to PERK inhibition include inhibiting the pathway downstream of P-eIF2α using a new molecule called ISRIB (Sidrauski et al., 2013; Tsai et al., 2018; Zyryanova et al., 2021). This compound has demonstrated neuroprotection and improved memory and cognition in various models of neurodegenerative diseases, including AD, ALS, and TBI (Bugallo et al., 2020; Chou et al., 2017; Halliday et al., 2015; Hosoi et al., 2016; Krukowski et al., 2020; Wong et al., 2018, 2019). However, not all studies inhibiting PERK have shown beneficial effects, with some of them showing enhanced apoptosis and no improvement in spatial learning and memory deficits (Athanasiou et al., 2017; Johnson & Kang, 2016). Given the existing evidence, modulation of PERK has emerged as a promising target for treating neurodegenerative diseases and associated cognitive impairment. However, the effects can vary depending on the pathological context and the specific signaling target that is inhibited, which may lead to negative side effects.

Lastly, evidence suggests that XBP1 also plays a role in cognitive function and synaptic plasticity. A decrease in XBP1 levels due to a genetic variation has been linked to psychiatric disorders in Japan (Cheng et al., 2014; Kakiuchi et al., 2003), and more recently to AD in China (Liu et al., 2013). Furthermore, a mouse model lacking the XBP1 gene displayed impaired learning and memory as well as altered synaptic transmission in the hippocampus (Hetz et al., 2008; Martínez et al., 2016). Conversely, increasing XBP1 expression in an AD model improved dendritic spine density and synaptic transmission in the hippocampus, ultimately reversing memory impairments (Cissé et

al., 2017) further supporting the idea that XBP1 is a crucial element in normal cognitive function and synaptic plasticity. In summary, modulation of the UPR branches has shown promise as a therapeutic strategy for alleviating cognitive symptoms in various neurodegenerative diseases. Nevertheless, more research is needed to determine the optimal timing and approach in each pathological context. This Thesis aims to contribute to a better understanding of ER stress modulation in HD, its relationship with cognition, and its potential as a therapeutic target.

2.2. DISSECTING THE ROLE OF THE ASTROCYTIC SECRETOME IN HD PATHOLOGY

2.2.1. Astrocytes in the central nervous system (CNS) homeostasis

The CNS is comprised mostly of astrocytes, which are the most abundant type of glial cell, accounting for 70% of all cells in the brain (Allen & Lyons, 2018; Nedergaard et al., 2003). They pose a greater challenge for categorization than other cell types due to their high heterogeneity. In fact, several categorization systems have been proposed, including those based on morphology (protoplasmic or fibrous), location (grey or white matter), or reactivity state (A1 neurotoxic or A2 neuroprotective) (Chai et al., 2017; Gokce et al., 2016; Khakh & Deneen, 2019). However, these classifications do not fully capture the complexity of astrocytes (Escartin et al., 2021). Recently, attempts have been made to establish astrocyte subtypes using transcriptional and proteomic profiles given that they exhibit regional differences, with unique gene and protein expression patterns (Batiuk et al., 2020; Diaz-Castro et al., 2019).

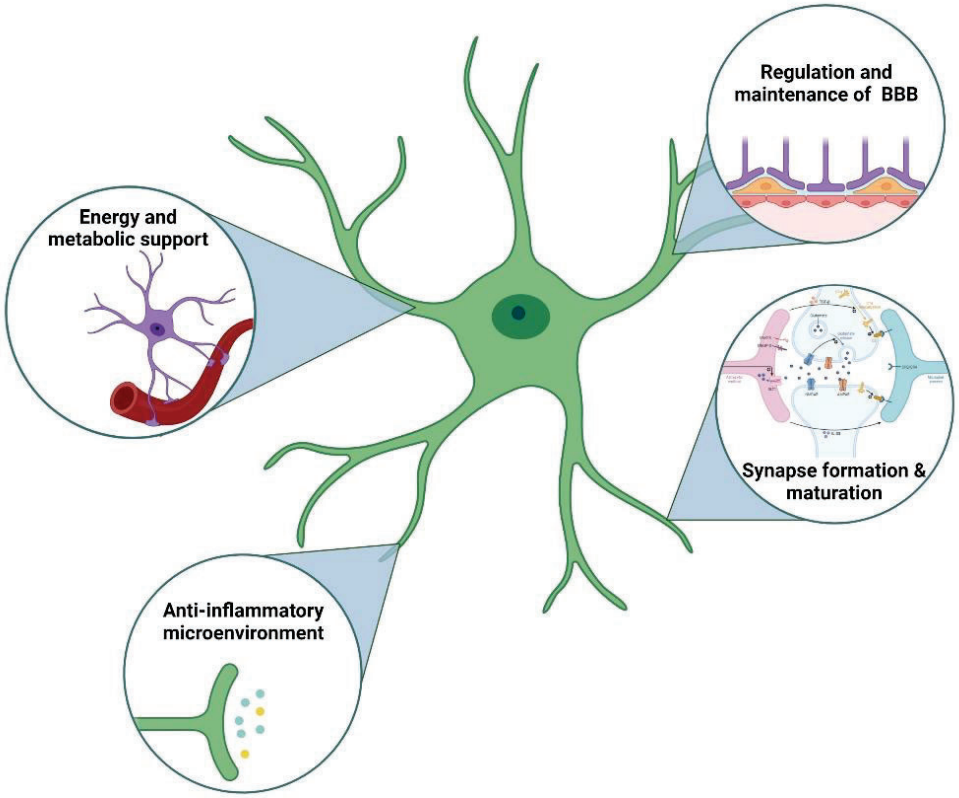


Figure 8. Astrocytes' functions in CNS homeostasis. Astrocytes play a crucial role in maintaining the normal function and stability of the CNS and have numerous functions including the regulation of blood flow and upholding the integrity of the blood-brain barrier. They also participate in regulating the formation, elimination, and functioning of synapses, as well as providing metabolic and energy support and promoting an anti-inflammatory microenvironment.

Although astrocytes cannot conduct action potentials, they are still excitable through changes in intracellular calcium concentration, allowing for communication with neurons and other astrocytes (Charles et al., 1991; Cornell-Bell et al., 1990; Shigetomi et al., 2008). They play important roles in maintaining K^+ and water homeostasis (Amiry-Moghaddam et al., 2004; Higashi et al., 2001), taking up glutamate (Danbolt, 2001), and forming networks through gap junctions (Abbott et al., 2006) (Figure 8). Moreover, astrocytes are also involved in maintaining the blood-brain barrier (BBB), regulating blood flow, and secreting various molecules to strengthen the tight junctions of the BBB (Koehler et al., 2009; Lécuyer et al., 2016). They also play a crucial role in energy and metabolic support (Pellerin et al., 2007), synapse formation and maturation with the release of TNFα, TGF-β, and IL-33 (Diniz et al., 2012; Stellwagen et al., 2005; Vainchtein et al., 2018), and maintain a healthy anti-inflammatory environment in the CNS by continually secreting anti-inflammatory factors such as TFG-β and IL-10 (Sofroniew, 2015). Therefore, astrocytes are essential to maintain proper neuronal function, so it's not surprising that neuron-astrocyte interactions are central to the development of many neurodegenerative diseases (Linnerbauer et al., 2020).

2.2.2. Alterations in astrocytes in HD

Astrocyte changes in HD patients

The hallmark of HD neuronal pathology is the presence of intracellular mHTT aggregates. These inclusions are not just found in neurons but also in cortical and striatal astrocytes and are detected to the same extent in adult-onset HD patient tissue (Jansen et al., 2017; Shin et al., 2005). Fibrillary astrocytosis, a defining feature of HD is absent in Grade 0 HD but progresses from mild to severe from Grade 1 to 4. Astrocytes imaged using GFAP immunostaining in HD brains display thicker processes and

hypertrophic somas compared to control striatal tissue. GFAP density increases twofold in Grade 2 HD tissue and above, and increased GFAP immunoreactivity has been noted in Grade 0-1 tissue (Myers et al., 1991; Rüb et al., 2016; J. P. Vonsattel et al., 1985). Besides, the loss of the excitatory amino acid transporter 2 (EAAT2) or glutamate transporter-1 (GLT-1), which is highly expressed in astrocytes, has been consistently observed in human HD *postmortem* tissue. Studies reveal a decrease in EAAT2 mRNA labeling that is dependent on the severity of the disease and starts early in the disease process impairing glutamate removal from the synaptic cleft after it has been released, contributing to excitotoxicity (Arzberger et al., 1997; Brymer et al., 2021; Faideau et al., 2010; Shin et al., 2005).

Astrocyte alterations in HD models

One key similarity between the astrocytic pathology in human HD brains and in HD mouse models is the progressive loss of GLT-1 and GLAST resulting in impaired glutamate uptake by astrocytes (Behrens et al., 2002; Estrada-Sánchez et al., 2009; Huang et al., 2010; Liévens et al., 2001; Milnerwood et al., 2010). In fact, the administration of ceftriaxone, a beta-lactam antibiotic that increases the levels of GLT-1, has been shown to mitigate several HD behavioral phenotypes and improve striatal glutamate uptake, suggesting that astrocyte physiology plays a critical role in the pathology of HD (B. R. Miller et al., 2008; Sari et al., 2010). Besides, the presence of cytoplasmatic and nuclear mHtt inclusions in astrocytes has also been observed in these models, with the proportion of astrocytes showing inclusions increasing over time (Jansen et al., 2017; Shin et al., 2005; Tong et al., 2014). For instance, studies have shown that expression of mHtt in astrocytes *in vitro* leads to the death of co-cultured striatal neurons (Shin et al., 2005), and *in vivo* reduces the expression of GLT-1, while promoting astrogliosis and HD-like symptoms (Bradford et al., 2010; Faideau et al., 2010; Haim, Ceyzériat, et al., 2015; Parsons et al., 2016). Similarly, a reduction in mHtt levels specifically in astrocytes has been shown to slow the progression of the disease with significant improvements in motor and psychiatric phenotypes as well as neuropathological and electrophysiological features (Wood et al., 2019). In addition to the previously mentioned alterations, astrocytes are also involved in maintaining the

homeostasis of K^+ in the brain influencing neurotransmitter release, cell proliferation, and apoptosis (Khakh et al., 2017; Liot et al., 2017; Zhang et al., 2018). However, in mouse models of HD, a decrease in Kir4.1 channel currents and membrane potential and conductance has been observed in astrocytes expressing mHtt (Ariano et al., 2005; Y. Ohno et al., 2018; Tong et al., 2014), which can be restored by adenoviral-mediated delivery of Kir4.1 into astrocytes (Tong et al., 2014). Additionally, astrocytes are instrumental in synthesizing cholesterol and antioxidants in the adult brain, which is also dysregulated in HD with decreased cholesterol metabolism and less antioxidant production (Haim, Carrillo-de Sauvage, et al., 2015; Leoni et al., 2008). Collectively, the findings from HD model studies investigating the role of astrocytes in HD pathology have consistently demonstrated that dysfunctional astrocytes contribute to the development and progression of HD pathology.

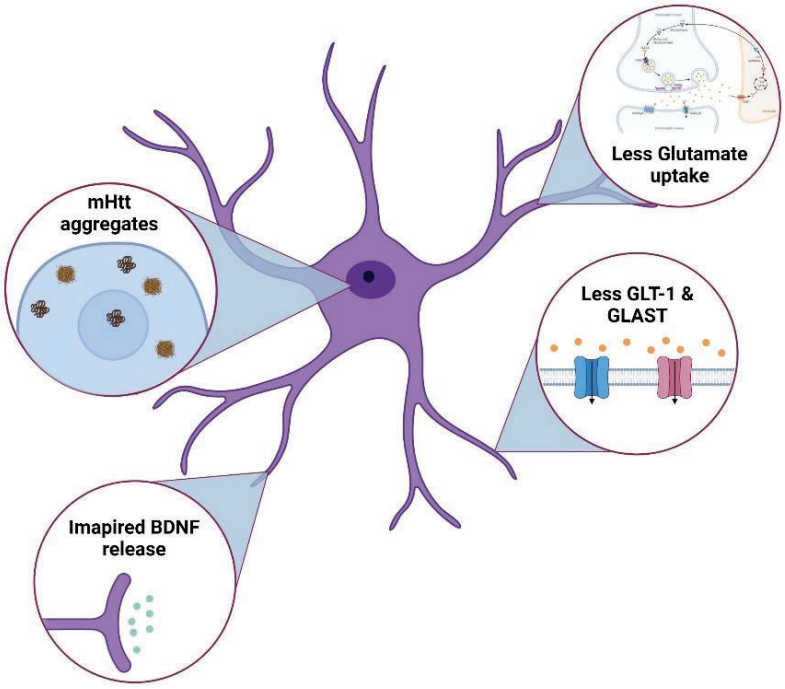


Figure 9. Alterations in HD astrocytes. The presence of mHtt in astrocytes results in a reduction in the glutamate transporters GLT-1 and GLAST, which impairs the absorption of extracellular glutamate. Furthermore, HD astrocytes display mHtt aggregates in their cytosol and nucleus and exhibit impaired BDNF release. These pathological processes may contribute to the targeted degeneration of neurons in the striatum observed in HD.

Astrocytes and BDNF in HD

Striatal neurons depend on BDNF for survival, provided mainly by the cortex (Anthony Altar et al., 1997; Baquet et al., 2004; Canals et al., 2004). A decrease in BDNF levels has been observed in HD human brains and animal models (Zuccato et al., 2001, 2005, 2011). This decrease is due to both transcriptional dysregulation caused by mHTT in the cortex and a decrease in BDNF transport to the striatum (Gauthier et al., 2004). Astrocytes play a crucial role in providing trophic support in the nervous system, including the release of BDNF (Miyamoto et al., 2015; Parpura & Zorec, 2010). However, the expression of mHtt in astrocytes can impair astrocytic function, including the release of BDNF (L. Wang, Lin, Wang, Wu, Han, Zhu, Difiglia, et al., 2012; L. Wang, Lin, Wang, Wu, Han, Zhu, Zhang, et al., 2012). Studies in mouse models have shown a reduction in BDNF released by astrocytes expressing mHtt, likely due to impaired docking of BDNF-containing vesicles (Hong et al., 2016). Besides, injecting an adenoviral vector into the striatum of R6/2 mice, which causes astrocyte-specific expression of BDNF, resulted in a delay in the onset of motor abnormalities (Arregui et al., 2011). Additionally, treatment with glatiramer acetate, which is known to increase BDNF expression, showed decreased neurodegeneration in R6/2 and YAC128 HD mice (Reick et al., 2016). Therefore, these findings suggest that mHTT expression in astrocytes contributes to the pathogenesis of HD and that astrocytes may be a good target for therapeutic intervention to increase BDNF levels and delay the onset of motor abnormalities.

2.2.3. Neuroinflammation in HD

When the CNS is injured or infected, it exhibits signs of inflammation that are identified by microglia and astrocytes. These cells produce prostaglandins, pro-inflammatory cytokines (e.g., TNFα, IL-6, IL-1β, and Interferon-γ), and other molecules that trigger the production of chemokines and adhesion molecules that attract immune cells and activate glial cells. However, an excessive response can lead to neuroinflammation (Shastri et al., 2013; Veerhuis et al., 2011). Initially, the term referred specifically to the inflammation of the CNS that was the result of the

infiltration of peripheral immune cells, due to conditions like viral or bacterial infections, stroke, or HIV encephalopathy among others. However, over time the definition has broadened to include neurodegenerative diseases, which show signs of inflammation such as increased cytokine expression and activation of glial cells, despite not exhibiting infiltrating immune cells (Aguzzi et al., 2013; Hensley, 2010). Factors such as aging, disease, drugs, and toxins can contribute to neuroinflammation playing a significant role in the progression of several neurodegenerative diseases (Schain & Kreisl, 2017; Smith et al., 2012).

Clinical features of neuroinflammation in HD

The accumulation of reactive astrocytes and microglia in the brains of HD patients has been documented by PET imaging and linked to HD pathology (Rosas et al., 2010; Tai et al., 2007a, 2007b). This activation can be detected even in presymptomatic patients up to 15 years before the expected onset of motor symptoms (Tai et al., 2007b), which is around the same time frame when increased levels of interleukin-6 (IL-6) and IL-8 are observed in the plasma of HD patients (Björkqvist et al., 2008). Besides, several proteins produced by innate immune cells have also been detected in the plasma of HD patients and correlate with HD progression including, IL-6, MMP-9, vascular endothelial growth factor (VEGF), and TGF-β1 (K. H. Chang et al., 2015; Dalrymple et al., 2007; Wild et al., 2011). In addition to plasma, Silvestroni and colleagues found that human HD *postmortem* tissue exhibits a distinct inflammatory signature compared to other neurodegenerative diseases (Hirsch et al., 2012; Möller, 2010) with elevated levels of TNFα and IL-1β specifically found in the striatum, and upregulation of MMP-9, IL-6, and IL-8 in the cortex and cerebellum (Silvestroni et al., 2009a). Moreover, other studies have found in cerebrospinal fluid (CSF) of HD patients signs of immune activation with upregulation of IL-6, IL-8, and TNFα (Björkqvist et al., 2008), and elevated levels of the signal transducer and activator of transcription (STAT)-5 in monocytes from HD patients (Träger et al., 2013).

INTRODUCTION

Astrocytes and Microglia in HD Neuroinflammation

Astrocytes play a key role in amplifying the initial inflammatory response initiated by microglia. Research has shown that astrocytes expressing mHTT are more likely to activate proinflammation. For instance, in the presence of an inflammatory stimulus, astrocytes from R6/2 mice exhibited a prolonged NF-kB activation and expressed proinflammatory factors such as TNFα and IL-1β (Hsiao et al., 2013). This resulted in greater harm to neurons and is a factor in the pathogenesis of HD. Besides, inhibiting TNFα improved motor function in R6/2 mice, with a decrease in mHtt aggregates and increased neuronal density (Alto et al., 2014; Hsiao et al., 2014). Also in HD, Danger-associated molecular patterns (DAMPs) produced by degenerating neurons, microglia, or astrocytes, activate intracellular signaling pathways within astrocytes, such as the JAK/STAT and NF-κB MAPK pathways which results in the formation of reactive astrocytes, and enhances the expression of cytoskeletal proteins and genes involved in the release of cytokines and chemokines (Palpagama et al., 2019). The exact role of these reactive astrocytes in the progression of HD is not fully understood yet, but it is believed that they may lose their neuroprotective properties and their ability to support neurons (Sofroniew, 2009). In the case of microglia, they comprise less than 10% of brain cells and are continuously monitoring the environment surrounding neurons and glial cells (Lawson et al., 1990; Ransohoff & Perry, 2009). In this case, mHTT enhances the expression of genes that produce pro-inflammatory cytokines, making microglia respond more strongly to harmful stimuli (O'Regan et al., 2021) releasing proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α leading to chronic inflammation and contributing to tissue damage and disease progression (Rocha et al., 2021; Savage et al., 2020; H. M. Yang et al., 2017). Overall, astrocytes, in conjunction with other glial cells such as microglia, play an indispensable role in maintaining a tightly regulated microenvironment that is crucial for proper CNS function. Previously believed to primarily provide nourishment and support to neurons, it is now evident that a tightly intertwined communication network between neurons and glia is necessary for optimal CNS function. Perturbations in this interplay have been linked to the pathogenesis of various neurodegenerative diseases, including HD. Importantly, the degree to which these neuroinflammatory changes are

detrimental to neurons and contribute to the progression of HD pathology is still not well understood. Therefore, our research aims to explore the role of neuron-astrocyte communication in the pathogenesis of HD. In this context, ARMS has recently gained attention as a potential regulator of astrocytic function (Jaudon et al., 2020, 2021). Thus, our Thesis aims to investigate the impact of ARMS on astrocytic-secretome alterations in HD and their contribution to HD motor symptoms.

2.2.4. ARMS/Kidins220

Discovery and structure

ARMS or Kidins220 protein (Ankyrin-Repeat Rich Membrane Spanning/kinase D-interacting substrate of 220 kDa) is a 1751 amino acid and 220 kDa protein characterized by having four transmembrane domains and long cytoplasmic C- and N-terminal tails (Figure 10). ARMS was initially described as a substrate for protein kinase D (Iglesias et al., 2000) and a protein that interacts simultaneously with $p75^{NTR}$ and Trk receptors (H. Kong et al., 2001). It is the fourth domain that interacts with TrkA's transmembrane domain (Arévalo et al., 2004), and forms a complex with this receptor and the cytoplasmic region of $p75^{NTR}$ by the C-terminal tail, specifically the PDZ-binding domain (Arévalo et al., 2004; M. S. Chang et al., 2004). This PDZ-binding domain also interacts with ephrin receptors (M. S. Chang et al., 2004; H. Kong et al., 2001). On the one hand, ARMS's N-terminal region contains eleven ankyrin domains recognized by the Trio protein, which is a guanine nucleotide exchange factor for Rho family GTPases (Neubrand et al., 2010). On the other, ARMS's C-terminal tail has a proline-rich region that binds the adapter protein CrkL, necessary for the sustained activation of the MAPK pathway (Arévalo et al., 2006). ARMS also has a Sterile Alpha Motif (SAM) domain involved in both intra- and intermolecular interactions and a Kinesin-Light Chain Interacting Motif (KIM) that binds the kinesin-1 motor complex (Bracale et al., 2007). Finally, ARMS interacts with other receptors, such as glutamate and VEGF receptors (Arévalo et al., 2010; Cesca et al., 2011). The large number of domains capable of interacting with other proteins make ARMS an important candidate to act as a signaling platform, as it can recruit specific adaptors and associate them with different receptors, such as Trk receptors, glutamate receptors, ephrin receptors, or VEGF receptors (Arévalo et al., 2010; Cesca et al., 2012; López-Menéndez et al., 2009; S. Luo et al., 2005).

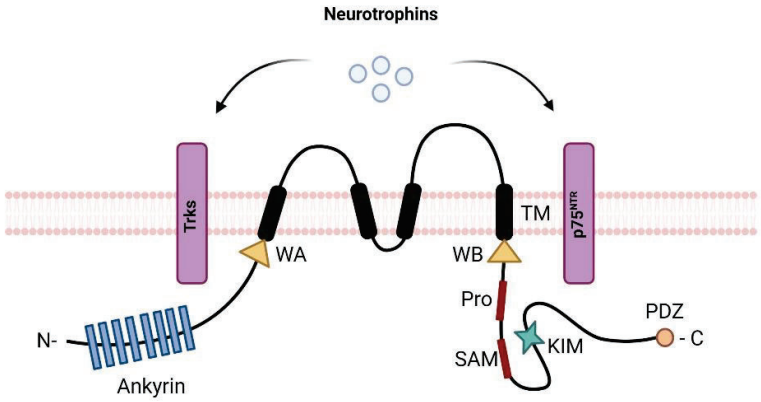


Figure 10. Structure of ARMS/Kidins220. Schematic representation of ARMS/Kidins220 structural domains: WA, Walter A. WB, Walter B. SAM, Sterile Alpha Motif domain. KIM, Kinesin-Light Chain Interacting Motif.

Distribution

ARMS is mainly expressed in the nervous system where it can modulate neuronal survival, physiology, differentiation, and synaptic plasticity (Neubrand et al., 2012). *In situ* hybridization studies revealed that ARMS is expressed in regions of the central nervous system with greater neural plasticity, such as the olfactory bulb, hippocampus, Purkinje cells, and spinal motor neurons (H. Kong et al., 2001). Within cells, ARMS is located in the plasma membrane and internal vesicles, and its expression is higher in regions of the plasma membrane that accumulate specific proteins and lipids involved in the regulation of cell signaling (Riol-Blanco et al., 2004).

Functional Role of ARMS in the CNS

In vivo, functional analysis of ARMS has been performed using transgenic animal models. Two independent lines of ARMS knock-out mice have been generated; homozygous knock-out mice ($ARMS^{-/-}$) that suffer from early embryonic lethality, and heterozygous knock-out mice ($ARMS^{+/-}$) with a 30-40% reduction of ARMS protein expression and normal development. These mice have impaired neuronal branching and synaptic capacity making them a useful model to study the effects of reduced ARMS protein expression in the nervous system (S. H. Wu et al., 2009). Besides, there is another knock-out mouse model that does not exhibit early embryonic lethality but

suffers from significant neuronal death and impaired response to neurotrophic stimuli in the later stages of embryonic development (Cesca et al., 2011, 2012). In addition, heterozygous ARMS$^{+/-}$ mice have increased basal synaptic transmission compared to wild-type animals in hippocampal slice preparations given that ARMS interacts with the glutamate receptors and its absence could be affecting the AMPA glutamate receptor subunit A1 (GluA1) (Arévalo et al., 2010). Thus, ARMS participates in fundamental aspects of neuronal physiology including cell survival, differentiation, and synaptic plasticity. In fact, these findings indicate that changes in the *KIDINS220* gene and/or the ARMS protein may be linked to various human pathologies. However, there is still limited research in this field. For example, ARMS has been shown to act as an oncogene in melanoma and neuroblastoma (Liao et al., 2007; Rogers & Schor, 2013b), and increased levels have been observed in AD patients (López-menéndez et al., 2013). Moreover, studies have found that the expression of the *KIDINS220* gene is altered in PD (Simunovic et al., 2009) and ASD patients (S. W. Kong et al., 2012) and more recently in schizophrenia patients too (Kranz et al., 2015). Thus, ARMS plays a crucial role in neuronal regulation, offering a promising avenue for therapeutic intervention in neurodegenerative and other neurological disorders. In the case of HD, increased levels of ARMS have been detected in two HD mouse models and in human HD brains, playing a crucial role in regulating the secretion of BDNF, a critical factor in the development of HD (López-Benito et al., 2016, 2018). Moreover, the *Kidins220* gene has two main isoforms in the adult brain, Kidins220-C32, and Kidins220-C33, which differ in their length and protein-protein interaction domains due to alternative exon splicing. Kidins220-C32 contains domains critical for neuronal functions, including neurotrophin pathway control, while Kidins220-C33 lacks these domains and, therefore, its physiological functions and distribution may greatly differ. In HD patients and the R6/1 mouse model, there is a selective downregulation of Kidins220-C33 in the striatum, which may influence the onset and/or progression of HD (Sebastián-Serrano et al., 2020). Thus, research has shown that ARMS plays a crucial role in neuronal differentiation, survival, and plasticity as one of the key effectors of neurotrophin pathways. However, the function of ARMS in glial cells has not been fully explored. Recent studies have shed light on the role of astrocytic ARMS in regulating glial Ca^{2+} dynamics, survival/death pathways, and astrocyte-neuron communication (Jaudon et

al., 2020, 2021), identifying ARMS as a novel player in the growing number of diseases associated with astrocytic dysfunctions. Therefore, the focus of this Thesis is to investigate the impact of ARMS on the astrocytic-secretome alterations in Huntington's disease and how they contribute to the motor deficits observed in HD.

AIMS

Despite the known and straightforward genetic cause of HD, the intricate molecular and cellular mechanisms that drive the degeneration and death of neurons are incredibly complex and remain uncharted. Unfortunately, this lack of knowledge has hindered the development of effective disease-altering therapies for 30 years since the causative mutation's discovery in 1993. Consequently, existing treatments only manage specific symptoms and fail to prevent the disease's inevitable neuropathological progression, making HD a cruelly devastating condition.

To address this critical knowledge gap, the primary objective of this thesis is to enhance our understanding of the therapeutic potential of targeting ER stress and the astrocytic secretome for pharmacological interventions in HD. To achieve this goal, we have identified the following specific aims:

1. To analyze the role of ER stress in contributing to the neuropathology and cognitive impairments of HD pathology.

2. To analyze the contribution of ARMS to the astrocytic secretome alterations in HD pathology.

METHODS

1. ANIMALS

For the *in vivo* studies, all animals used were males and were housed together in numerical birth order in mixed genotypes. For the *in vitro* studies, both male and female mice were included in the experiments. Genotyping was determined by PCR analysis from a tail biopsy. The animals were housed with access to food and water *ad libitum* in a colony room kept at 19-22 °C and a 40-60% humidity under a 12:12 hour light/dark cycle. All animal-related procedures were performed in compliance with the National Institute of Health Guide for the care and use of laboratory animals and were approved by the local animal care committee of the Universitat de Barcelona (448/17 and 315/18) and the Generalitat de Catalunya (9878 P2), following the directive of the European Commission (2010/63/EU) and Spanish (RD53/2013) guidelines for the care and use of laboratory animals.

1.1. R6/1 mouse model

The R6/1 heterozygous transgenic mouse model expresses the N-terminal fragment of the human mutant huntingtin (*mHtt*) exon 1 with CAG repeats ranging from 115 to 150 (Mangiarini et al., 1996) and was obtained from The Jackson Laboratory® (Bar Harbor, ME, USA Cat. #006471). They were maintained in a BL/6:CBA background together with their non-transgenic wild-type (WT) littermates that served as controls.

1.2. GRP78$^{+/-}$ mouse model

Heterozygous GRP78$^{+/-}$ mice were very kindly given by Dr. Manuel Guzmán's laboratory and were previously purchased from The Jackson Laboratory® (Bar Harbor, ME, USA Cat. #019549). To study the function of Grp78 in HD, we cross-mated R6/1 mice with heterozygous GRP78$^{+/-}$ mice and generated a new double-mutant R6/1: GRP78$^{+/-}$ mouse line that, therefore, expresses a pathogenic fragment of the human *mHtt* exon 1 and carries a single functional *Grp78* allele. Note that very early embryonic lethality occurs in homozygous GRP78$^{+/-}$ mice (S. Luo et al., 2006).

2. *IN VIVO* ANIMAL PROCEDURES

2.1. AAV-mediated shARMS/Kidins220 expression vectors

To knock down ARMS/Kidins220 expression, we tested different short hairpin RNAs (shRNAs) for ARMS and validated the efficiency of the shRNA 2 to eventually be cloned into adeno-associated viruses (AAVs). The AAV2/5 plasmids and infectious AAV viral particles containing the GFP expression cassette with scrambled (SCB) shRNA (shSCB) or ARMS shRNA (shARMS) under the GFAP promoter were generated by the "Unitat de Producció de Vectors" at the Center of Animal Biotechnology and Gene Therapy at the Universitat Autònoma de Barcelona, Barcelona, Catalonia (CABTEG).

2.2. Intrastriatal injection of adeno-associated vectors

AAV2/5-GFAP-shRNA-GFP or AAV2/5-GFAP-shARMS-GFP viral particles were used to downregulate ARMS protein expression in astrocytes. 8-week-old WT and R6/1 mice were deeply anesthetized with a mixture of oxygen and isoflurane (3-4% for induction and 1-2% for maintaining anesthesia) and placed on a stereotaxic apparatus for bilateral intra-striatal injections (1,6µL; 15×10^9 genome copies). Two injections per hemisphere were administered in the striatum at the following coordinates relative to bregma: (1) anteroposterior (AP), +0.8; mediolateral (ML), +/- 1.8; and (2) anteroposterior (AP), +0.3; mediolateral (ML), +/- 2; and dorsoventral (DV), -2.6 below the Dural surface. Viral vectors were injected using a 10µL-Hamilton syringe at an infusion rate of 0.250µL/min. The needle was left in place for 3 minutes to ensure complete diffusion and then slowly retracted from the brain. Both hemispheres were injected with the same shRNA. Four weeks after the injection motor coordination and learning were evaluated (See experimental timeline in Figure 11).

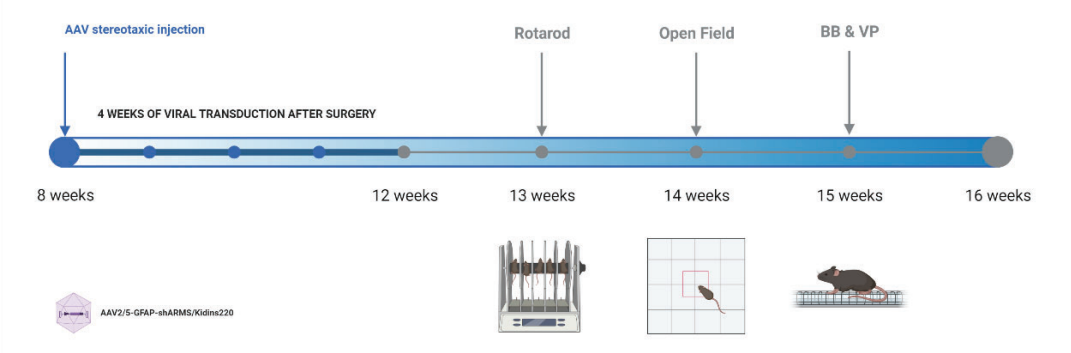


Figure 11. Experimental timeline. Scheme displaying the protocol used to evaluate the effect of knocking down the expression of ARMS/Kidins220 in astrocytes in R6/1 pre-symptomatic mice.

2.3. Surgery, cannula implantation, and GSK2606414 treatment *in vivo*

WT and R6/1 mice were anesthetized with a mixture of oxygen and isoflurane (4% isoflurane for induction and 2% for maintenance) and small holes were drilled in the skull to target the lateral ventricle at the following coordinates: - 0.22mm AP, + 1mm ML, and - 2.4mm DV. Stainless steel guide cannulas (26-G, Plastics One Inc®, Roanoke, VA, USA) were lowered and secured to the skull using surgical screws and acrylic dental cement. Dummy cannulas with the same length to that as the guide cannula were inserted to prevent clogging. One week after surgery, mice were pre-treated with the PERK inhibitor GSK2606414 (MCE®, NJ, USA Cat. #HY-18072) (Axten et al., 2012) (20µM) during 3 alternate days, and then a daily drug administration routine was performed for one week during the behavioral analysis (see the experimental procedure in Figure 12). GSK2606414 was dissolved in DMSO and further diluted in PBS to a final DMSO concentration of 0.5%. Mice received daily 1µL of vehicle or 1µL of 20µM GSK2606414 using infusion cannulas 0.5mm longer than guide cannulas. Drugs were infused at 0.5 µl/min in awake and freely moving mice. After infusion, the cannula was left for an additional 3 minutes to ensure proper diffusion and avoid reflux.

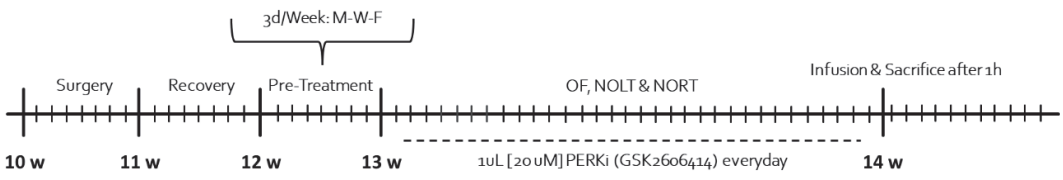


Figure 12. Experimental timeline. Scheme displaying the protocol used to evaluate the effect of reducing PERK activity administering GSK2606414 intraventricularly.

3. BEHAVIORAL ASSESSMENT

Cognitive functions and spontaneous locomotor activity were evaluated in R6/1 and R6/1:GRP78$^{+/-}$ mice at 12-16 weeks of age. Motor learning was assessed by the accelerating rotarod task (ARTP) while spatial and recognition long-term memory was assessed by the novel object location and recognition tasks (NOLT and NORT) respectively. Spontaneous locomotor activity was determined in the Open-field test. Protocols were performed as previously described (Brito, Giralt, Enriquez-Barreto, Puigdellívol, Suelves, Zamora-moratalla, et al., 2014).

3.1. Evaluation of cognitive functions

3.1.1. Novel object Location Test

Exploration took place in a quadrangular open field (40 × 40cm sides, 40cm high). The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19-22 °C, with a humidity of 40-60%. Mice were first habituated to the arena in the absence of objects with spatial cues attached to the walls for 15 min for 2 consecutive days. Spontaneous locomotor activity and time/distance in the periphery/center were measured during this phase. On the third day (NOLT training), 2 identical objects (A and A') were placed in two adjacent corners of the arena, and mice were allowed to explore for 10 min. 24 hours later (NOLT testing), one copy of the familiar object (A) was placed in the same location whereas the other object (A") was placed in the corner diagonally opposite. During testing, mice were allowed to explore for 5 min. The arena was rigorously cleaned between animals to avoid odors. Animals were tracked and recorded with SMART® Junior software (Panlab, Spain). Exploration times were recorded and used to calculate the Discrimination index as follows:

$$\frac{time\ exploring\ novel\ object - time\ exploring\ familiar\ object}{time\ exploring\ both\ objects} \times 100$$

3.1.2. Novel object Recognition Test

Exploration took place following the same standards as indicated before in the NOLT. Mice were first habituated to the arena in the absence of objects for 15 min for 2 consecutive days. Spontaneous locomotor activity and time/distance in the periphery/center were measured during this phase. On the third day (NORT training), mice were allowed to explore two identical objects (B and B') for 10 min before returning to their home cage. 24 hours later (NORT testing), one copy of the familiar object (B) and a new object (C) were placed in the same location as during the training session, and animals were allowed to explore for 5 min. The arena was rigorously cleaned between animals to avoid odors. Animals were tracked and recorded with SMART® Junior software (Panlab, Spain). Exploration times were recorded and used to calculate the Discrimination index as indicated above.

3.2. Evaluation of motor function

3.2.1. Open-field Test

The Open-field test consisted of a white quadrangular arena (40 × 40cm sides, 40cm high) with opaque white walls without external cues. Mice were placed in the center of the arena and were allowed to move freely for 15 min for two consecutive days while being tracked with SMART® Junior software (Panlab, Spain). The arena was rigorously cleaned between trials to eliminate odors. For analysis, spontaneous locomotor activity was measured as the total distance traveled and results show the average distance traveled during the two consecutive days.

3.2.2. Accelerating Rotarod

The ARTP was performed as described in (Puigdellívol et al., 2015). Animals were placed on a motorized rod and rotation speed was gradually increased from 4 to 40 rpm over the course of 5 min. Time latency was recorded when the animal was unable to keep up with the increasing speed and fell. The task was performed four times per day for three consecutive days for a total of 12 trials. Different trials within the same

day were separated by 1 h. Results show the average fall latencies per trial during the 3 days of training.

3.2.3. Fixed Rotarod

The fixed rotarod test was performed to assess motor coordination instead of learning. In this format of the task, there are two main phases: the training phase and the testing one. During the training phase, mice are placed on the rod at a fixed speed of 10 rpm twice per day (spaced 1h in time) for 3 consecutive days and the number of falls is recorded until reaching a latency time of 60 seconds on top of the rod. Only those animals that learned to stay on the rod will proceed to the testing phase. In this case, testing was performed weekly at 10, 16, and 24 rpm, and the number of falls in 60 seconds was recorded.

3.2.4. Balance beam

Mice were placed on a flat-surfaced wooden-squared beam (50 cm long and 13mm wide) and a rounded metal beam (50 cm long and 9mm wide) divided into 5 cm frames. Each beam was placed horizontally, 40-50 cm above the bench surface with nesting materials resting beneath to cushion any falls. The test consisted of two sessions, the training, and the testing, separated by at least 4 h. Animals were allowed to walk for 2 min along each beam, and the number of slips, the time to cross 30 frames, and the total number of crossed frames were measured in each session.

3.2.5. Vertical Pole

The test takes place during 3 consecutive days consisting of 2 days of training followed by a testing day with 3 trials per day separated by 1 hour in time. The pole apparatus consists of a 50 cm-tall pole wrapped in tape to facilitate walking on it, resting in a home cage with nesting materials. Mice are placed just below the top of the vertical pole with their head pointed upwards and are tasked to descend to the ground without pausing. The subject's ability to descend to the floor by turning its body around

without falling is used to assess its locomotor function or dysfunction. The time to completely turn and the time to climb down the pole were measured every trial. The trials when mice fell from the pole were counted and the ones when mice descended the pole without complete reorientation were counted as error trials and analyzed separately.

4. CELL CULTURES

4.1. Primary astrocytic cultures

Striatums obtained from mouse pups at postnatal day 2 (P2) were dissected, mechanically dissociated, and seeded onto 6-well plates pre-coated with 0.1 mg/mL poly-D-lysine (Sigma-Aldrich®, Cat. #P0899) in MEM medium (Gibco®, Cat. #31095-029) supplemented with 20% FBS (PAN Biotech®, Cat. #P40-37500), 90 mM glucose (Sigma®, Cat. #G5400) and 1% penicillin/streptavidin (Gibco®, Cat. #15090-046). At DIV5 cultures were left overnight shacking to detach other cell types from the astrocytic culture and the medium was changed the following day. Cells were passaged at DIV7 with trypsin, centrifuged for 10 minutes at 500 g, and seeded at different densities depending on the experiment (Table 1) onto new coverslips or plates. The medium was changed every 3-4 days and cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Embryos' genotype was determined by PCR analysis from a tail biopsy.

Table 1. Cell density of astrocytic cultures at passage DIV7.

CELL NUMBER	PLATE	DENSITY	EXPERIMENT
20,000 cells	12mm coverslip	10,000 cells/cm^2	Immunocytochemistry, DIV14
200,000 cells	6-well plate	20,000 cells/cm^2	ACM to treat neurons, DIV21
250,000 cells	p60	12,000 cells/cm^2	Western Blot, DIV14
100,000 cells	6-well plate	10,000 cells/cm^2	Western Blot, DIV21
60,000 cells	6-well plate	6,000 cells/cm^2	Western Blot, DIV28

4.2. Primary neuronal cultures

Mechanically dissociated striatal cells from previously dissected E17.5 WT and R6/1 mice embryos were plated onto 12 mm coverslips in 24-well plates for immunocytochemistry, and onto 12-well plates for Western blot analysis, respectively. Plates were pre-coated with 0.1 mg/mL poly-D-lysine (Sigma-Aldrich®, Cat. #P0899), and dissociated cells were cultured with Neurobasal Medium (Gibco®, Cat. #21103-049) supplemented with 1% Glutamax (Gibco®, Cat. #35050-038) and 2% B27 (Gibco®, Cat. #17504-044) for 14 days in vitro (DIV) (Table 2). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Embryos' genotype was determined by PCR analysis from a tail biopsy.

Table 2. Cell density of neuronal cultures.

CELL NUMBER	PLATE	DENSITY	EXPERIMENT
80,000 cells	12mm coverslip	40,000 cells/cm^2	Treatment with ACM
80,000 cells	12mm coverslip	40,000 cells/cm^2	Immunocytochemistry
750,000 cells	6-well plate	80,000 cells/cm^2	Western Blot

5. CELL BIOLOGY METHODS

5.1. Treatments *in vitro*

To stimulate astrocytes and promote secretion, primary striatal astrocytes at DIV21 were treated with potassium chloride (KCl) 50 mM dissolved in ACSF buffer containing 1mM HEPES-NaOH (pH 7.4), 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5.6 mM Glucose for 1 hour at 37 °C in a humidified atmosphere containing 5% CO_2. After treatment, cells were harvested for Western blot (see section 6.1.2 in this chapter) and supernatants were collected for further analysis.

5.2. Lentivirus and AAV transduction

Primary striatal astrocytes at DIV14 were transfected with lentiviruses expressing different small hairpin RNA (shRNA) constructs for ARMS (sh1, sh2, and sh3) or with sh-scramble. Following 6 days of transduction, the levels of ARMS were analyzed in astrocytic lysates by Western blot using specific antibodies against ARMS and GFP.

To knock down ARMS expression in astrocytes, primary striatal astrocytes at DIV9 were transduced using a medium containing AAV2/5-GFAP-shRNA-GFP or AAV2/5-GFAP-shARMS-GFP at 200,000 multiplicities of infection (MOI). 24 hours later, the medium was changed and GFP expression was visualized at DIV21 with an inverted microscope to confirm efficient transduction before proceeding to the KCl stimulation.

5.3. Neuronal Branching and viability assessment

To evaluate the effect of the ARMS downregulation in astrocytes on neuronal morphology and viability, WT and R6/1 primary neurons at DIV14 were treated with the ACM from ARMS knockdown primary striatal astrocytes after treatment with KCl. 24 hours later, cells were fixed and stained with MAP2 (see section 8.1 in this chapter) and DAPI. Digital images of 5 randomized fields per coverslip were acquired with a 20x objective in a ZEISS Axio Observer Z1 epifluorescence microscope. For the experiment, 5 different embryos were analyzed per condition with two replicates each. Automated quantification of branching and cell viability of these images was performed using the free open-source Cell Profiler® 4.2.5 and Cell Profiler Analyst® 3.0.4 software (http://cellprofiler.org/) using specific custom-made pipelines as previously described (Barriga et al., 2017). All images were background corrected to enhance the neuronal body by applying a threshold module. The intensity of the threshold determined whether each pixel was treated as a region of interest or background. Nuclei were detected from the DAPI channel with the “IdentifyPrimaryObjects” module. An adaptive threshold mask was set to obtain MAP2-positive staining. After filtering for nuclei inside the MAP2 mask, “IdentifySecondaryObjects” with nuclei as the seed was used to define the neuronal soma and dendrites. The “MorphologicalSkeleton” and

"MeasureSkeleton" modules were then applied to obtain measurements of the number of trunks, non-trunks, endpoints, and total tree length of each cell.

6. MOLECULAR BIOLOGY METHODS

6.1. Protein extraction

6.1.1. Extraction from mouse brain samples

Animals were sacrificed by cervical dislocation at different ages. Brains were quickly removed, dissected, frozen in dry ice, and stored at -80 °C until use. Brain tissue was homogenized by sonication in lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 50 Mm NaF, 1% NP-40, 10% glycerol supplemented with 1mM sodium orthovanadate and protease inhibitor mixture (Sigma-Aldrich®, Cat. #P8340), centrifuged for 15 minutes at 16.000g at 4 °C and the supernatants were collected.

6.1.2. Extraction from primary cultures

To obtain protein extracts, primary cultures were washed with PBS and incubated with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40 and supplemented with 1mM sodium orthovanadate, and protease inhibitor cocktail (Sigma-Aldrich®, Cat. #P8340). Cells were harvested using a scraper and disaggregated by pipetting up and down through an insulin syringe. Finally, samples were centrifuged at 13,200 g at 4 °C for 15 min and supernatants were collected.

6.2. Protein quantification

Protein concentration was determined with a colorimetric assay using the Detergent-Compatible Protein Assay kit (Bio-Rad®, Cat. #5000116) as indicated in the manufacturer's instructions. Absorbance was measured at 650-750 nm using the spectrophotometer Synergy™ 2 Multi-Detection Microplate Reader (BioTek) in duplicates and only those with a standard deviation lower than 0.5 were considered for further analysis.

6.3. Western Blot

Protein extracts (15-20µg) were denatured in SDS sample buffer (62.5mM Tris-HCl (pH=6.8), 2% (w/v) SDS, 10% glycerol, 140mM ß-mercaptoethanol, and 0.1% (w/v) bromophenol blue) and boiled at 100 ºC for 5 min. They were resolved in 6%-15% polyacrylamide gels (SDS-PAGE) at 30mA for 1h. Proteins were then transferred to a nitrocellulose membrane (Amersham® by GE Healthcare Life Sciences®, Cat. #10600002) for 1.5 hours at 100 V at 4 ºC to avoid excessive warming. After 1h incubation in blocking buffer containing 10% non-fat powdered milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20) membranes were blotted overnight (o/n) at 4 °C with the following primary antibodies (Table 3):

Table 3. Primary antibodies for Western blot. A list of primary antibodies is provided as well as their source, molecular weight, dilution, and reference number.

Antigen	MW (kDa)	Host species	Dilution	Source	ID
Arc	55	Mouse	1:500	Santa Cruz	sc-17839
ARMS/kidins220	220	Rabbit	1:500	-	-
ATF6	90	Mouse	1:1000	Novus Biological	NBP1-40256SS
BDNF	17-35	Mouse	1:1000	Icosagen	3C11
c-fos	62	Rabbit	1:1000	Cell Signalling	4384
CHOP	27	Rabbit	1:1000	Novus Biological	NBP2-13172
CREB	43	Rabbit	1:1000	Cell Signalling	9197S
P-CREB(S133)	43	Rabbit	1:1000	Millipore	06-519
Egr1	75	Rabbit	1:1000	Cell Signalling	4154S
GFP	36	Rabbit	1:1000	ThermoFisher	A11122
Grp78	78	Rabbit	1:2000	Abcam	ab21685
IRE1α	110	Rabbit	1:1000	Novus Biological	NB100-2324SS
P-IRE1α(S724)	110	Rabbit	1:1000	Novus Biological	NB100-2323SS
LC3B	14-16	Rabbit	1:1000	Cell Signalling	27755
p62	62	Mouse	1:1000	Abcam	ab56416
PERK	170	Rabbit	1:1000	Cell Signalling	3192
P-PERK(T980)	170	Rabbit	1:1000	ThermoFisher	MA5-15033

PSD95	95	Rabbit	1:2000	Cell Signalling	34505
P-PSD95(T19)	95	Rabbit	1:1000	Abcam	ab16496

Membranes were then rinsed three times for 10 min with TBS-T and incubated for 1h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Table 4). After washing for 30 min with TBS-T, membranes were developed using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology®, Cat. #sc-2048) in a ChemiDoc imaging system (Bio-Rad®).

Table 4. Secondary antibodies for Western blot. A list of secondary antibodies is provided as well as their source, molecular weight, dilution, and reference number.

Antigen	Dilution	Source	ID
Anti-goat IgG	1:3000	Promega	V8051
Anti-mouse IgG	1:3000	Promega	W4021
Anti-rabbit IgG	1:3000	Promega	W4011

Loading control antibodies (Table 5) were incubated for 20 min at RT and then washed and developed as the other primary antibodies. ImageLab® Software Version 6.0 (2017) was used to quantify band intensity relative to the loading control.

Table 5. Primary antibodies for loading control for Western blot. A list of primary antibodies is provided as well as their source, molecular weight, dilution, and reference number used for the western blot analysis.

Antigen	MW (kDa)	Host species	Dilution	Source	ID
Actin	42	Mouse	1:50000	Sigma	A3854
Tubulin	55	Mouse	1:50000	Sigma	6074

6.4. Enzyme-linked immunosorbent assay (ELISA)

Quantification of BDNF protein levels in supernatants was directly measured with an ELISA kit for mouse BDNF (Cloud-Corp®, Cat. #SEA011Mu) according to the manufacturer's instructions. In brief, the flat-bottomed 96-well microplate was pre-coated with an antibody specific to BDNF. Standards (7-500 pg/ml) and samples were then added to the appropriate microplate wells with a biotin-conjugated antibody specific to BDNF. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. After the addition of the TMB substrate, only those wells that contain BDNF, biotin-conjugated antibody, and enzyme-conjugated Avidin exhibited a color change. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of BDNF in the samples is then determined by comparing the O.D. of the samples to the standard curve.

6.5. Mouse cytokine array profiler

Secretome samples from ARMS knockdown astrocytes exposed to KCl (50 mM for 1 hour) were assessed using a proteome profiler array: Mouse Cytokine Array Kit (R&D Systems®, UK, Cat. #ARY006). The array detects 40 mouse cytokines, chemokines, and acute phase proteins simultaneously.

The Proteome Profiler Mouse Cytokine Array Kit is a membrane-based sandwich immunoassay. Samples are mixed with a cocktail of biotinylated detection antibodies and then incubated with the array membrane which is spotted in duplicate with capture antibodies to specific target proteins. Captured proteins are visualized using chemiluminescent detection reagents and developed using a ChemiDoc imaging system (Bio-Rad®). The signal produced is proportional to the amount of analyte bound. An average signal of pixel density was determined for the pair of duplicate spots and the background was subtracted.

6.6. Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from primary striatal astrocytic cultures from P2 WT and R6/1 pups using the RNeasy Lipid Tissue Mini Kit (QIAGEN®, Cat. #74804). The concentration of RNA was measured with Nanodrop 1000 spectrophotometer (ThermoFisher). 500 ng of purified RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. #436814). The cDNA synthesis was performed at 25 °C for 10 min, at 37 °C for 120 min, and a final step at 85 °C for 5 min in a final volume of 20 µl as instructed by the manufacturer. Then, cDNA was analyzed by quantitative RT-PCR using PrimeTime qPCR Assays (Integrated DNA Technologies) (Table 6). Quantitative PCR was performed in a final volume of 12 µl on 96-well plates with Premix Ex Taq (Takara Biotechnology, Cat. #RR037A). Reactions included Segment 1: 1 cycle of 30 seconds at 95 °C and Segment 2: 40 cycles of 5 seconds at 95 °C and 20 seconds at 60 °C. All RT-PCR assays were run in duplicate. To provide negative control and exclude contamination by genomic DNA, the PrimeScript RT enzyme was omitted in the cDNA synthesis step, and samples were subjected to the PCR reaction in the same manner as each probe. RT-PCR data were quantified using the comparative quantitation analysis program of 64 MxProTM quantitative PCR software version 3.0 (Stratagene) and actinβ gene expression was used as a housekeeping gene.

Table 6. Probes used for gene expression analysis. For each gene, information about the reference assay and source is shown.

GENE	ASSAY	SOURCE
Actinβ	Mm.PT.39a.22214843.g	Integrated DNA Technologies
Kidins220	Mm.PT.58.29496516	Integrated DNA Technologies
BDNF	Mm.PT.58.8157970	Integrated DNA Technologies

7. HISTOLOGY

7.1. Tissue fixation

Animals were deeply anesthetized and immediately perfused intracardially with 4% (weight/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) after perfusion with ice-cold PBS for 5 min. Brains were dissected out and postfixed for 48-72h shacking at 4 °C in the same solution. Coronal sections (30µm) of the whole brain were obtained using a vibratome (Leica® VT1000), collected as free-floating, and maintained in PBS with 0.02% sodium azide at 4 °C until their posterior processing.

7.2. Nissl staining and stereology

Nissl staining was performed in floating sections (30µm in width) separated 240µm each, after incubation with Cresyl violet solution (0.1 g/L) for 45 min. Then, sections were dehydrated in increasing ethanol concentrations (70% - 100%) and finally mounted with DPX. Bright-field images were acquired at ×4 with an AF600 Leica microscope, and hippocampal volumes were estimated following the Cavalieri method as described in (Brito, Giralt, Enriquez-Barreto, Puigdellívol, Suelves, Zamora-Moratalla, et al., 2014).

8. IMMUNOFLUORESCENCE

8.1. Immunocytochemistry

Primary neuronal or astrocytic cultures grown on 12mm coverslips were fixed at DIV14 with 4% PFA for 10 min, rinsed in PBS, and treated with 0.1 M glycine for 15 min to quench formaldehyde crosslinking. Coverslips were washed three times with PBS and then permeabilized with 0.1% saponin for 10 min. Cells were blocked with PBS containing Normal Horse Serum (NHS) 15% for 30 min at RT and incubated overnight at 4 °C with the appropriate primary antibodies diluted in NHS 5% in PBS (Table 7).

Table 7. Primary antibodies used for immunocytochemistry. A list of primary antibodies is provided as well as their source, dilution, and reference number.

ANTIGEN	SPECIES	DILUTION	SOURCE	ID
GFAP-488	-	1:250	Invitrogen	53-9892-82
GLAST-PE	-	1:50	Miltenyi	130-118-344
MAP2	Mouse	1:500	Sigma-Aldrich	M 1406

After incubation, coverslips were washed three times to remove the remaining primary antibody and incubated for 1h at RT with the appropriate fluorescent secondary antibodies (Table 8) diluted in NHS 5% in PBS. Finally, coverslips were rinsed and mounted on microscope slides with DAPI-Fluoromount-G® (Southern Biotech, Cat. #0100-20).

Table 8. Secondary antibodies used for immunocytochemistry. A list of secondary antibodies is provided as well as their source, dilution, and reference number.

ANTIGEN	DILUTION	SOURCE	ID
Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG	1:100	Jackson ImmunoResearch	715-545-150
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG	1:100	Jackson ImmunoResearch	711-545-152
Cy™3 AffiniPure Donkey Anti-Mouse IgG	1:100	Jackson ImmunoResearch	715-165-150

8.2. Immunohistofluorescence

Free-floating coronal sections were washed twice with PBS and incubated with 50 mM NH_4Cl for 30 min to block tissue auto-fluorescence. Following three washes with PBS, the sections were permeabilized and blocked with antibody buffer containing PBS with 0.3% Triton X-100 (PBST 0.3%), 0,02% sodium azide, 2% bovine serum albumin (BSA) and 3% normal goat serum (NGS, Pierce Biotechnology®) for 1 hour at room temperature with constant shaking. Brain slices were then incubated overnight at 4 °C with the following primary antibodies:

Table 9. Primary antibodies used for immunohistochemistry. A list of primary antibodies is provided as well as their source, dilution, and reference number.

ANTIGEN	SPECIES	DILUTION	SOURCE	ID
GFAP	Mouse	1:500	Sigma Aldrich	G3893
ARMS/kidins220	Rabbit	2µg/µl	-	-
GFP	Rabbit	1:500	ThermoFisher	A11122

The remaining primary antibody was washed away with PBST 0.3% and samples were incubated with appropriate secondary antibodies for 2 hours at room temperature. Nuclei were stained for 10 minutes with 4',6-diamidino-2-phenylindole (DAPI) (1:5000, Sigma Aldrich®, Cat. #D9542). Brain sections were mounted on the slides, dried, and covered with Mowiol (Merck®, Darmstadt, Germany) to incorporate the coverslip.

Table 10. Secondary antibodies used for immunohistochemistry. A list of secondary antibodies is provided as well as their source, dilution, and reference number.

ANTIGEN	DILUTION	SOURCE	ID
Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG	1:100	Jackson ImmunoResearch	715-545-150
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG	1:100	Jackson ImmunoResearch	711-545-152
Cy™3 AffiniPure Donkey Anti-Mouse IgG	1:100	Jackson ImmunoResearch	715-165-150

8.3. Imaging and Analysis

Immunofluorescence was examined by confocal microscopy using a Leica TCS SP5 confocal microscope (Leica® Microsystems) or Zeiss LSM880 confocal microscope (Zeiss®). Digital images were obtained with 63.0x 1.40 OIL or 63.0x glycerol objective depending on the experiment. For each image, z-stacks were taken from the entire three-dimensional structure. The digital zoom and size of the stack depended on the experiment.

9. IMMUNOPEROXIDASE STAINING FOR mHtt AGGREGATES

For EM48 immunohistochemistry, sections were first incubated for 30 minutes in 0.01 M sodium citrate buffer (pH 6) in a water bath at 80 °C for antigen retrieval. Then, endogenous peroxidases were blocked in PBS containing 5% H_2O_2 for 45 minutes, and nonspecific protein interactions were also blocked with PBS containing 3% normal horse serum. Tissue was incubated overnight at 4 °C with anti-EM48 (1:500, Merck Millipore®, Cat. #MAB5374), diluted in PBS containing 0.02% sodium azide and 3% normal horse serum. Sections were then incubated at room temperature for 2 hours with a biotinylated anti-mouse antibody (1:200; Pierce; ThermoFisher Scientific®), diluted in PBS containing 3% normal horse serum. Sections were washed three times in PBS for 5 minutes each and incubated for 1.5 hours at room temperature in Reagent A (Avidin) and Reagent B (Biotinylated HRP), each of them diluted at 1:56 in PBS, from the ABC kit (Pierce, ThermoFisher Scientific®, Cat. #32020). The immunohistochemical reaction was developed by incubating the samples for 7 minutes in diaminobenzidine (DAB), diluted at 1× in PB 0.1 M and 5% H_2O_2. Sections were mounted in gelatinized slides and were left at room temperature to dry completely to finally mount them with DPX. Three coronal hippocampal sections per animal spaced 240µm apart were chosen for the analysis. Bright-field images from 100% of the CA1 were acquired at 40× magnification with an AF600 Leica microscope. Automated quantification of the number of nuclear huntingtin aggregates in the CA1 hippocampal region and striatum was performed using the Trainable Weka Segmentation Fiji Plugin within the Image J® software (a specific pipeline file was created).

10. DENDRITIC SPINE ANALYSIS: GOLGI-COX STAINING

Following the manufacturer's instructions, we performed the Golgi-Cox impregnation using the Rapid Golgistain Kit (FD Neurotechnologies®, Cat. #PK401). Mice were killed by cervical dislocation at 18-19 weeks of age, brains were quickly removed, and both hemispheres were separated and incubated in a mix of solution A/B for 2 weeks. Then, brains were changed to solution C for 7 days and eventually processed using a cryostat to obtain 100µm sections mounted in gelatin-coated slides left to dry for 24 h. Sections

were stained with the kit's provided solutions and finally, sections were dehydrated and mounted with DPX. We obtained bright field images using a Leica AF6000 epifluorescence microscope with a ×63 numerical aperture objective for dendritic spine analysis. Segments of apical dendrites from hippocampal CA1 pyramidal neurons and MSNs were selected and spine density was measured manually in the stacks using the ImageJ Plug-in Cell Counter. Spines were marked in the appropriate focal plane preventing double counting and counted in dendritic segments ranging from 20 to 60µm in length.

11. STATISTICAL ANALYSIS

All raw data were processed and analyzed using Excel® Microsoft Office and GraphPad Prism® software version 8.0. Results are expressed as the mean ± standard error of the means (SEM). Outliers were identified with the ROUT method and the normality of data was analyzed following the Shapiro-Wilk test. Statistical analysis was performed using the unpaired Student's *t*-Test (two-tailed) for comparisons between two groups. For multi-component variables comparison, One-way or Two-way ANOVA was performed followed by the appropriate *post hoc* tests as indicated in the figure legends. P values below 0.05 were considered statistically significant.

Schematic illustrations were created with BioRender.com

RESULTS

1. EMERGING ROLE OF THE GRP78-PERK AXIS IN HUNTINGTON'S DISEASE COGNITIVE IMPAIRMENTS.

ER stress is a common pathological feature of neurodegenerative diseases including HD that leads to the activation of the UPR signaling cascade to ensure correct proteostasis and cellular health (Hetz & Saxena, 2017; Riccardi et al., 2015). Upon activation, UPR sensors induce an initial protective phase that, if unresolved, will eventually lead to a pro-apoptotic stage triggering the intrinsic apoptotic pathway within the cell (Tabas & Ron, 2011; Urra et al., 2013). Particularly, chronic upregulation of UPR markers has been detected in HD cellular and mouse models as well as in HD patients (Carnemolla et al., 2009; Duennwald & Lindquist, 2008; Leitman et al., 2013, 2014; Reijonen et al., 2008; Shacham et al., 2019). However, how the different UPR branches are affected depending on the brain region, and the potential impact on memory formation and synaptic function, two major neuropathological hallmarks of HD, remain to be addressed.

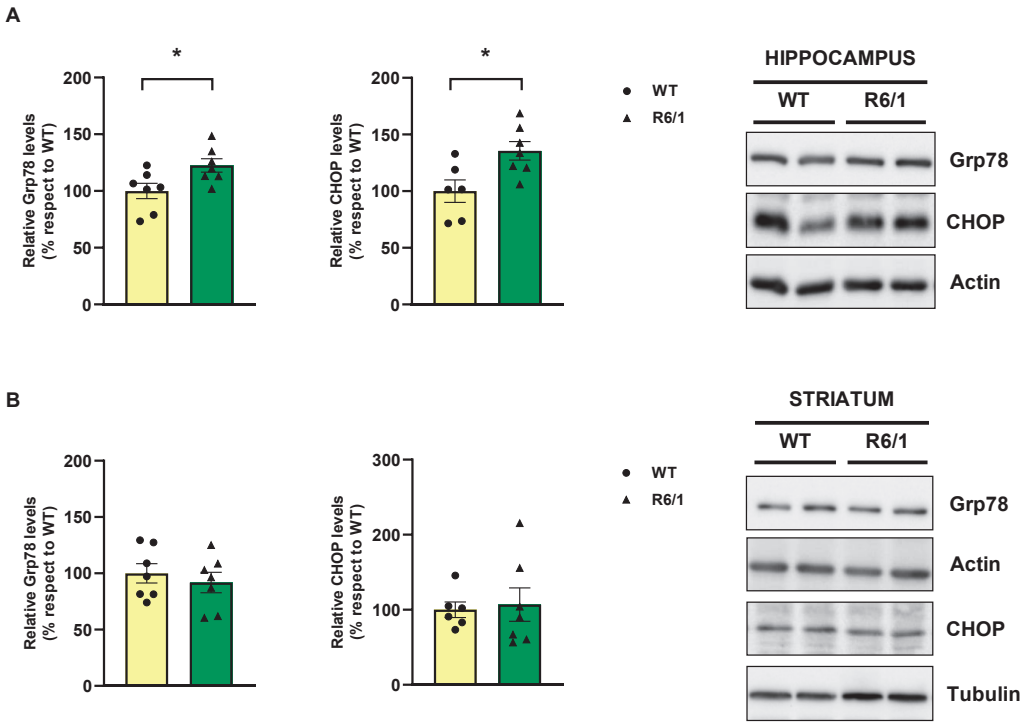
Therefore, with this first study, we aimed to obtain a detailed and comprehensive picture of the ER stress response in HD, and the particular role it plays in the cognitive alterations associated with this devastating neurodegenerative disorder.

1.1. ER stress assessment in the R6/1 mouse model.

To characterize the ER stress response in HD, we first analyzed in the striatum, cortex, and hippocampus of R6/1 mice the levels of two critical ER stress-related proteins, namely GRP78, the main chaperone that acts as an ER stress sensor (Casas, 2017; M. Wang et al., 2009) and CHOP, a key pro-apoptotic player that upregulates under chronic ER stress conditions (Tabas and Ron, 2011; Urra et al., 2013; Cai et al., 2015).

1.1.1. GRP78 and CHOP levels are significantly increased in the hippocampus of R6/1 mice at pre-symptomatic disease stages.

Western blot analysis was performed on hippocampal, striatal, and cortical extracts obtained from R6/1 mice at the age of 12 weeks, right before cognitive deficits appear (Figure 13). Increased protein levels of GRP78 and CHOP were found in the hippocampus of R6/1 mice compared to WT mice (Figure 13A). In contrast, no significant changes were detected either for GRP78 or CHOP within the striatum of R6/1 mice (Figure 13B), while a slight but significant decrease was only observed for cortical GRP78 levels (Figure 13C). These data demonstrate a region-specific upregulation of the ER stress response in the hippocampus of R6/1 mice manifested as an increase in the protein levels of both GRP78 and CHOP at pre-symptomatic stages of the disease.



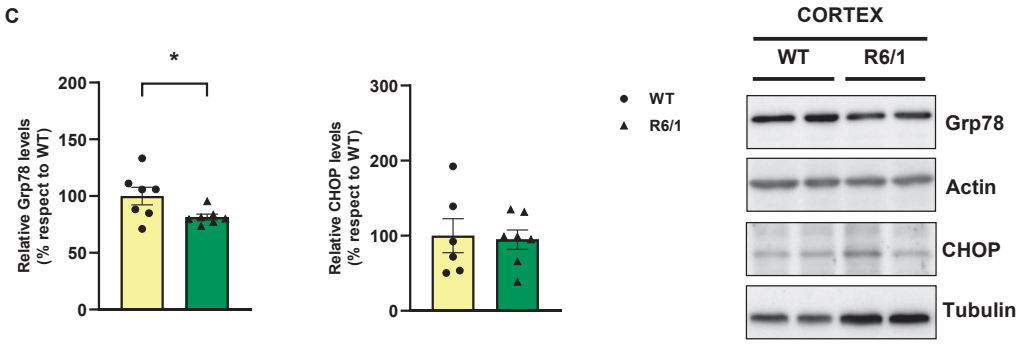


Figure 13. GRP78 and CHOP levels are significantly increased in the hippocampus of R6/1 mice at pre-symptomatic disease stages. Representative immunoblots and densitometric analysis showing GRP78 and CHOP levels in total lysates from **A** hippocampal, **B** striatal, and **C** cortical extracts of WT and R6/1 mice at 12 weeks of age. Actin and tubulin were used as the loading control. Histograms represent the relative protein levels expressed as a percentage of WT values. *P<0.05: compared to WT by Student's *t*-test. All data represent the mean ± SEM (n = 6-7 mice/group).

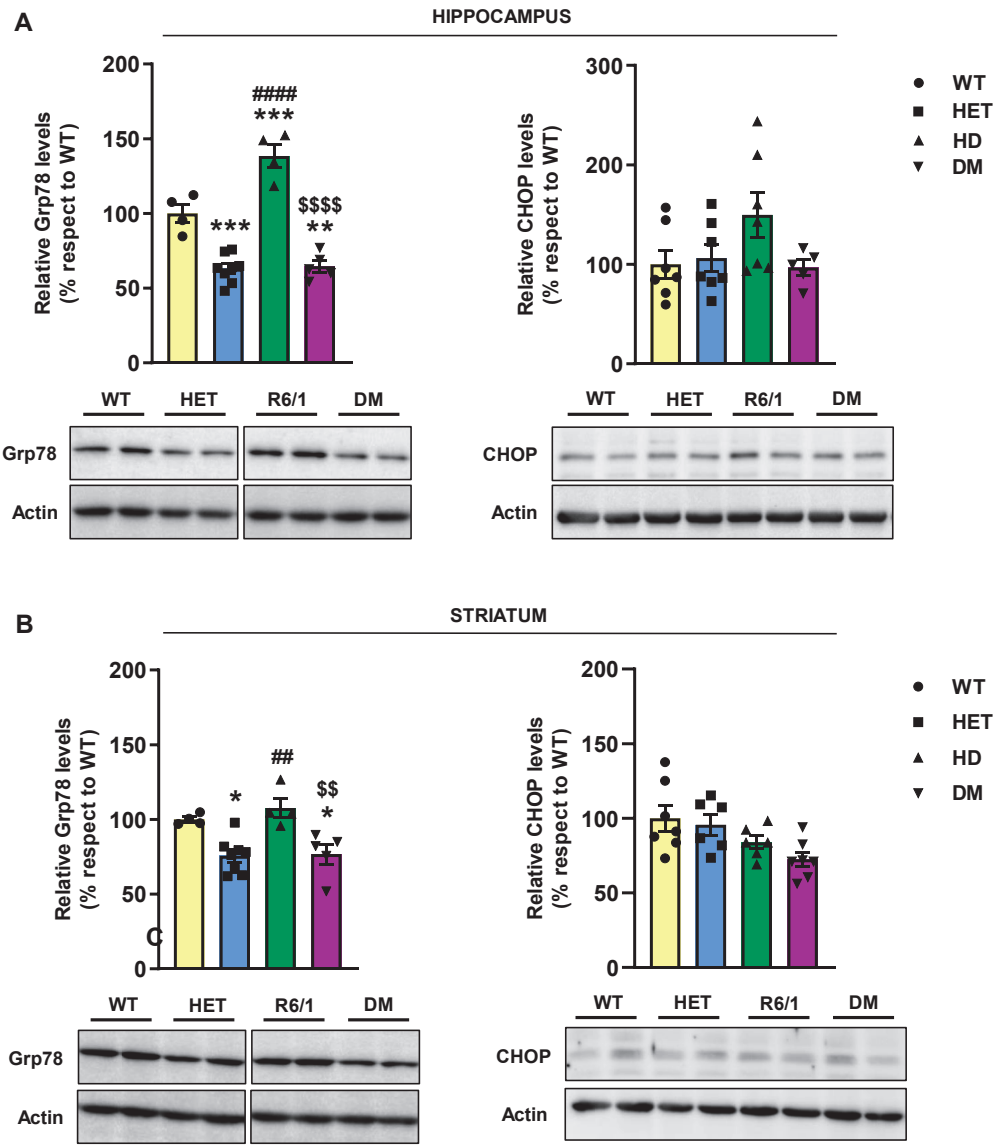
1.2. Genetic manipulation of GRP78 in R6/1 mice.

To further explore the contribution of GRP78 to the neuropathology and cognitive impairments in HD, we generated a new double-mutant (R6/1:GRP78$^{+/-}$) mice line, hereafter DM, by cross-mating R6/1 mice with heterozygous GRP78$^{+/-}$ mice (S. Luo et al., 2006).

1.2.1. GRP78 levels are reduced in the hippocampus of R6/1:GRP78$^{+/-}$ mice.

To assess the ER stress response in the resultant DM mice, we first analyzed the levels of GRP78 and CHOP in hippocampal, striatal, and cortical extracts at 12 weeks of age by Western blot. Our results revealed a significant reduction in the hippocampal levels of GRP78 in DM mice similar to those found in heterozygous GRP78$^{+/-}$ mice, together with normalization for CHOP expression to WT levels (Figure 14). Next, we determined the levels of GRP78 and CHOP in striatal and cortical extracts. No significant changes for GRP78 were detected between R6/1 and WT mice in those areas. However, we observed a slight but significant reduction in the striatum of DM mice when compared to WT mice. Finally, no differences in CHOP expression were detected between

genotypes in either the striatum or the cortex. These results demonstrate that the genetic reduction of GRP78 expression in R6/1 mice normalizes hippocampal CHOP protein levels suggesting an improvement in the overall ER stress response.



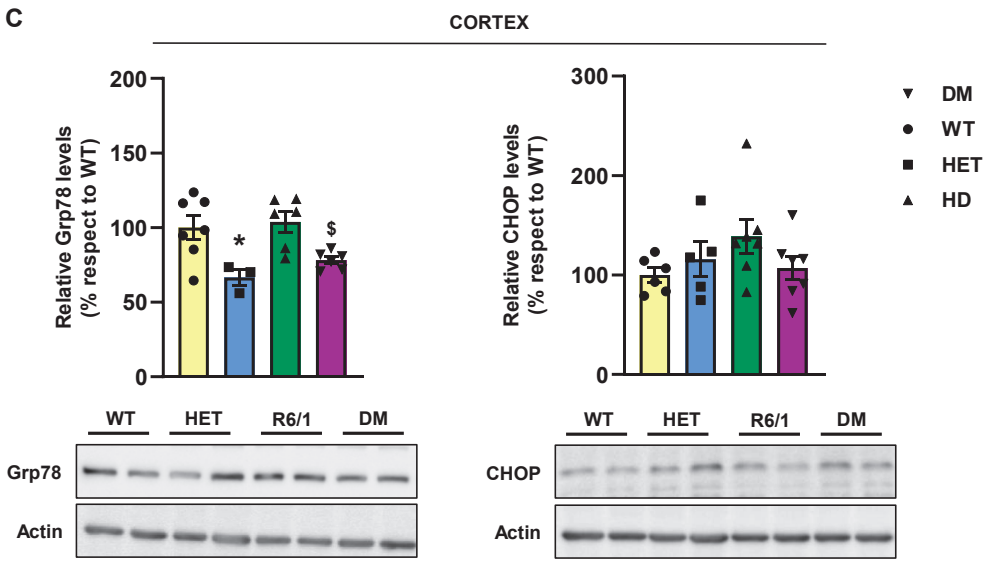


Figure 14. Grp78 levels are reduced in the hippocampus of DM (R6/1:GRP78$^{+/-}$) mice. Representative immunoblots and densitometric analysis showing GRP78 and CHOP levels in total lysates from **A** hippocampal, **B** striatal, and **C** cortical extracts of WT, heterozygous GRP78$^{+/-}$ (HET), R6/1 and double-mutant R6/1:GRP78$^{+/-}$ (DM) mice at 12 weeks of age. Actin was used as the loading control. Histograms represent the relative protein levels expressed as a percentage of WT values. *P<0.05, **P<0.01, ***P<0.001: compared to WT. ##P<0.01, ####P<0.0001: compared to HET. $$P<0.01, $$$$P<0.0001: compared to R6/1. All data were analyzed by One-way ANOVA followed by Tukey's test and represent the mean ± SEM (n = 4-7 mice/group).

1.2.2. Genetic reduction of GRP78 expression in R6/1 mice prevents hippocampal-dependent cognitive deficits.

Long-term spatial and recognition memory was then evaluated in DM mice at 13 weeks of age by the Novel Object Location and Recognition Tasks (NOLT/NORT) respectively (Figure 15). At this age, R6/1 mice display long-term memory deficits (Anglada-Huguet et al., 2014; Giralt et al., 2013; Giralt, Saavedra, et al., 2011, 2012b; Miguez et al., 2015). During the training sessions, all mice were presented with two similar objects (A and A') that they explored equally showing no preference for either location or object (Figure 15B and 15C). 24 hours later when long-term memory was assessed, R6/1 mice were unable to discriminate between the old and new location (NOLT; Figure 15B) nor between the familiar and novel object (NORT Figure 15C). Notably, DM mice exhibited

preserved spatial and recognition memory with a similar percentage of time exploring the new location as well as discrimination indexes comparable to WT mice.

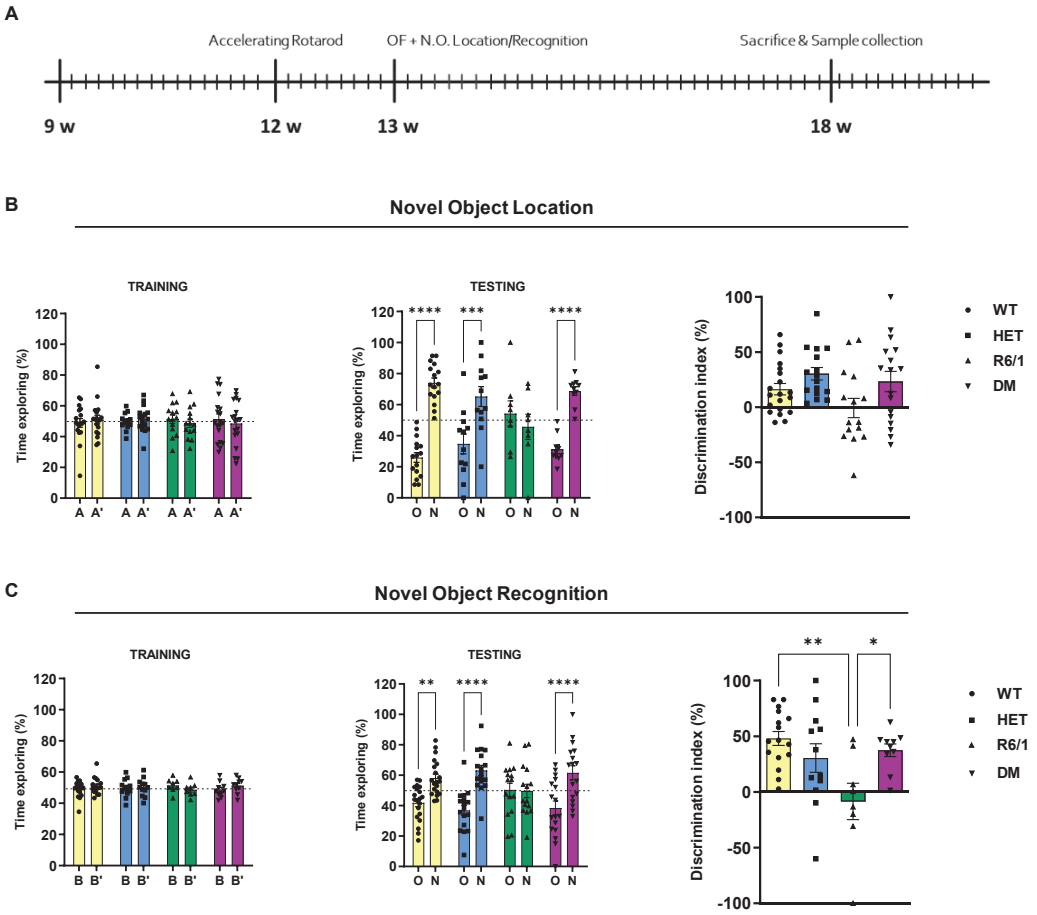
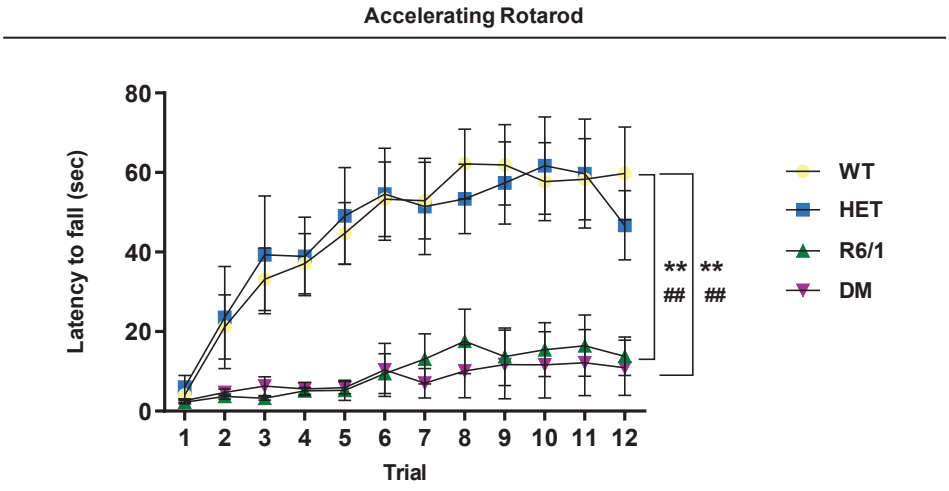


Figure 15. Genetic reduction of GRP78 levels prevents hippocampal-dependent cognitive deficits in R6/1 mice. A Timeline representing the experimental behavioral design. Performance in spatial **B** (NOLT) and recognition **C** (NORT) memory tests. **Left:** Exploration time during training and testing sessions expressed as a percentage (%). The dashed line marks the chance level of exploration. Data were analyzed by Two-way ANOVA followed by Sídák's Test. **P<0.01, ***P<0.001, ****P<0.0001 compared to the percentage of time exploring the familiar object. **Right:** Discrimination indexes are expressed as a percentage (%). Data were analyzed by One-way ANOVA followed by Tukey's test. *P<0.05, **P<0.01. All data represent the mean ± SEM (n>8 mice/group).

1.2.3. Genetic reduction of GRP78 expression in R6/1 mice does not affect motor learning or spontaneous locomotor activity.

Given the beneficial effect of GRP78 reduction in cognitive deficits, we next aimed to evaluate the impact on motor performance. To this aim, motor learning and spontaneous locomotor activity were evaluated using the accelerating rotarod task and the open-field tests respectively (Figure 16). Performance on a gradually accelerating rotarod was evaluated at 12 weeks of age and both R6/1 and DM mice showed poor motor learning ability over trials compared to either WT or heterozygous GRP78$^{+/-}$ mice (Figure 16A). When the spontaneous locomotor activity was assessed in the open-field test, once again both R6/1 and DM mice traveled less distance in the arena over the course of 15 minutes compared to WT or heterozygous GRP78$^{+/-}$ mice (Figure 16B). To exclude anxiety-like behaviors due to the reduction of GRP78 expression, we calculated the percentage of time spent in the periphery versus the center of the open-field arena and found no significant differences between genotypes. These results suggest that by lowering GRP78 levels in R6/1 mice, hippocampal-dependent long-term memory deficits can be prevented while no beneficial effect can be observed in motor learning or spontaneous locomotor activity.

A



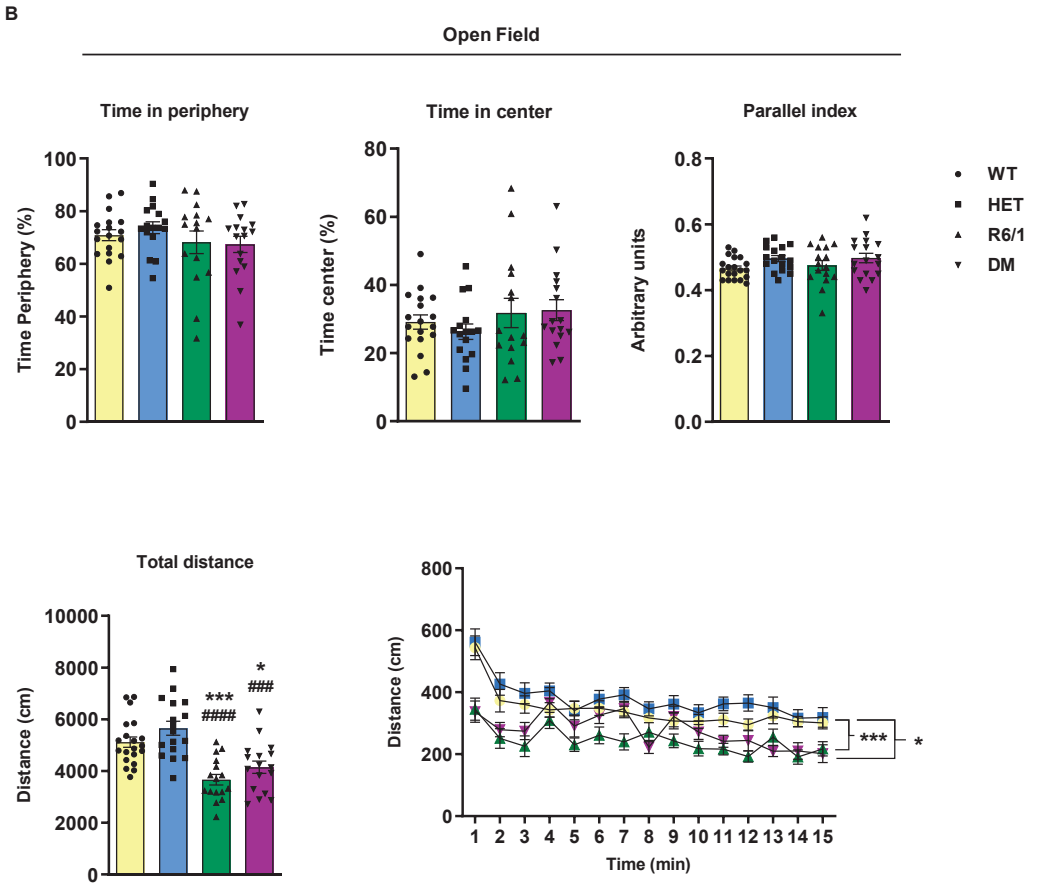


Figure 16. Motor learning and spontaneous locomotor deficits are not prevented in R6/1 mice after the genetic reduction of GRP78 levels. A Latency to fall in the accelerating rotarod task at 12-13 weeks of age. **P<0.01: compared to WT. ##P<0.01: compared to HET. Data were analyzed by Two-way ANOVA followed by Tukey's test. All data represent the mean ± SEM (n = 18-20 mice/group). **B** Anxiety-like behavior was measured by the time spent in the periphery/center of the open field arena. Spontaneous locomotor behavior was analyzed by measuring the total distance traveled and parallel index. Data were analyzed by One-way ANOVA followed by Tukey's test. *P<0.05, **P<0.01, ***P<0.001: compared to WT mice. ##P<0.01, ###P<0.001, ####P<0.001: compared to HET mice. All data represent the mean ± SEM (n = 12-15 mice/group).

1.2.4. Genetic reduction of GRP78 expression in R6/1 mice partially prevents motor coordination symptoms.

To evaluate motor coordination, performance in the rotarod was assessed weekly in a different cohort of animals from 10 to 18 weeks of age at fixed speeds of 10, 16, and 24 rpm after a training phase at a fixed speed of 10 rpm conducted at 9 weeks of age (Figure 17). At the end of the training phase, only those mice that learned to stay on

top of the rod were tested thereafter. Motor coordination was severely impaired in R6/1 mice starting at 13 weeks of age, worsening thereafter when compared to WT mice throughout trials. Interestingly, DM mice slightly reduced the number of falls compared to R6/1 mice, partially improving overall motor coordination, and delaying the onset of motor coordination deficits.

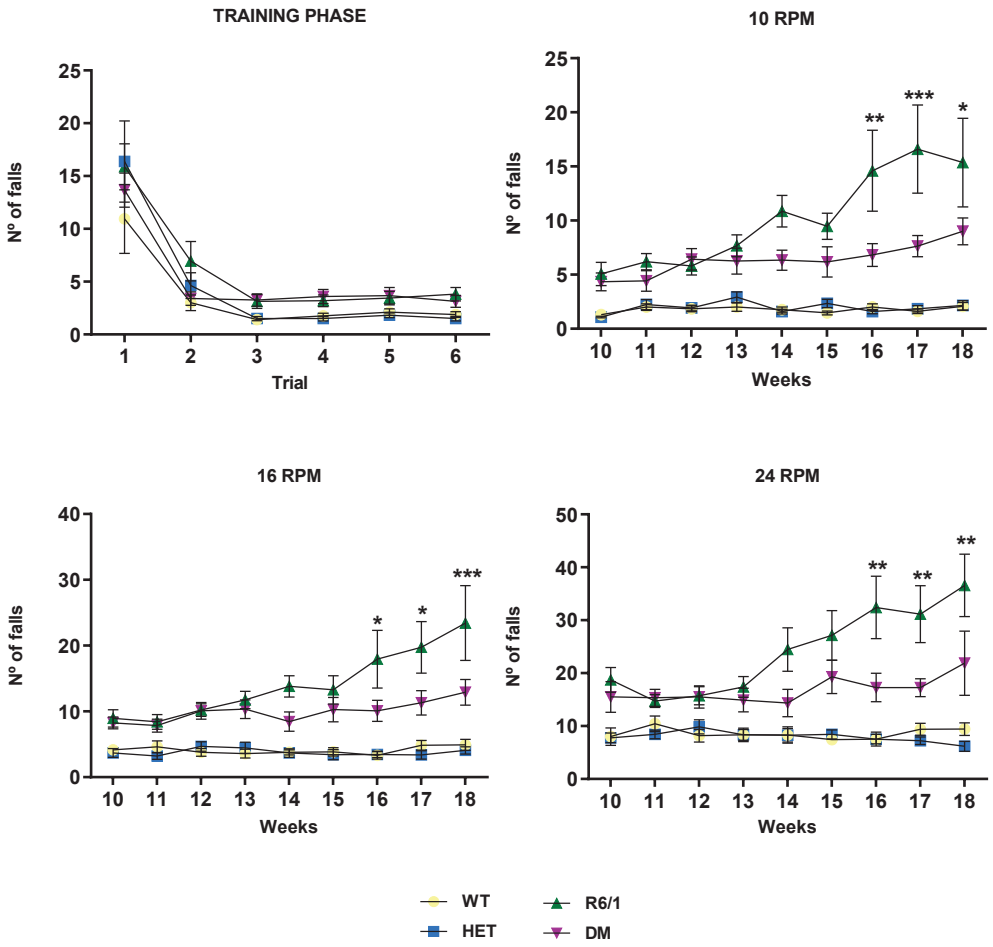


Figure 17. Genetic reduction of GRP78 expression in R6/1 mice partially prevents motor coordination symptoms. Motor coordination was assessed by performing the fixed rotarod test. At 9 weeks old, the number of falls in 60 seconds was recorded at a fixed speed of 10 rpm. Testing was performed weekly at 10, 16, and 24 rpm, and the number of falls was recorded. Data were analyzed by Two-way ANOVA followed by Tukey's test. *P<0.05,**P<0.01, ***P<0.001: DM compared to R6/1 mice. All data represent the mean ± SEM (n = 12-15 mice/group).

1.3. Genetic reduction of GRP78 levels improves hippocampal and striatal neuropathology.

Given that the genetic reduction of GRP78 in R6/1 mice had a positive impact on hippocampal-dependent cognitive disturbances and slightly improved motor coordination deficits, we next focused our research on how GRP78 may contribute to hippocampal and striatal neuropathology.

1.3.1. Genetic reduction of GRP78 levels does not alter the overall gross hippocampal or striatal volumes.

Striatal volume loss is a pathological HD hallmark that occurs in HD brains due to extensive atrophy and loss of medium spiny neurons (MSN) (Graveland et al., 1985; J. P. Vonsattel et al., 1985a). On the contrary, hippocampal volumes do not differ significantly between HD and control groups (Glikmann-Johnston et al., 2021). While the R6/1 model has an absence of MSN cell death, striatal volume loss is still observed due to the smaller soma size of MSNs (Barriga et al., 2017; Hansson et al., 1999). In line with these data, we found a significant reduction in striatal volume in R6/1 mice compared to WT mice at the age of 18 weeks. No significant reduction was observed for DM mice, suggesting that genetic reduction of GRP78 levels prevents striatal volume loss at this age in DM mice (Figure 18A). Conversely, we observed no significant differences in hippocampal volumes between genotypes, indicating no gross hippocampal anatomical changes in either R6/1 or DM mice (Figure 18B).

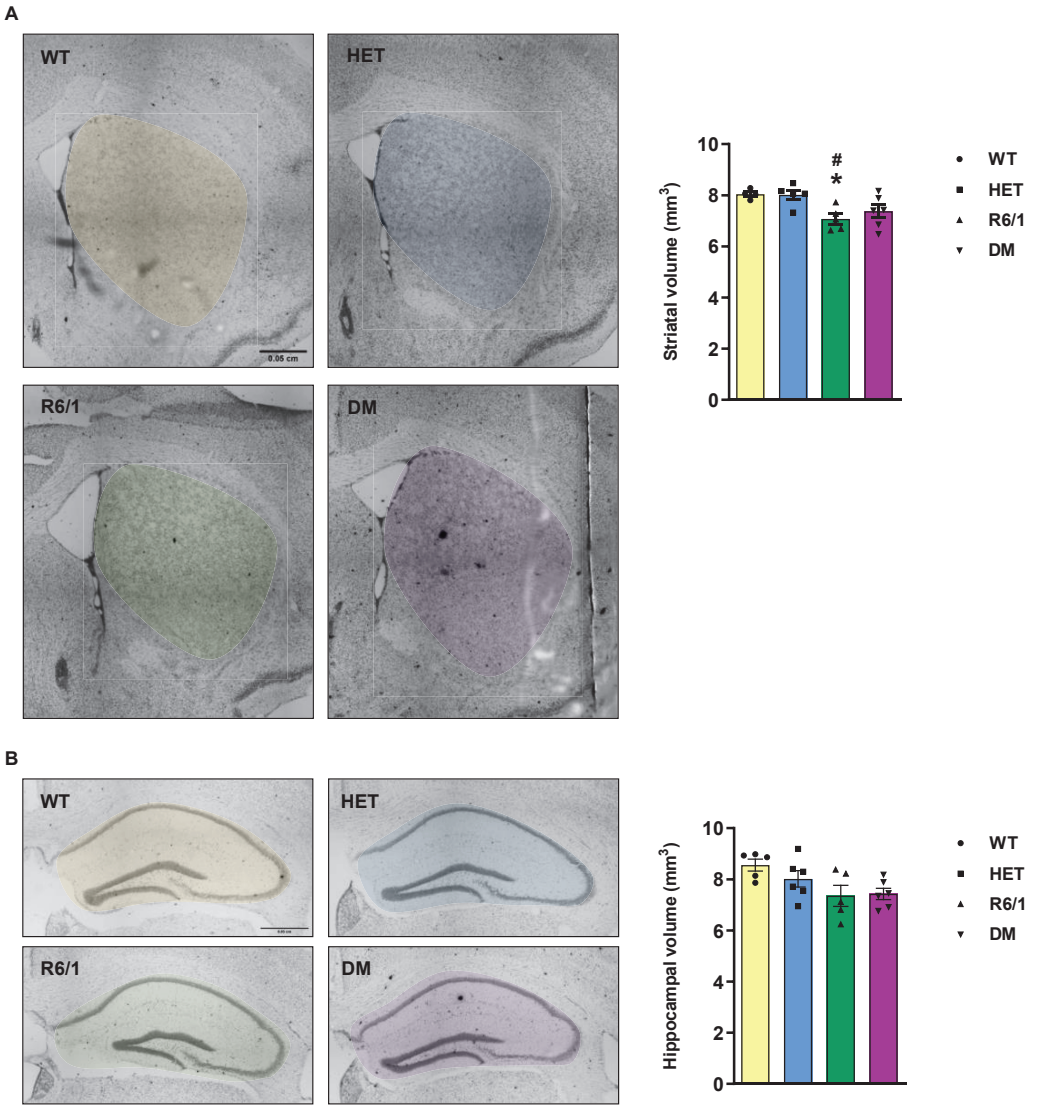
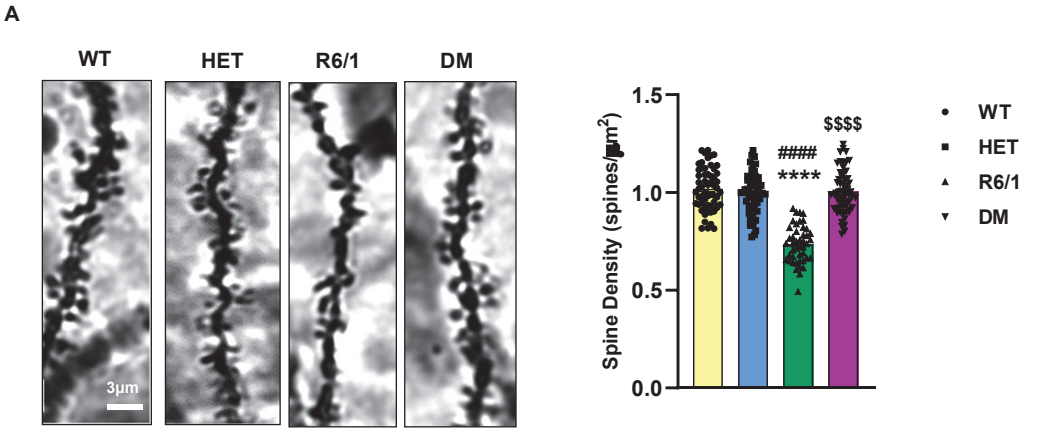


Figure 18. Genetic reduction of GRP78 levels does not alter the overall gross hippocampal or striatal volumes. A Striatal and **B** hippocampal volumes of Nissl-stained coronal sections were stereologically quantified in 18-week-old WT, heterozygous GRP78$^{+/-}$ (HET), R6/1 and double-mutant R6/1:GRP78$^{+/-}$ (DM) mice. Data were analyzed by One-way ANOVA followed by Tukey's test. All data represent the mean ± SEM (n = 5-6 mice/group). Scale bar 0.05cm.

1.3.2. Genetic reduction of GRP78 levels prevents dendritic spine loss in R6/1 hippocampal CA1 pyramidal neurons and striatal MSN.

Previous studies have reported dendritic spine loss in both HD patients (Ferrante et al., 1991; Graveland et al., 1985) and mouse models of HD (Guidetti et al., 2001; Murmu et al., 2013; Spires et al., 2004) which has been associated with cognitive impairments. Thus, to investigate the potential role of GRP78 in modulating structural synaptic changes in the striatum and hippocampus of HD, we conducted a dendritic spine density analysis by Golgi staining in both apical CA1 pyramidal neurons and MSNs (Figure 19). Our results showed in both areas a significant reduction in spine density in R6/1 mice compared to WT mice. Notably, the reduction of GRP78 expression in DM mice completely recovered spine density in the hippocampus (Figure 19A) and only partially in the striatum (Figure 19B), suggesting that improvements in spatial and recognition memory as well as in motor coordination in DM mice could be related to the recovery of dendritic spine density.



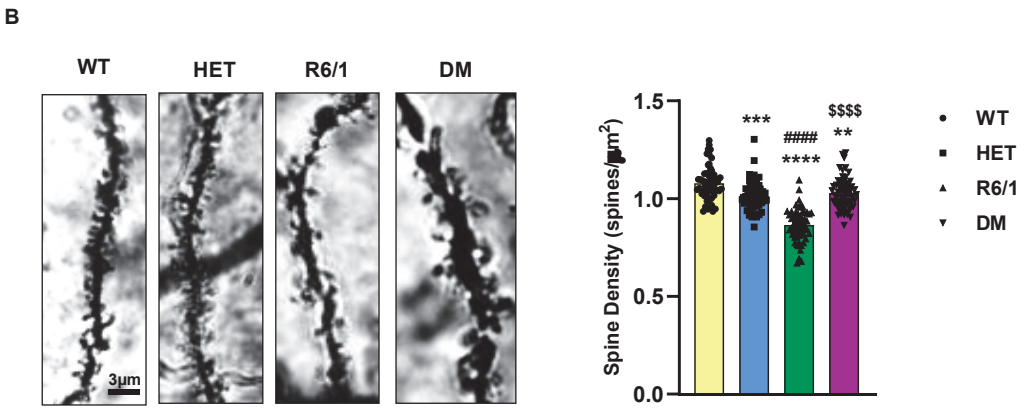


Figure 19. Genetic reduction of GRP78 levels prevents dendritic spine loss in R6/1 hippocampal CA1 pyramidal neurons and striatal MSN. Representative photomicrographs of **A** apical dendrites from CA1 pyramidal neurons and **B** striatal dendrites from MSN from WT, heterozygous GRP78$^{+/-}$ (HET), R6/1 and double-mutant R6/1:GRP78$^{+/-}$ (DM) mice. Histograms show quantitative analysis of dendritic spine density per micrometer of dendritic length. Data were analyzed by One-way ANOVA followed by Tukey's test. **P<0.01, ***P<0.001, ****P<0.0001: compared to WT. $$$$ P<0.0001: compared to R6/1. #### P<0.0001: compared to HET. All data represent the mean ± SEM (>50 dendrites/group: n = 4 mice/group). Scale bar 3µm.

1.3.3. Genetic reduction of GRP78 levels in R6/1 mice decreases the formation of hippocampal and striatal intranuclear inclusions.

The presence of mHtt aggregates is another well-known neuropathological hallmark of HD (G. Bates, 2003; DiFiglia et al., 1997; J. P. Vonsattel et al., 1985). Therefore, to evaluate whether GRP78 contributes to mHtt aggregate formation, we performed an immunohistochemical analysis of the EM48 antibody staining in coronal slices of the hippocampus and striatum from 18–19-week-old R6/1 and DM mice. As previously described by our group (Anglada-Huguet et al., 2014; García-Forn et al., 2018), we observed a very strong positive nuclear staining for EM48, along with the presence of intranuclear inclusions (NIIs) in the hippocampal CA1 region and the striatum of R6/1 mice (Figure 20). Remarkably, DM mice exhibited a significant and prominent reduction in aggregate density in both areas suggesting that the reduction of GRP78 in R6/1 mice may participate in delaying the onset of striatal and hippocampal HD neuropathology.

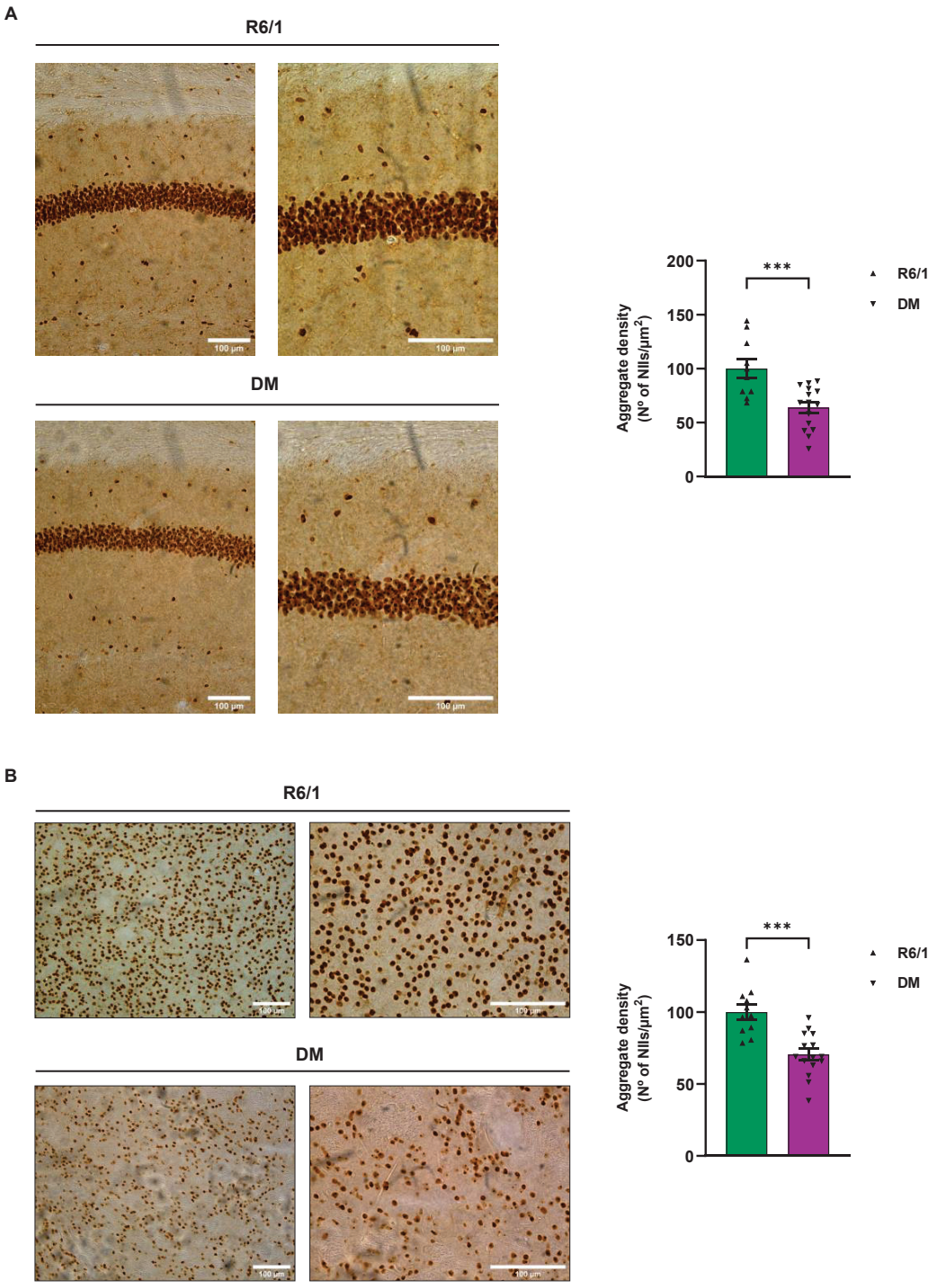


Figure 20. The formation of hippocampal and striatal intranuclear inclusions is also diminished by the genetic reduction of GRP78 in R6/1 mice. Representative photomicrographs at 10X and 20X showing nuclear EM48 immunostaining in the **A** hippocampal CA1 region and **B** striatum of R6/1 and double-mutant R6/1:GRP78$^{+/-}$ (DM) mice. Scale bar 100 µm. Histograms represent the aggregate density of EM48 positive neuronal intranuclear inclusions (NIIs). Each symbol represents the mean density of aggregates from one

CA1 hemisphere. ***P <0.001 compared to R6/1 mice by Student's *t*-test. Data represent the mean ± SEM (All CA1 NIIs were quantified in each hemisphere. n = 5-6 mice/group).

1.4. Specific activation of the PERK pathway in the hippocampus of R6/1 mice at pre-symptomatic stages.

Recent evidence indicates that the activation of the UPR sensor PERK could be the downstream mechanism that explains how the ER stress response leads to cognitive impairment (Shacham et al., 2021). Building upon the observed positive effect of the GRP78 reduction on memory deficits in R6/1 mice, we next investigated the potential role of the PERK pathway activation in HD cognition.

1.4.1. Phosphorylated PERK is significantly increased in the hippocampus of R6/1 mice at pre-symptomatic disease stages.

To this aim, the three major UPR sensor proteins: PERK, ATF6, and IRE1α were analyzed by Western blot in the hippocampus of 12-week-old R6/1 mice. The activation of PERK and IRE1α was evaluated by measuring its phosphorylation at Thr980 and Ser724, respectively, while ATF6 activity was calculated by measuring its proteolytic cleavage (Figure 21). Western blot analysis in hippocampal extracts from WT and R6/1 mice detected a significant increase in p-PERK levels in R6/1 mice compared to age-matched WT mice whereas p-IRE1α and ATF6 levels (full-length and cleaved forms) remained unaltered. This suggests a specific and selective activation of the PERK axis in the hippocampus of R6/1 mice in the early stages of the disease.

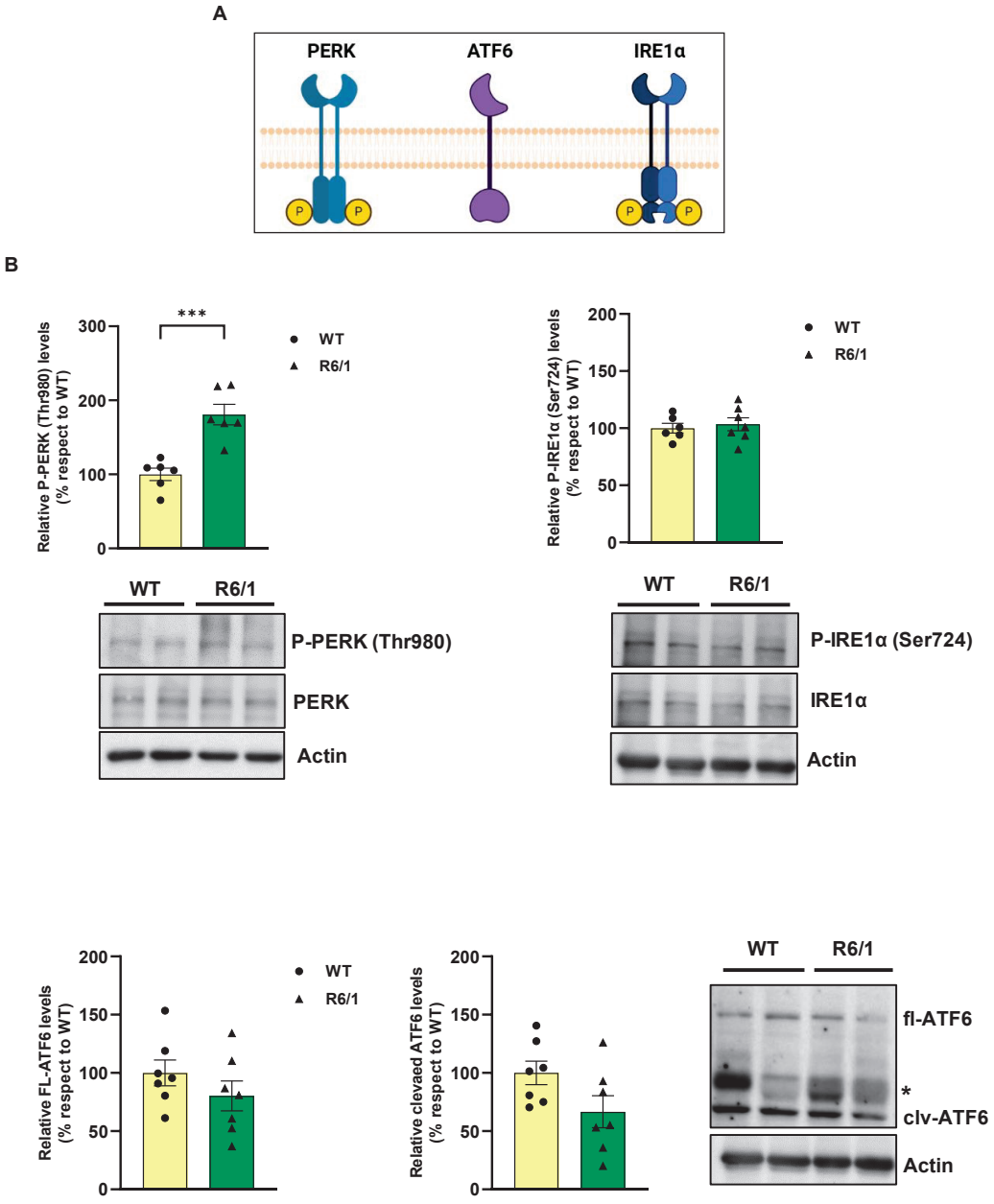
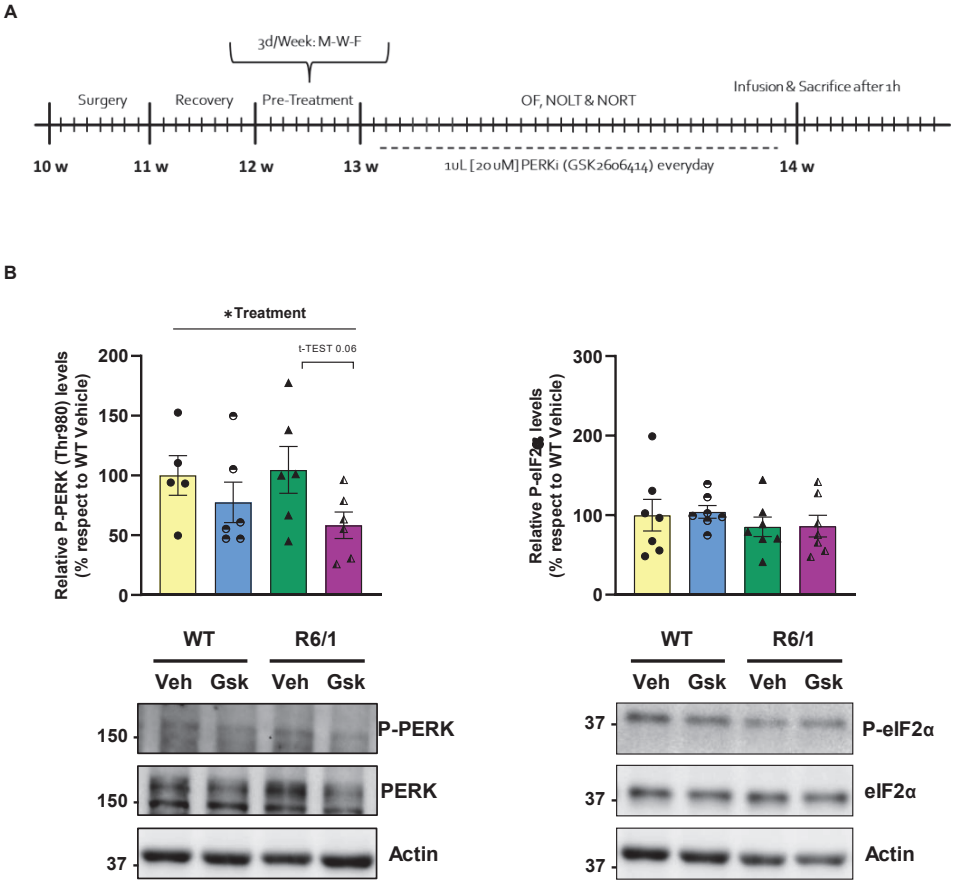


Figure 21. P-PERK levels are significantly increased in the hippocampus of R6/1 mice at pre-symptomatic disease stages. A Representative illustration showing the three main UPR sensors. **B** Representative immunoblots and densitometric analysis showing P-PERK (Thr980), PERK, P-IRE1α (Ser724), IRE1α, fl-ATF6 (full-length) and clv-ATF6 (cleaved) levels in total lysates from hippocampal extracts of WT and R6/1 mice at 12 weeks of age. (*) indicates an unspecific band recognized by the antibody. Actin was used as the loading control. Histograms represent the relative protein levels expressed as a percentage of WT values. ***P<0.001: compared to WT by Student's *t*-test. All data represent the mean ± SEM (n = 6-7 mice/group).

1.4.2. Treatment with the PERK inhibitor GSK2606414 prevents long-term memory deficits in R6/1 mice.

Given the aberrant increase in PERK activity found in the hippocampus of R6/1 mice, we next addressed whether PERK inhibition could prevent memory impairments in R6/1 mice (Figure 22). To achieve this aim, PERK activity was reduced by infusing intraventricularly the PERK inhibitor GSK2606414 (20µM) on 3 alternate days before the cognitive evaluation at 12 weeks of age, and then daily during the behavioral assessment (Figure 22A). First, we evaluated the effect of GSK2606414 on PERK phosphorylation by Western blot. We observed a significant treatment effect on p-PERK levels with decreased phosphorylation both in WT and R6/1 mice (Figure 22B). Since the role of PERK in hippocampal-dependent memory has been associated with the activation of the eukaryotic translation initiation factor eIF2α (Ma et al., 2013; Moreno et al., 2012), we next analyzed the phosphorylation of eIF2α at Ser51 in the vehicle and GSK2606414-treated R6/1 mice (Figure 22B).



Surprisingly, no significant changes were detected in p-eIF2α between either genotypes or treatments, suggesting that the PERK/eIF2α axis is not involved in R6/1 memory disturbances. Next, the effect of PERK inhibition on long-term spatial and recognition memory was evaluated with the novel object location and recognition tests (NOLT/NORT), respectively (Figure 22C). In line with our previous results (Figure 15), vehicle-treated R6/1 mice were unable to discriminate between new locations or objects in the NOLT and NORT tasks. On the contrary, R6/1 mice treated with the PERK inhibitor showed a similar percentage of time exploring both the novel locations and objects compared to vehicle-treated WT mice. Interestingly, and given the critical role of PERK in cognitive functions (Sharma et al., 2018; Zhu, Henninger, et al., 2016), GSK2606414-treated WT mice worsened their performance in both tasks compared to WT vehicle-treated mice as they could not accurately discriminate new locations or objects. Altogether, these results suggest an important contribution of ER stress on recognition and spatial memory deficits in R6/1 mice through the hyperactivation of PERK signaling.

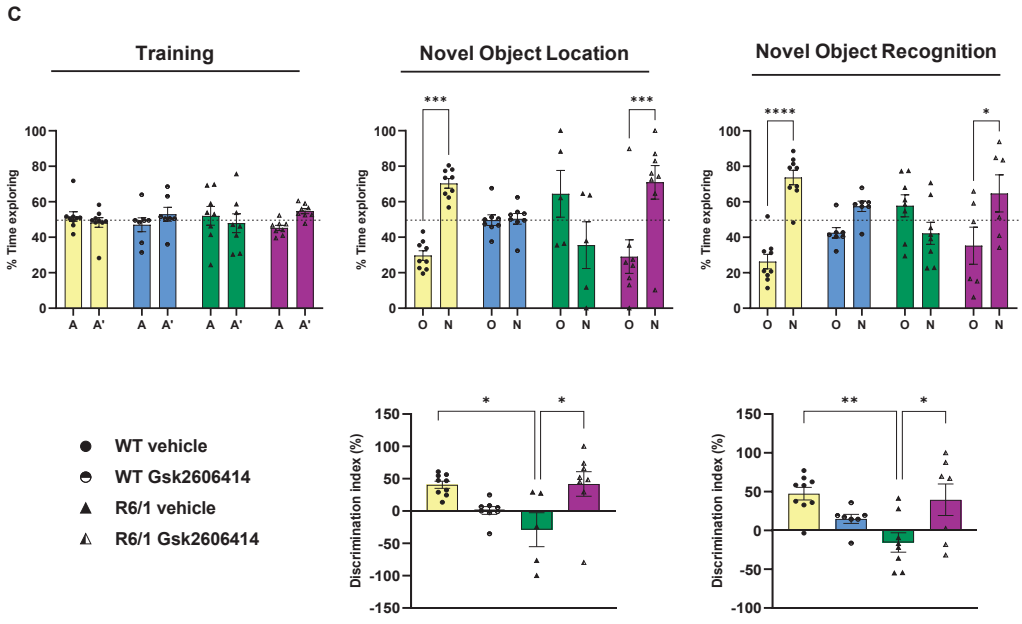


Figure 22. **Treatment with the PERK inhibitor GSK2606414 prevents long-term memory deficits in R6/1 mice. A** Timeline representing the experimental design. **B** Representative immunoblots and densitometric analysis showing hippocampal levels of P-PERK (Thr980), PERK, P-eIF2α (Ser51), and eIF2α from GSK2606414-treated mice. Actin was used as the

loading control. **C** Performance in spatial and recognition memory tests. Exploration time during training and testing sessions is expressed as a percentage (%). The dashed line marks the chance level of exploration. Data were analyzed by Two-way ANOVA followed by Sídák's test. *P<0.05, ***P<0.001, ****P<0.0001 compared to the percentage of time exploring the familiar object. All data represent the mean ± SEM (*n* = 7-9 mice/group). Discrimination indexes are expressed as percentages (%). Data were analyzed by One-way ANOVA followed by Tukey's test. *P<0.05, **P<0.01. All data represent the mean ± SEM (*n* = 7-9 mice/group).

1.4.3. Treatment with the PERK inhibitor GSK2606414 slightly improves spontaneous locomotor activity.

We also evaluated whether the inhibition of PERK in R6/1 mice had any major impact on motor performance (Figure 23). To this aim, spontaneous locomotor activity was evaluated in the open-field test. Our results reveal a slight but significant effect in treated R6/1 mice when the mean speed in the periphery was measured, while no positive effect was observed in the total distance traveled (Figure 23A). To exclude anxiety-like behaviors due to the reduction of PERK activation, we calculated the percentage of time spent in the periphery versus the center of the open-field arena and found no significant differences between genotypes or treatments (Figure 23B). All in all, these results suggest that PERK activity is not critically involved in the spontaneous locomotor deficits of R6/1 mice.

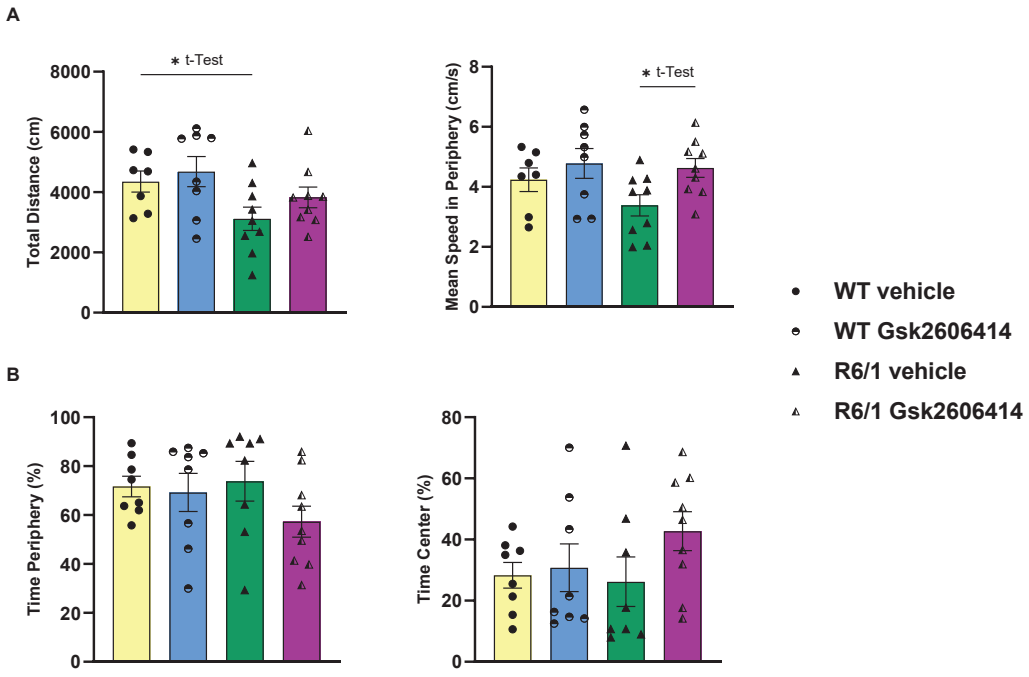


Figure 23. Treatment with the PERK inhibitor GSK2606414 slightly improves spontaneous locomotor activity. A Spontaneous locomotor behavior was analyzed by the Open-field Test measuring the total distance traveled and mean speed. **B** Anxiety-like behavior was measured by the time spent in the center of the open field arena during two consecutive days (15 min/day). Data were analyzed by One-way ANOVA followed by Tukey's test. All data represent the mean ± SEM (n = 7-9 mice/group).

1.4.4. Pharmacological inhibition of PERK restores hippocampal dendritic spine density in R6/1 mice.

Our previous results suggest that treatment with GSK2606414 prevents memory decline in R6/1 mice independently of eIF2α phosphorylation. Therefore, we were interested in defining other molecular mechanisms by which the inactivation of PERK could be exerting its beneficial effects. Since the reduction of GRP78 expression had a positive impact on hippocampal structural synaptic plasticity, we next evaluated whether the inhibition of PERK could have a similar effect. Notably, GSK2606414 treatment in R6/1 mice restored the dendritic spine loss in CA1 pyramidal neurons. However, in accordance with the observed negative effect of PERK inhibition in spatial and recognition memories in WT mice, we found a reduced number of dendritic spines in treated WT mice compared to the WT vehicle. Overall, these results indicate an involvement of the GRP78/PERK axis in hippocampal structural synaptic alterations and, therefore, in HD cognitive deficits.

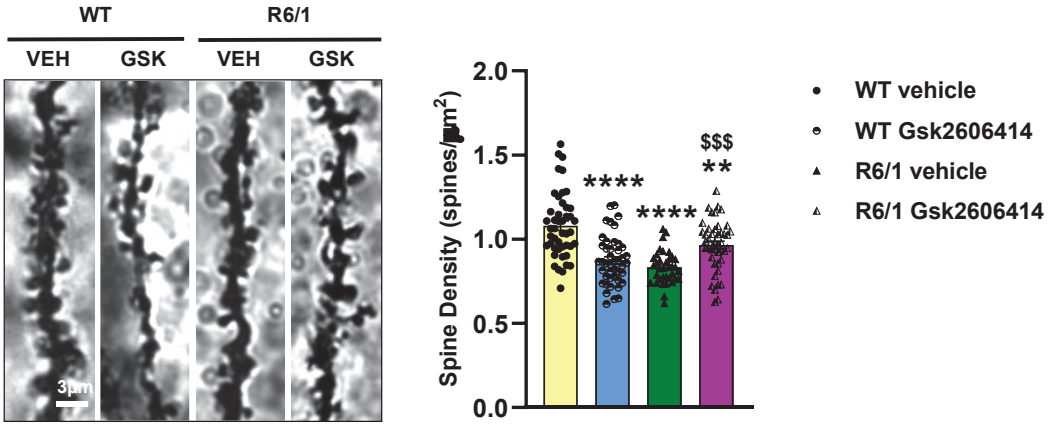


Figure 24. Pharmacological inhibition of PERK ameliorates hippocampal dendritic spine pathology in R6/1 mice. Representative photomicrographs showing apical dendrites from CA1 pyramidal neurons. The histogram shows a quantitative analysis of dendritic spine density per

micrometer of dendritic length. Data were analyzed by One-way ANOVA followed by Tukey's test. **P<0.01, ****P<0.0001: compared to WT vehicle. $$$ P<0.001: compared to R6/1 vehicle. All data represent the mean ± SEM (>45 dendrites/group: n = 4 mice/group). Scale bar 3µm.

1.4.5. Treatment with GSK2606414 upregulates the protein levels of memory-related genes in R6/1 mice.

To get further insight into the molecular mechanism underlying improved cognition following inhibition of PERK, the expression of some synaptic-related proteins and memory-related genes were evaluated in the hippocampus of the vehicle and treated R6/1 mice. Phosphorylation of PSD95 by PERK at T19 residue has been reported to negatively affect the stability of dendritic spines (T. Sen et al., 2017). Therefore, we first analyzed the levels of total and p-PSD95 in WT and R6/1 hippocampal extracts. No significant changes were found when comparing genotypes or treatments, suggesting that this pathway is unlikely involved in the observed HD cognitive deficits (Figure 25A). Next, we examined the levels of CREB and BDNF, two important proteins critical for synaptic function and memory formation in HD (Giralt et al., 2013; Puigdellívol et al., 2016). No differences were found when comparing genotypes or treatments (Figure 25B and C).

Finally, we analyzed the levels of some immediate early genes known to be activated by neuronal activity and to regulate memory and synaptic plasticity (Herdegen & Leah, 1998; Korb & Finkbeiner, 2011; Minatohara et al., 2016) and whose expression has been associated with spatial and recognition memory deficits in HD (Giralt, Saavedra, et al., 2012b; Valor et al., 2013). Thus, the protein levels of Arc, c-fos, and Egr1 were determined in vehicle and GSK2606414-treated WT and R6/1 mice. We found an increase in both Arc and c-fos protein levels in GSK2606414-R6/1 mice compared to vehicle-treated R6/1 mice (Figure 25D and E). On the contrary, inhibition of PERK was not able to rescue the significant decrease in Egr1 protein levels observed in R6/1 mice compared to WT mice (Figure 25F). Altogether, these data suggest that inhibition of PERK positively affects memory function in HD by acting on both structural and synaptic plasticity.

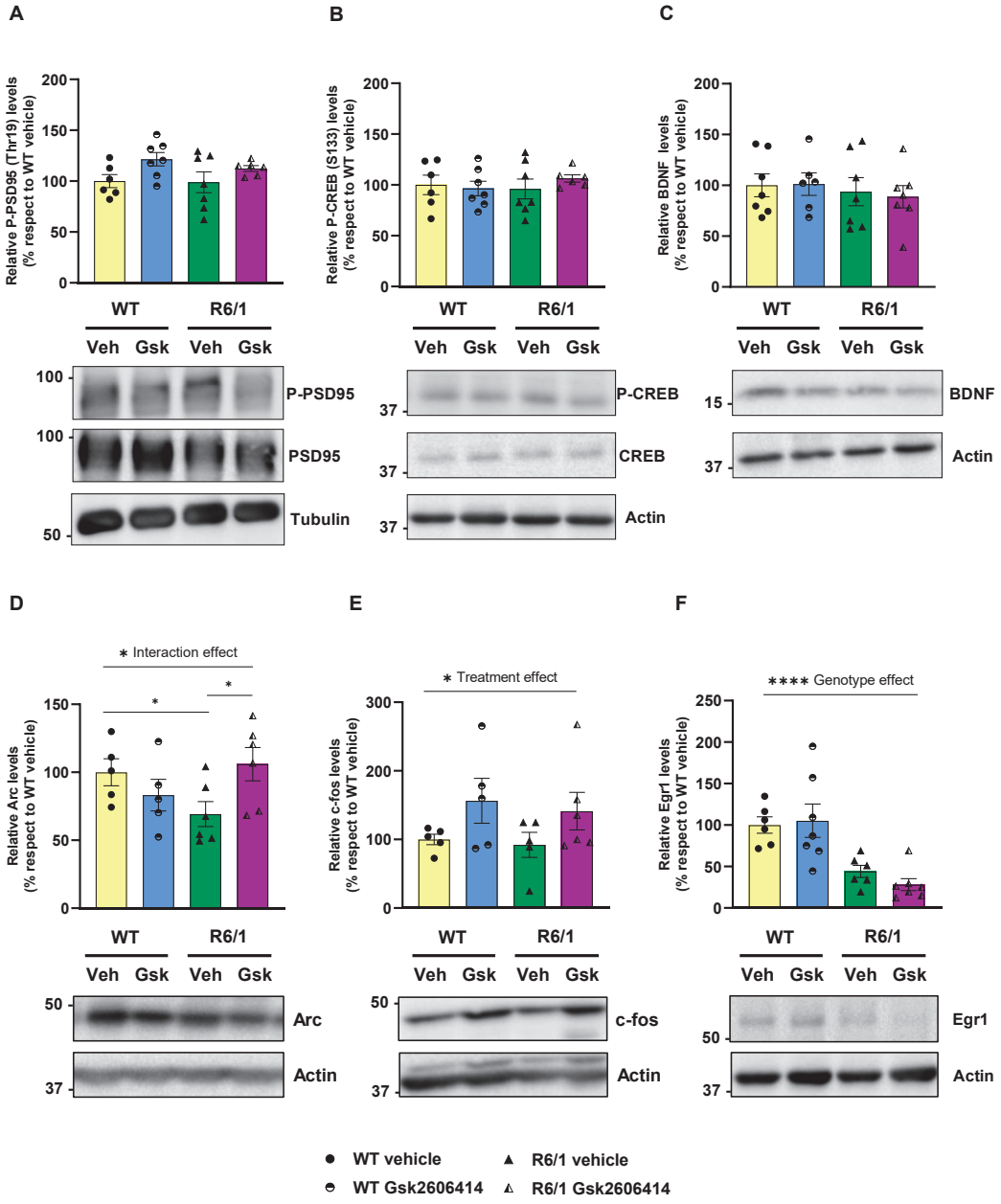


Figure 25. Treatment with GSK2606414 upregulates the expression of memory-related genes in R6/1 mice. Representative immunoblots and densitometric analysis showing **A** P-PSD95(Thr19), PSD95 **B** P-CREB(Ser133), CREB, **C** BDNF, **D** Arc, **E** c-fos, and **F** Egr1 levels in total lysates from hippocampal extracts of the vehicle and treated-WT and R6/1 mice at 14 weeks of age. Actin and tubulin were used as the loading control. Histograms represent the relative protein levels expressed as a percentage of WT vehicle values. Data were analyzed by Two-way ANOVA followed by Tukey's Test or *t*-Test *P<0.05 and represent the mean ± SEM (n = 5-7 mice/group).

2. ASTROCYTES AT THE HUB OF STRIATAL NEURONAL DYSFUNCTION IN HD: DISSECTING THE ROLE OF ARMS ON THE ASTROCYTIC SECRETOME.

Most aspects of the nervous system function rely on the neuron-glia crosstalk, particularly astrocytes (Schiera et al., 2020). This communication implies the release of different factors that may influence both cell types and modulate synaptic transmission and neuronal function (Robertson, 2018). However, most of our knowledge about the molecular mechanisms underlying HD pathology has been gained from studies conducted on neurons.

This second project is aimed to study the contribution of the astrocyte-neuron crosstalk in HD. We hypothesize that changes in the HD astrocyte-secretome may contribute to neuronal vulnerability and dysfunction by altering astrocyte-neuron communication. In particular, we postulate that aberrant ARMS/Kidins220 expression, a multi-functional scaffolding protein highly expressed in the nervous system (Scholz-Starke & Cesca, 2016) will promote deficient astrocyte BDNF secretion and induce an abnormal astrocytic inflammatory signature leading to a loss of neuroprotection, and therefore, neuronal dysfunction and degeneration. We hope that this project will contribute to changing the "*neuro-centric*" perspective that currently applies to research in neurodegenerative diseases by postulating that associated symptoms are not neuron-autonomous events but a failure of the astrocyte-neuron communication.

2.1. Study of ARMS levels and distribution in cellular HD models.

ARMS was first discovered in 2000 (Iglesias et al., 2000) and is predominantly expressed in the nervous system, modulating neuronal processes of survival, differentiation, and synaptic plasticity (Neubrand et al., 2012). Although research on ARMS is scarce, recent studies have suggested that alterations in ARMS expression are associated with different human pathologies, including various forms of cancer (Liao et al., 2007, 2011) and neurological and neuropsychiatric diseases such as Alzheimer's

(López-Menéndez et al., 2009), Parkinson's (Simunovic et al., 2009), autism (S. W. Kong et al., 2012), or schizophrenia (Kranz et al., 2015). In the context of HD, our group has demonstrated elevated hippocampal levels of ARMS in HD animal models as well as in HD human samples (López-Benito et al., 2018). However, no studies on ARMS expression in cell-specific populations in HD have been reported. Therefore, given the important role of astrocytes as a source of neurotrophic factors and modulators of neuroinflammation (Albini et al., 2023; Kwon & Koh, 2020; Miyazaki & Asanuma, 2020), in this second project, we have studied the role of ARMS in HD astrocyte functions and evaluated its therapeutic potential in the treatment of motor deficits in HD.

2.1.1. Primary astrocytes express significantly higher levels of ARMS than neurons.

No data regarding the differential expression of ARMS in neurons and glia have been reported. Given the particular vulnerability of the striatum in HD, we first analyzed by Western blot the protein levels of ARMS in DIV14 cultured striatal neurons and astrocytes obtained from mouse striatum (Figure 26). Our results showed regardless of the genotype, a significant increase in ARMS protein expression in astrocytes compared to neurons, indicating that it is highly more abundant in this cell type.

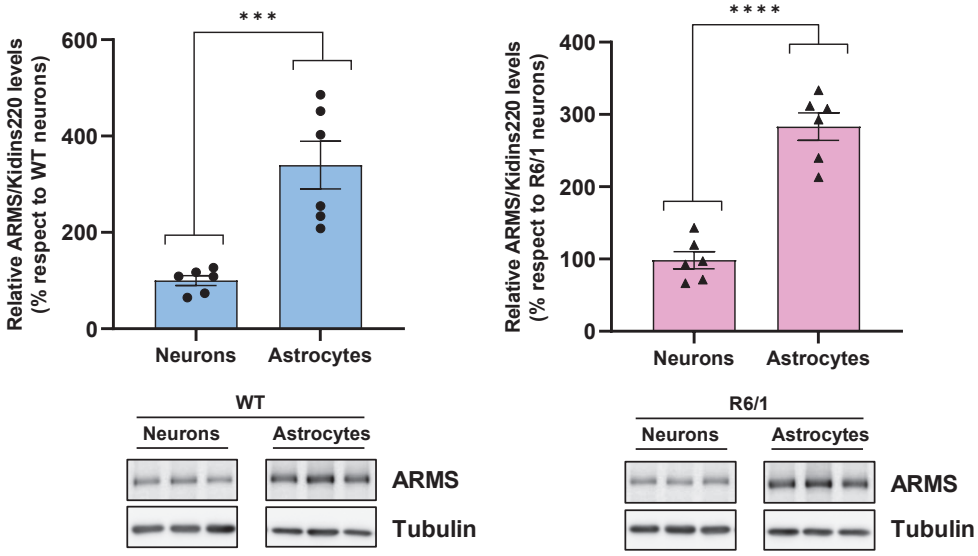


Figure 26. Primary astrocytes express significantly higher levels of ARMS than neurons. Representative immunoblots and densitometric analysis showing ARMS and actin levels in total lysates from WT and R6/1 striatal primary neurons and astrocytes at 14 DIV. Tubulin was used as the loading control. Histograms represent the relative protein levels expressed as a percentage of WT or R6/1 neuronal values. ***P<0.001, ****P<0.0001: compared to WT or R6/1 neurons by *t*-test. All data represent the mean ± SEM (n = 6 pups-embryos/group).

2.1.2. R6/1 striatal primary astrocytes express significantly higher levels of ARMS than WT striatal primary astrocytes.

Since our previous findings indicated that ARMS expression is more predominant in astrocytes than neurons, we next determined whether ARMS was differentially expressed in R6/1 compared to WT astrocytes. To achieve this, we analyzed ARMS mRNA and protein levels in astrocytes at DIV14. First, we measured the gene expression levels of ARMS (*kidins220*) using RT-qPCR, which revealed no significant differences between R6/1 and WT astrocytes (Figure 27A). However, when ARMS protein levels were analyzed by Western blot, we observed a significant increase in R6/1 astrocytes compared to WT astrocytes (Figure 27B).

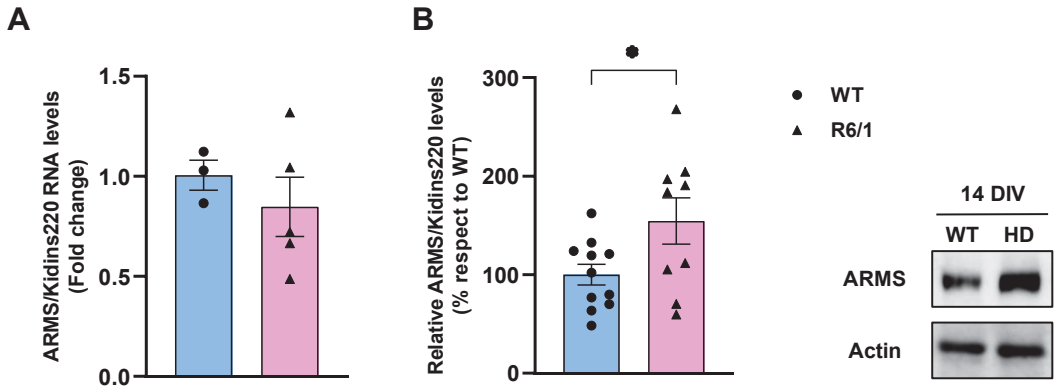


Figure 27. R6/1 striatal primary astrocytes express significantly higher levels of ARMS than WT striatal primary astrocytes. A The mRNA levels of *Kidins220* were measured by quantitative RT-qPCR in WT and R6/1 striatal primary astrocytes at DIV14. mRNA levels were normalized to *Actin* and relativized to WT as fold change. **B** Representative immunoblots and densitometric analysis showing ARMS levels in total lysates from WT and R6/1 striatal primary astrocytes at DIV14. Actin was used as the loading control. The histogram represents the relative protein levels expressed as a percentage of WT values. *P<0.05: compared to WT by Student's *t*-test. All data represent the mean ± SEM (n>9 pups/group).

2.2. Modulation of ARMS levels *in vivo*

Since ARMS expression is abnormally elevated in R6/1 striatal astrocytes, we next aimed to determine if normalization of ARMS expression in astrocytes can impact motor behavior in R6/1 mice.

2.2.1. Evaluation of viral transduction efficiency in striatal primary astrocytes.

To address this question, we first determined the most efficient small hairpin RNA construct to downregulate ARMS protein levels in astrocytes. We used lentiviral vectors to integrate different shRNA constructs for ARMS (sh1, sh2, sh3), and at DIV6 striatal primary astrocytes were transduced with 50 µl of the different shRNA plasmids. 24 hours later, the protein levels of ARMS and GFP were analyzed by Western Blot to prove knock-down effectiveness (Figure 28). Our results demonstrated that the sh2-ARMS construct proved optimal transfection efficiency determined by the protein levels of GFP and the best ability to downregulate ARMS protein expression. Therefore, based on these data the sh2-ARMS construct was selected for the generation of AAV2/5-GFAP-shARMS viruses.

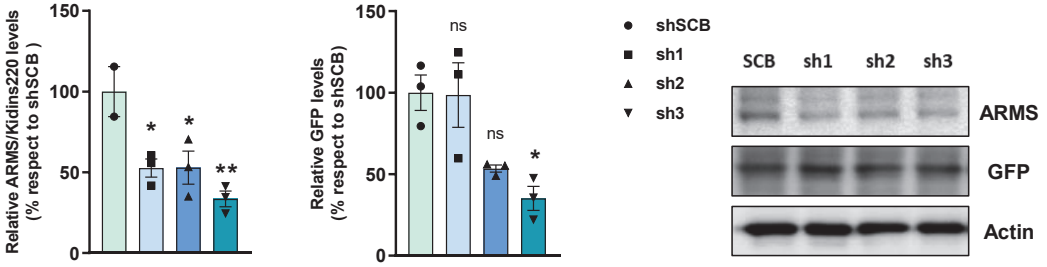


Figure 28. Efficacy of the shARMS lentiviruses in striatal primary astrocytes. Modulation of ARMS levels. Representative immunoblots and densitometric analysis showing ARMS and GFP levels in total lysates from WT striatal primary astrocytes after transduction. Actin was used as the loading control. Histograms represent the relative protein levels expressed as arbitrary units or as a percentage of WT shSCB values. *P<0.05, **P<0.01: compared to WT shSCB by One-way ANOVA followed by Tukey's test. All data represent the mean ± SEM (n=3 pups/group).

2.2.2. Evaluation of viral transduction efficiency in the striatum of R6/1 mice.

To evaluate the impact of knocking down astrocytic ARMS on the HD motor behavioral deficits, AAV2/5-GFAP-shRNA-GFP or AAV2/5-GFAP-shARMS-GFP viral particles were bilaterally injected into the striatum of 8-week-old WT and R6/1 mice at pre-symptomatic stages of the disease and motor learning and coordination were evaluated 4 weeks after the viral injections (Figure 29A). First, the AAVs injection was validated by analyzing the GFP staining in striatal brain slices from injected mice. As shown in Figure 29B, GFP immunostaining detected as green cells was observed in the whole striatum which demonstrated a good transduction efficiency of AAVs.

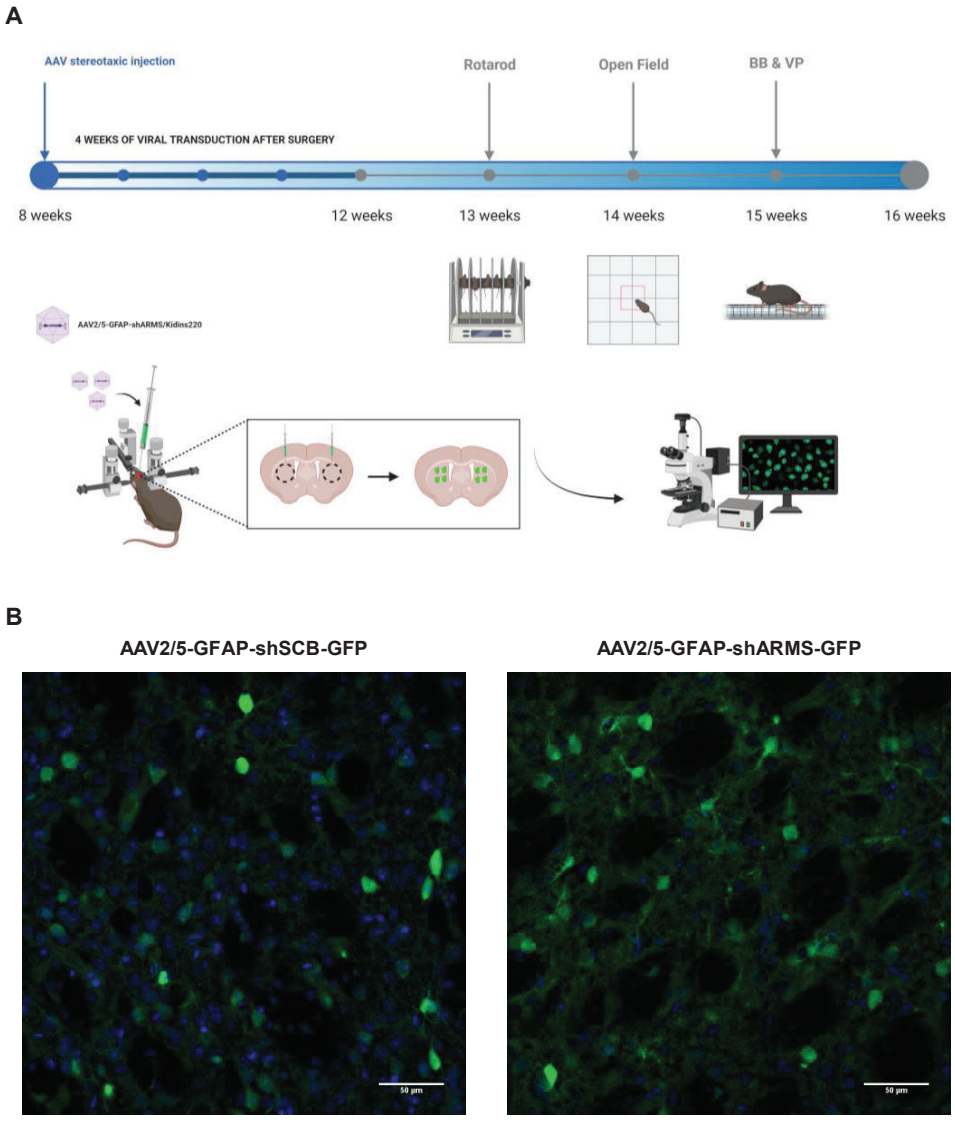


Figure 29. Evaluation of viral transduction efficiency in the striatum of R6/1 mice. A Scheme displaying the protocol used to evaluate the effect of reducing the expression of ARMS in R6/1 pre-symptomatic mice. AR, accelerating rotarod; FR, fixed rotarod; OF, open field; BB, balance beam; VP, vertical pole. **B** Representative images obtained by immunohistofluorescence against GFP (green) and DAPI (blue) showing transduced GFAP striatal cells 1 month after injections. Scale bar 50µm.

2.2.3. Efficacy of AAV2/5-GFAP-shARMS to knockdown ARMS expression in striatal astrocytes in R6/1 mice.

To analyze the effectiveness of AAV2/5-GFAP-shARMS-GFP to downregulate astrocytic ARMS levels, immunohistochemistry against ARMS and GFAP was performed in striatal brain slices from WT and R6/1 mice at 16 weeks of age after the behavioral analysis (Figure 30A).

Pictures show ARMS expression (green) in GFAP+ astrocytes (red). Quantification of ARMS was performed as the ratio of colocalized particles/area of ARMS in GFAP astrocytes over the total area of GFAP astrocytes (Figure 30B). We observed that the colocalization of ARMS and GFAP area in R6/1 sh-SCB mice was higher than in WT sh-SCB mice, which goes in accordance with the increased levels of ARMS detected in R6/1 primary astrocytes. Supporting a good transduction efficacy of AAV-shARMS, R6/1 sh-ARMS mice showed a significant decrease in the ratio of colocalization of both the area and particles of ARMS in GFAP astrocytes. Similarly, we detected in WT sh-ARMS mice a significant reduction in the colocalization of the particles of ARMS in GFAP astrocytes. These data suggested that AAV2/5-GFAP-shARMS-GFP viral particles are able to downregulate astrocytic ARMS levels in R6/1 mice.

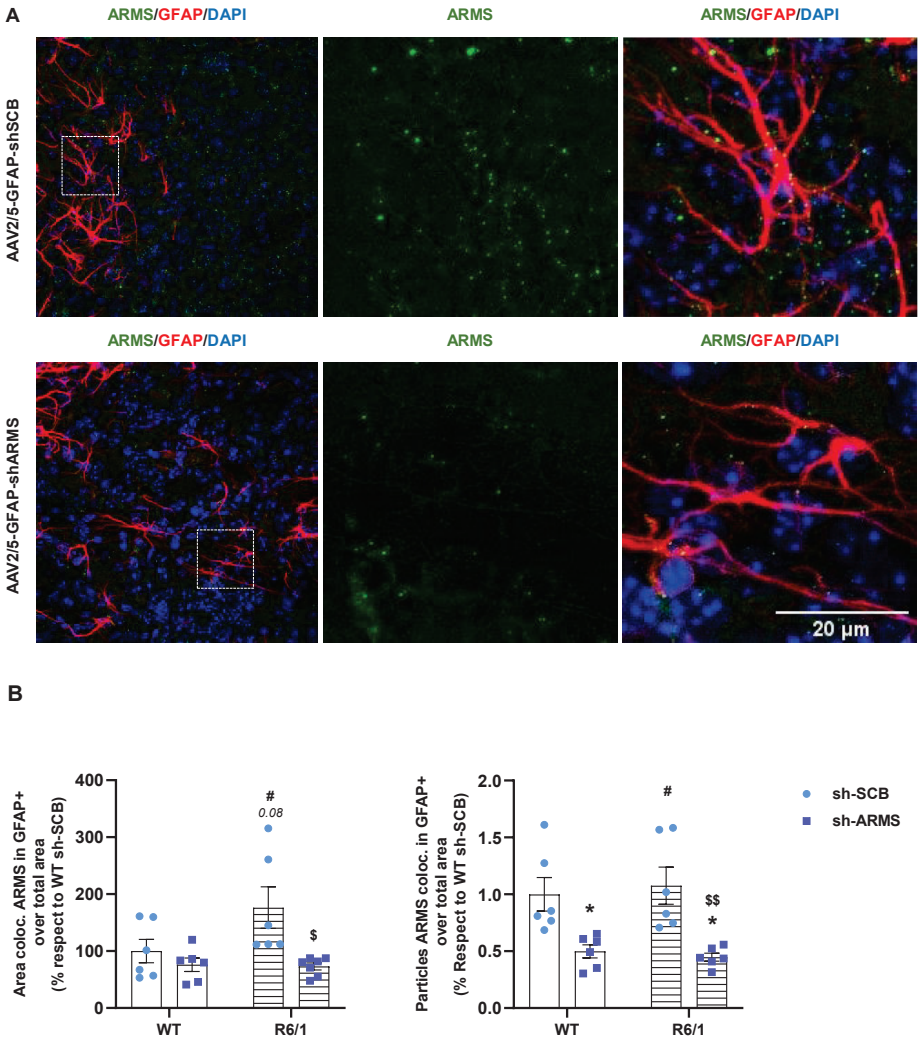


Figure 30. Efficacy of AAV2/5-GFAP-shARMS to knockdown ARMS expression in striatal astrocytes in R6/1 mice. A Representative images obtained by immunohistofluorescence against ARMS (green), GFAP (red), and DAPI (blue) showing reduced ARMS expression in GFAP striatal cells in R6/1 mice after behavior at 16 weeks of age. Scale bar 20µm. The white dotted square represents the zoom-in for the following pictures. **B** Histograms represent the ratio of the colocalized particles and area of ARMS in GFAP over the total area of GFAP+ astrocytes. *P<0.05: compared to WT sh-SCB, #P<0.05: compared to WT sh-ARMS, and $P<0.05: compared to R6/1 sh-SCB by Two-way ANOVA followed by Tukey's test. All data represent the mean ± SEM (n = 6 images/group). >60 stacks were quantified per image.

2.2.4. ARMS knockdown in striatal astrocytes partially improves motor learning and coordination in R6/1 mice.

Once we confirmed the effective knockdown of ARMS expression in astrocytes, a cohort of 40 animals (20 WT and 20 R6/1) were injected with AAV2/5-GFAP-shRNA-GFP or AAV2/5-GFAP-shARMS-GFP at the age of 8 weeks and motor learning, coordination, and spontaneous locomotor behavior were evaluated 4 weeks after. First, we assessed motor learning using the accelerating rotarod task. As shown in (Figure 31A), at the age of 12-13 weeks, R6/1 mice injected with sh-SCB (R6/1 sh-SCB) were unable to maintain the balance on the rotarod while WT mice (WT sh-SCB and WT sh-ARMS) learned the rotarod task with increased latency to fall over trials. Notably, injection of AAV2/5-GFAP-shARMS-GFP in R6/1 mice (R6/1 sh-ARMS) significantly ameliorated motor learning deficits, showing a significantly better performance compared to R6/1 sh-SCB mice.

To study motor coordination, the fixed rotarod test was performed on the same cohort of mice that had been previously tested on the accelerating rotarod task. Given that R6/1 mice injected with sh-SCB (R6/1 sh-SCB) were unable to learn the task during the training session, we only performed two trials in the fixed rotarod for all genotypes and conditions at a fixed speed of 10 rpm. Our results showed a significant improvement in the number of falls at 10 rpm in R6/1 sh-ARMS mice compared to R6/1 sh-SCB mice indicating a significant improvement in motor coordination deficits (Figure 31B). Finally, to study the role of astrocytic ARMS in spontaneous locomotor activity, we measured the traveled distance in the open field for 30 minutes. In accordance with previous studies from our group (Giralt, Carretán, et al., 2011), R6/1 sh-SCB mice traveled less distance than WT sh-SCB mice. Nevertheless, we could not detect any effect of the AAV2/5-GFAP-shARMS-GFP treatment neither in WT nor R6/1 mice (Figure 31C) indicating that ARMS doesn't have a robust role in spontaneous locomotor behavior. Altogether, these data demonstrate the contribution of the astrocytic ARMS to motor learning and coordination in R6/1 mice.

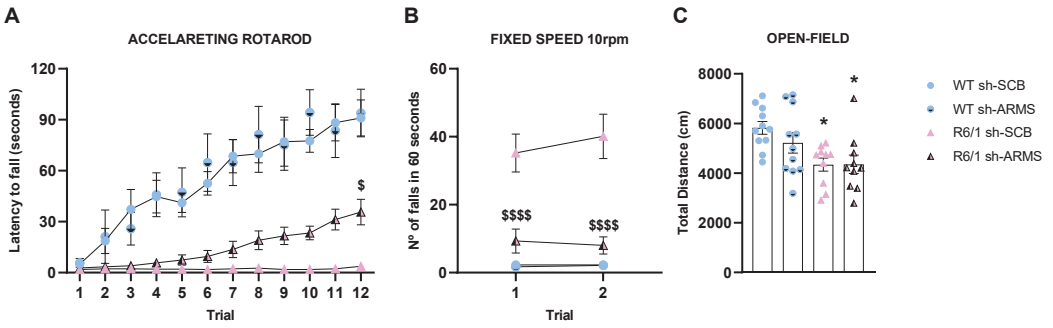
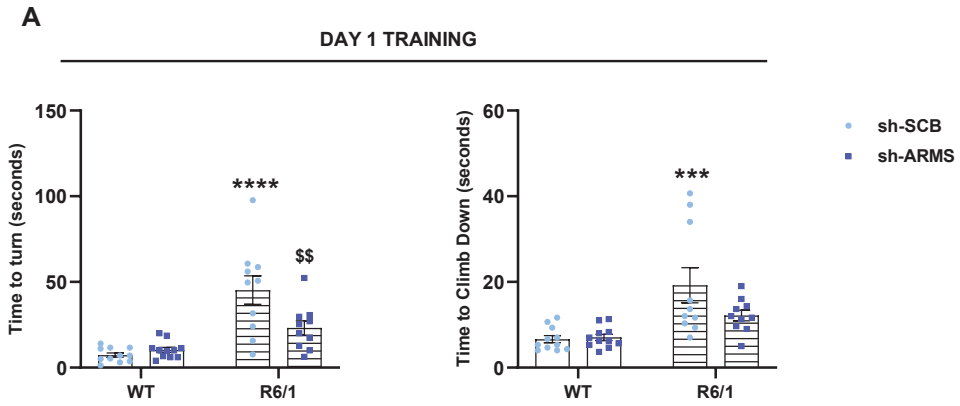


Figure 31. ARMS knockdown in striatal astrocytes partially improves motor learning and coordination in R6/1 mice. A Latency to fall in the accelerating rotarod task at 12-13 weeks of age. **B** After the accelerated paradigm, the number of falls at 10rpm for 60 seconds was recorded twice. **C** Spontaneous locomotor behavior was analyzed by the open-field test measuring the total distance traveled. *P<0.05: compared to WT sh-SCB, $P<0.05, $$$$P<0.0001: compared to R6/1 sh-SCB. Data were analyzed by Two-way ANOVA followed by Tukey's test. All data represent the mean ± SEM (n = 10-11 mice/group).

2.2.5. ARMS knockdown in striatal astrocytes partially improves fine motor coordination and balance in R6/1 mice.

Given the beneficial effect of knocking down astrocytic ARMS in motor learning and coordination in R6/1 mice, we next evaluated fine motor coordination and balance skills with the vertical pole, and balance beam tests respectively. Regarding the vertical pole task, both WT and R6/1 mice were able to turn and climb down the pole. However, R6/1 sh-SCB mice were much slower to do so in both training sessions and during testing. Interestingly, R6/1 mice injected with sh-ARMS improved the time to turn during training and testing sessions indicating improved fine motor coordination (Figure 32).



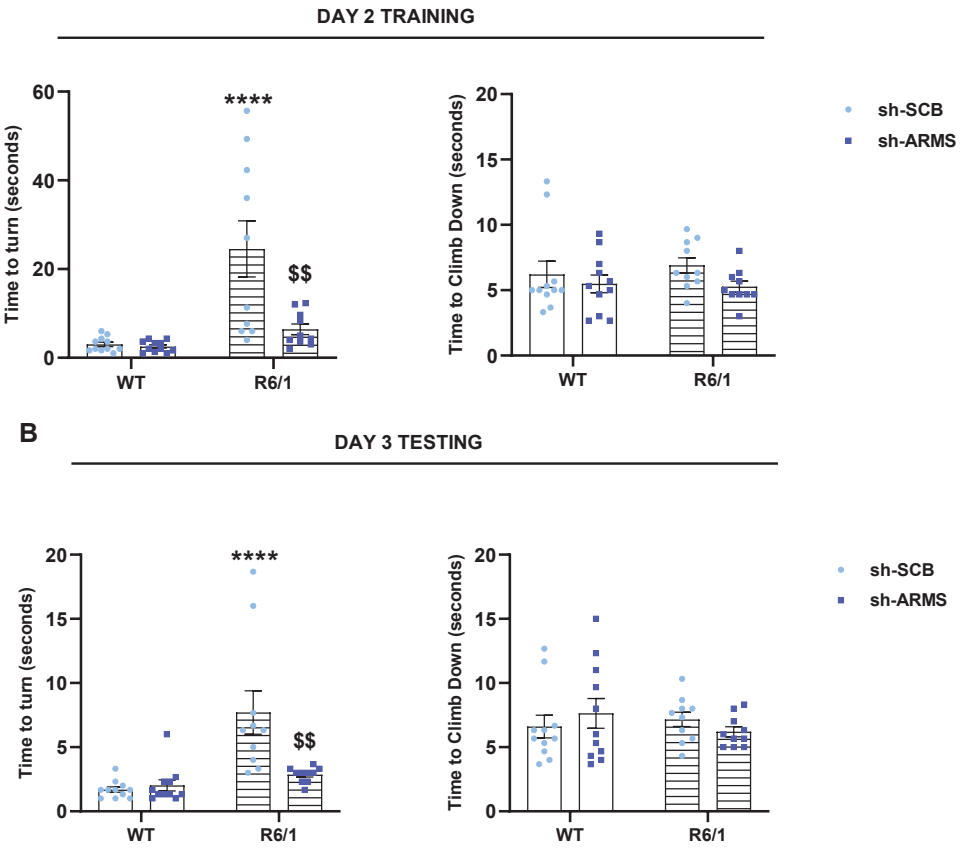


Figure 32. ARMS knockdown in striatal astrocytes partially improves fine motor coordination in the vertical pole test. The time to turn (left) and time to descend (right) the vertical pole were recorded after placing the mice upwards on the top of the pole. Three trials were conducted, and data represent the mean ± SEM (n=10-11 mice/group). ***P<0.001, ****P<0.0001: compared to WT sh-SCB mice. $$P<0.01: compared to R6/1 sh-SCB mice. Data were analyzed by Two-way ANOVA followed by Tukey's test.

To assess balance skills, we employed the balance beam test consisting of two sessions - the training and the testing, with different levels of task difficulty achieved by varying the beam shape (rounded and squared). Both WT and R6/1 mice successfully completed both beams, but R6/1 mice, irrespective of treatment, were significantly slower in crossing both the squared and rounded beams and crossed fewer frames in two minutes compared to WT mice (Figure 33). However, our findings indicated that R6/1 mice injected with AAV2/5-GFAP-shARMS-GFP (R6/1 sh-ARMS) had fewer slips than R6/1 mice injected with sh-SCB (R6/1 sh-SCB) in the rounded beam during both the training and testing sessions. Altogether, these data demonstrate that the

reduction of ARMS expression in astrocytes slightly improves balance deficits in R6/1 mice.

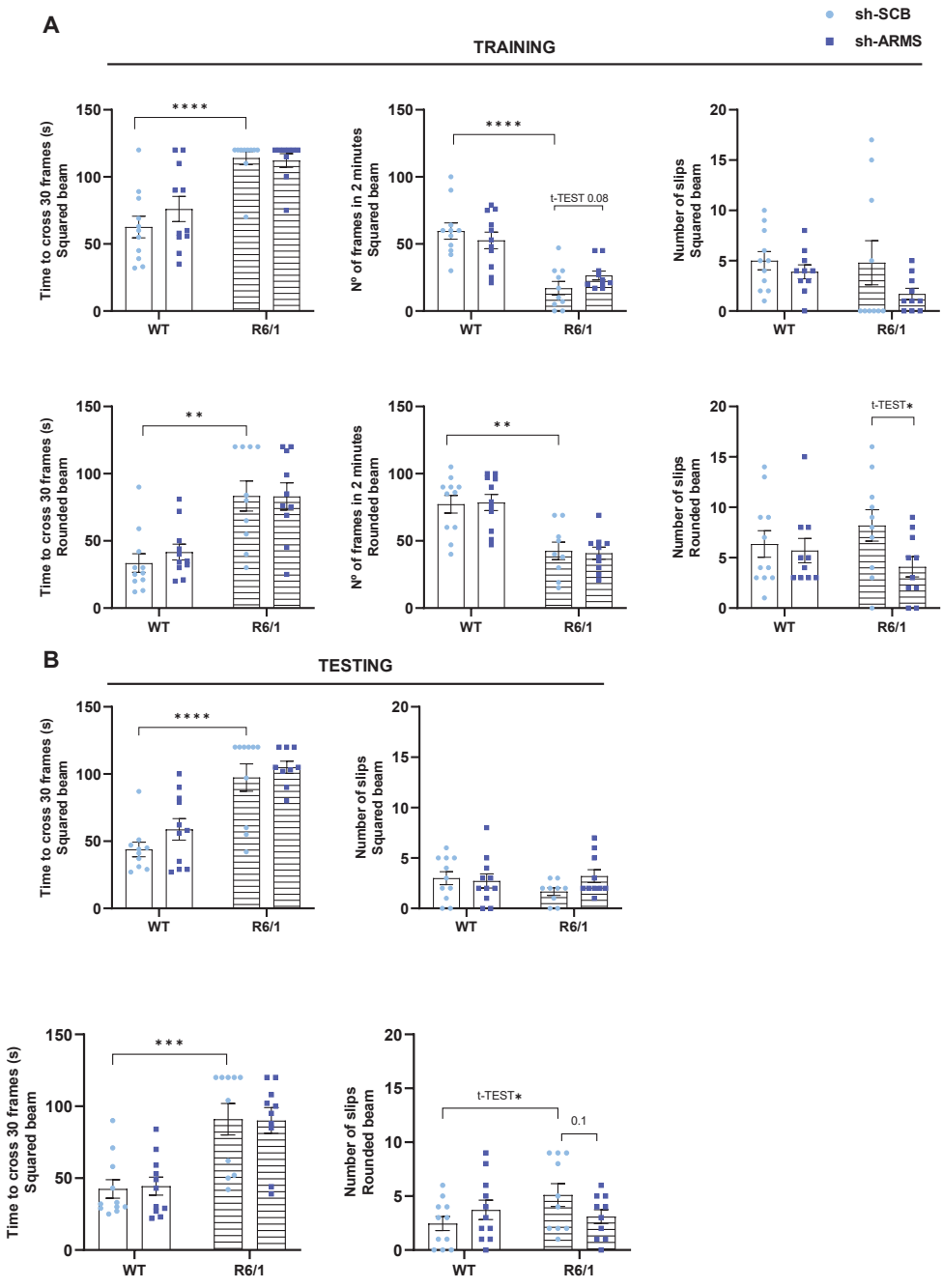


Figure 33. ARMS knockdown in striatal astrocytes partially improves balance skills in R6/1 mice. The time to cross 30 frames, the number of frames crossed in 2 minutes and the number of slips in the squared and rounded beams were recorded during training **A** and testing **B** sessions. Data represent the mean ± SEM (n=10-11 mice/group). **P<0.01, ***P<0.001, ****P<0.0001: compared to WT sh-SCB mice. Data were analyzed by Two-way ANOVA followed by Tukey's test or by Student's *t*-test.

2.3. Role of ARMS in astrocytic function: secretome modulation.

Our current findings suggest that the expression of ARMS is significantly elevated in R6/1 astrocytes contributing to motor behavioral deficits. Following astrocytic ARMS knockdown, we have observed a moderate improvement in motor learning and coordination deficits, as well as a partial improvement in fine motor behavior and balance in R6/1 mice. Therefore, we next aimed to identify the molecular mechanisms underlying this beneficial effect.

2.3.1. Modulation of ARMS levels in striatal primary astrocytes with AAV2/5-GFAP-shARMS.

In order to investigate the underlying mechanisms of the positive impact of modulating ARMS in astrocytes on HD motor deficits, we focused on its potential involvement in the release of BDNF, cytokines, and chemokines by astrocytes. To this aim, we move to primary astrocytic cultures obtained from the striatum of R6/1 and WT mice.

First, we tested the AAV's (AAV2/5- GFAP-shARMS-GFP) efficacy to infect primary astrocytes and knock down ARMS expression. WT and R6/1 striatal primary astrocytes were transduced at DIV9 with AAV2/5-GFAP-shARMS-GFP or AAV2/5- GFAP-shSCB-GFP at 250.000 MOI overnight. At DIV21, GFP expression was visualized with an inverted microscope to confirm efficient transduction, and astrocytes were collected for Western blot analysis (Figure 34). We found a significant reduction of approximately 40-50% of ARMS expression both in WT and R6/1 astrocytes when transduced with AAV2/5-GFAP-shARMS-GFP compared to AAV2/5- GFAP-shSCB-GFP proving the ability to successfully knock down astrocytic ARMS.

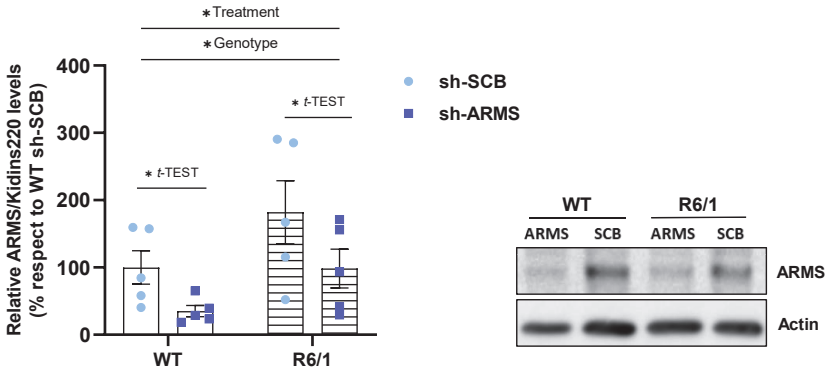


Figure 34. Modulation of ARMS levels in striatal primary astrocytes with AAV2/5-GFAP-shARMS. Representative immunoblots and densitometric analysis showing ARMS levels in total lysates from WT and R6/1 striatal primary astrocytes at DIV21. Actin was used as the loading control. Histograms represent the relative protein levels expressed as a percentage of WT sh-SCB values compared by Two-way ANOVA followed by Tukey's test. After Two-way ANOVA, we performed the Student's *t*-test between genotypes. *P<0.05: compared to WT or R6/1 sh-SCB values. All data represent the mean ± SEM (n = 5 pups/group).

2.3.2. Knocking down ARMS in striatal primary astrocytes increases the release of BDNF.

Astrocytes play a crucial role in sustaining and modulating the function of nearby neurons by releasing brain-derived neurotrophic factor (BDNF) through exocytosis (Miyamoto et al., 2015; Parpura & Zorec, 2010; X. Wu et al., 2008). Previous studies have shown decreased BDNF levels in HD cellular and mouse models (B. Lu et al., 2005; Zuccato & Cattaneo, 2007), while approaches to increase BDNF levels alleviate neuropathology and symptoms in animal models of HD (Giralt, Carretán, et al., 2011; Zuccato et al., 2005). Deficient BDNF-regulated secretion associated with increased expression of ARMS has been previously reported by our group in HD mouse models (López-Benito et al., 2018). Therefore, since we found increased ARMS levels in HD astrocytes and previous studies have reported compromised exocytosis of BDNF vesicles in primary HD astrocytes (Hong et al., 2016), we aimed to study the role of astrocytic ARMS in BDNF release. To this aim, we stimulated primary striatal astrocytes with 50 mM KCl for 1 h, and BDNF release was measured in the medium using ELISA analysis (Figure 35A). Similar BDNF release was found between WT and R6/1 sh-SCB primary astrocytes. However, treatment with AAV2/5-GFAP-shARMS-GFP increased the release of BDNF only in R6/1 astrocytes, indicating that the aberrantly increased ARMS expression in R6/1 astrocytes impedes the release of BDNF (Figure 35B). Therefore, our findings suggest that astrocytic ARMS may play a crucial role in the pathology of HD by interfering with the release of BDNF.

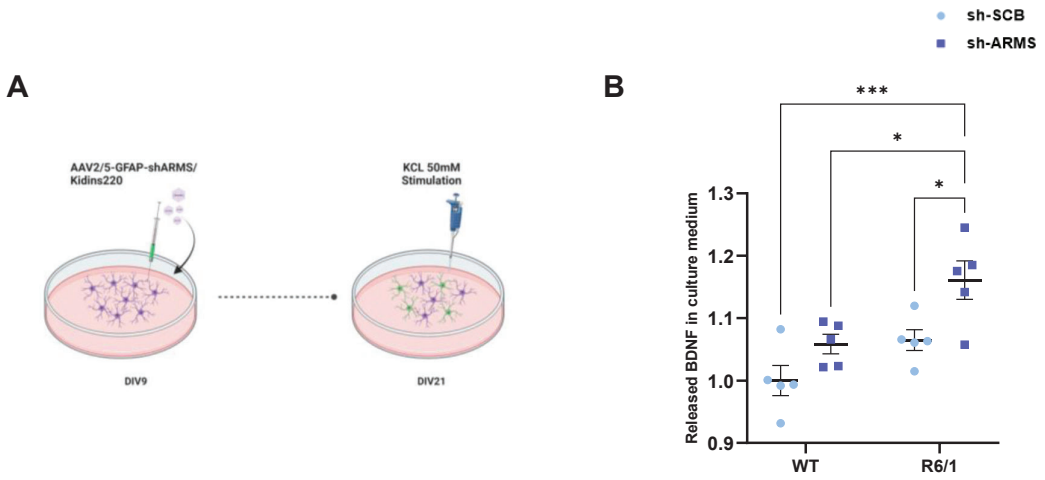


Figure 35. Knocking down ARMS in striatal primary astrocytes increases the release of BDNF. **A** Schematic representation of the experimental timeline followed to evaluate the effect of the treatment with AAV2/5-GFAP-shARMS and KCl 50mM for 1h on the release of BDNF by striatal *in vitro* astrocytes. **B** Representative histogram showing BDNF levels in supernatants from WT and R6/1 striatal astrocytes at DIV21 after treatment with AAV2/5-GFAP-shARMS and challenged with KCl 50mM for 1h. *P<0.05, ***P<0.001. Data were analyzed by Two-way ANOVA followed by Tukey's test and represent the mean ± SEM (n = 5 supernatants/condition).

2.3.3. Knocking down ARMS in striatal primary astrocytes affects the release of cytokines.

In addition to releasing BDNF, activated astrocytes can also secrete cytokines and chemokines with either neuroprotective or neurotoxic effects, contributing to the pathogenesis of HD (Hsiao et al., 2013; Linnerbauer et al., 2020; Rocha et al., 2016; Stephenson et al., 2018). Besides, increased production of inflammatory molecules such as Interleukins (IL-6, IL-8, IL-10) and tumor necrosis factor α (TNFα) have been observed in HD (Rocha et al., 2016). Therefore, we aimed to investigate the role of astrocytic ARMS on cytokine release. To accomplish this, we used a mouse cytokine array profiler capable of detecting 40 different cytokines, chemokines, and acute-phase proteins simultaneously. We obtained the astrocytic conditioned medium (ACM) from primary WT and R6/1 astrocytes at DIV21 after ARMS knockdown and stimulation with 50 mM KCl for 1h and the cytokine profile was analyzed (Figure 36A). We successfully detected CXCL12, INF-Y, IL-1α, IL-16, TIMP-1, and TNFα in the secretomes and analyzed their occurrence (Figure 36B/C). Our analysis revealed that both WT and R6/1 sh-SCB

striatal primary astrocytes primarily release CXCL-12, followed by TIMP-1, TNFα, and to a lesser extent IL-1α, INF-Y, and IL-16. Notably, the most significant changes observed after the modulation of astrocytic ARMS corresponded with the most prominent cytokines found in the secretomes (Figure 37).

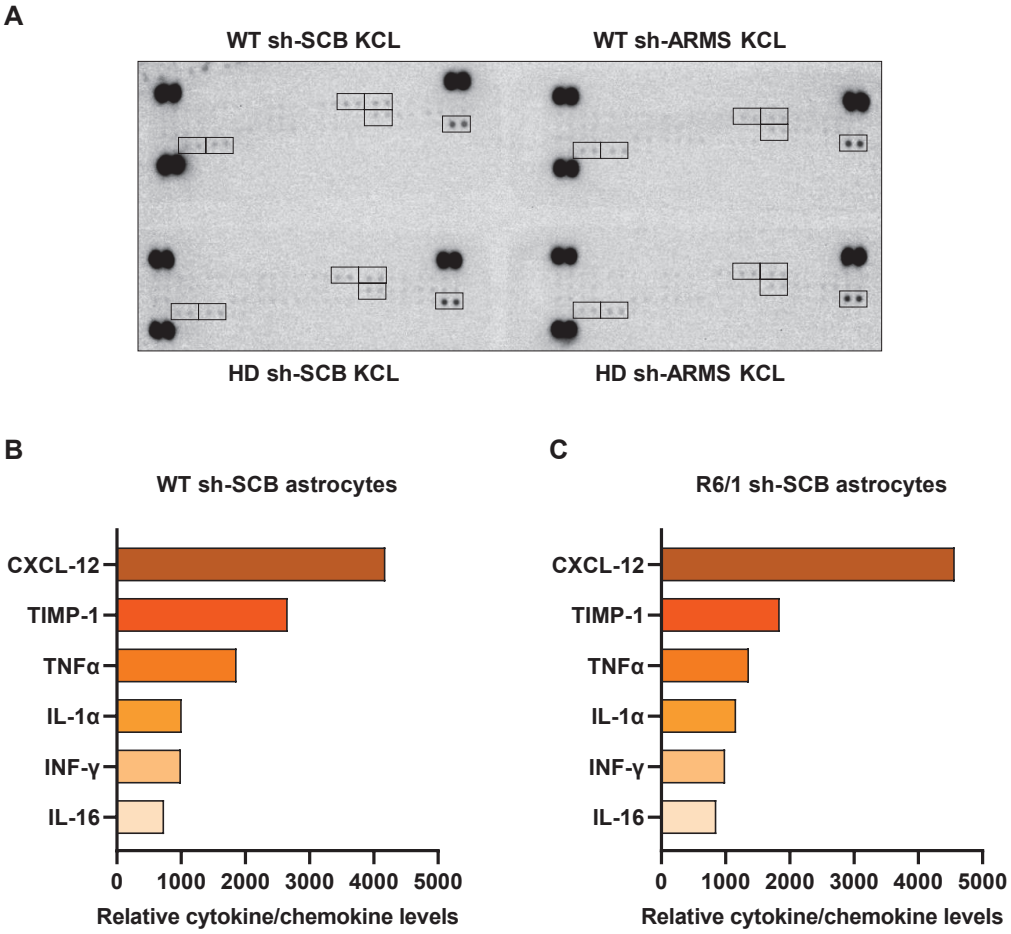


Figure 36. Mouse Cytokine Array and occurrence of cytokines and chemokines in the secretomes from WT and R6/1 sh-SCB striatal primary astrocytes. A Representative images from the individual Mouse Cytokine Array from R&D Systems® performed with the supernatants of WT and R6/1 sh-SCB striatal astrocytes at 21 DIV after being challenged with KCl 50 mM for 1h. **B** and **C** Representative histograms showing CXCL12, INF-Y, IL-1α, IL-16, TIMP-1, and TNFα relative levels in the supernatants from WT and R6/1 sh-SCB striatal astrocytes.

Thus, we observed that INF-Y levels remained unchanged in both genotypes and treatment conditions, indicating that ARMS cannot modulate their release. However, we found that IL-16 levels were affected only in WT astrocytes by treatment with sh-ARMS, resulting in an increased release. Additionally, both WT and R6/1 sh-ARMS astrocytes showed significantly decreased release of TIMP-1 and TNFα into the medium and increased release of CXCL12 levels, suggesting that secretion of these cytokines and chemokines is indeed modulated by ARMS expression. Finally, we found that WT sh-ARMS astrocytes released more IL-1α compared to WT sh-SCB astrocytes, while no differences were observed in R6/1 astrocytes (both sh-SCB and sh-ARMS). Based on our findings, we can suggest that ARMS plays a regulatory role in the secretion of cytokines and chemokines.

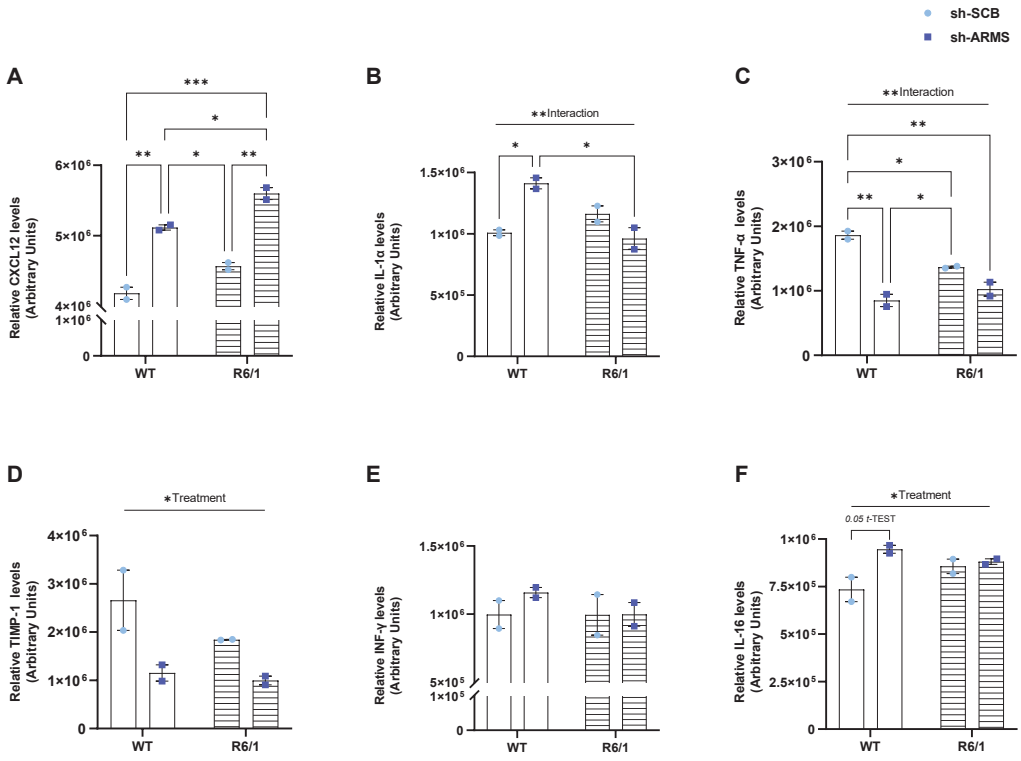


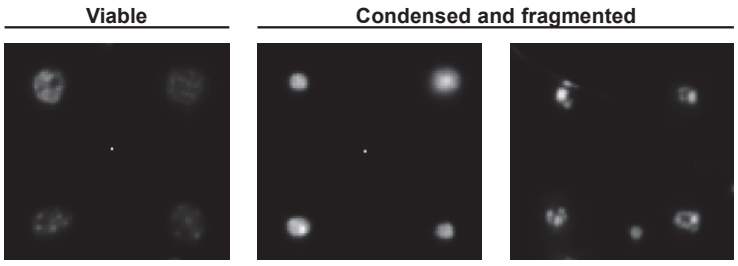
Figure 37. Knocking down ARMS in striatal primary astrocytes affects the release of cytokines. Representative histograms showing **A** CXCL12, **B** IL-1α, **C** TNFα, **D** TIMP-1, **E** INF-Y, and **F** IL-16 levels in the supernatants from WT and R6/1 striatal primary astrocytes. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Data were analyzed by Two-way ANOVA followed by Tukey's test and represent the mean ± SEM (n = 1 array/condition incubated with ≥5 different secretomes).

2.3.4. ACM from knockdown ARMS astrocytes does not affect neuronal viability.

Based on our findings, it appears that the abnormal levels of astrocytic ARMS hinder the release of BDNF from astrocytes and contribute to an atypical astrocytic inflammatory profile. As a next step, we aimed to investigate whether the changes in the HD astrocyte-secretome may have an impact on neuronal vulnerability by modifying neuron-astrocyte communication. To examine this, we exposed both WT and R6/1 primary neurons to ACM obtained from ARMS knockdown astrocytes stimulated with 50 mM KCl for 1 hour.

To assess neuronal viability, neurons were stained with MAP2 antibody and DAPI, and results were analyzed using the Cell Profiler® and Cell Profiler Analyst® software. We divided the nuclei from MAP2+ cells into two groups: viable or condensed and fragmented (Figure 38A). Using a confusion matrix generated by Cell Profiler®, we calculated the classification accuracy to categorize MAP2+ nuclei into these two groups. We found that MAP2+ R6/1 nuclei had a 93% accuracy (Figure 38B), while WT MAP2+ nuclei had an 87% accuracy (Figure 38C), demonstrating the software's effectiveness in categorizing all nuclei. No significant differences in the percentage of viable and condensed nuclei between WT or R6/1 neurons treated with sh-SCB or sh-ARMS ACM were found, indicating that the secretome from astrocytes with decreased levels of ARMS does not affect neuronal viability.

A



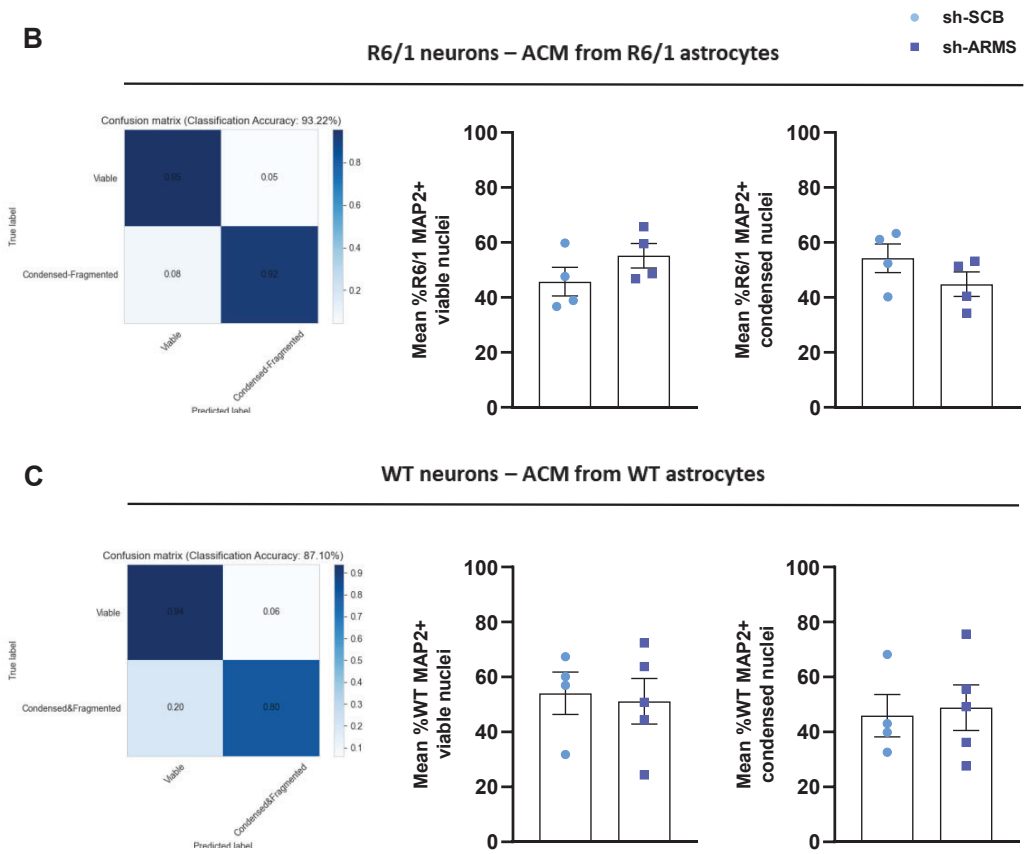


Figure 38. ACM from knockdown ARMS astrocytes does not affect neuronal viability. A Representative images obtained by immunofluorescence against DAPI (blue) showing the viable, condensed, and fragmented nuclei from WT and R6/1 neurons after treatment with ACM. **B** and **C** Confusion matrixes showing the classification accuracy of the automated trainable machine learning software used to classify nuclei. Histograms represent the percentage of R6/1 and WT MAP2+ viable or condensed nuclei. Data were analyzed by Student's *t*-test and represent the mean ± SEM (n=4-5 embryos/group and 2 technical replicates/embryo). Each point represents the mean of all the MAP2+ nuclei in 10 randomized pictures (10-15 MAP2+ neurons/picture).

2.3.5. ACM from knockdown ARMS astrocytes improves neuronal morphological features.

After studying neuronal viability, we wanted to investigate whether the changes in the HD astrocyte-secretome could translate into modifications in neuronal morphological features. We analyzed the arborization complexity of neurons through an automated image analysis pipeline (Figure 39) that measured the mean number of MAP2+ trunks (primary dendrites), non-trunks (intermediate branches), endpoints (neurites), and the

total tree length as previously described by our group (Barriga et al., 2017). Interestingly, WT neurons treated with WT sh-ARMS ACM showed similar numbers of trunks, a tendency to increase the number of non-trunks and endpoints, and a significantly higher total tree length compared to those treated with WT sh-SCB ACM. These findings suggest that reducing ARMS levels in WT astrocytes may have a slightly positive impact on WT neuronal morphological features (Figure 39A).

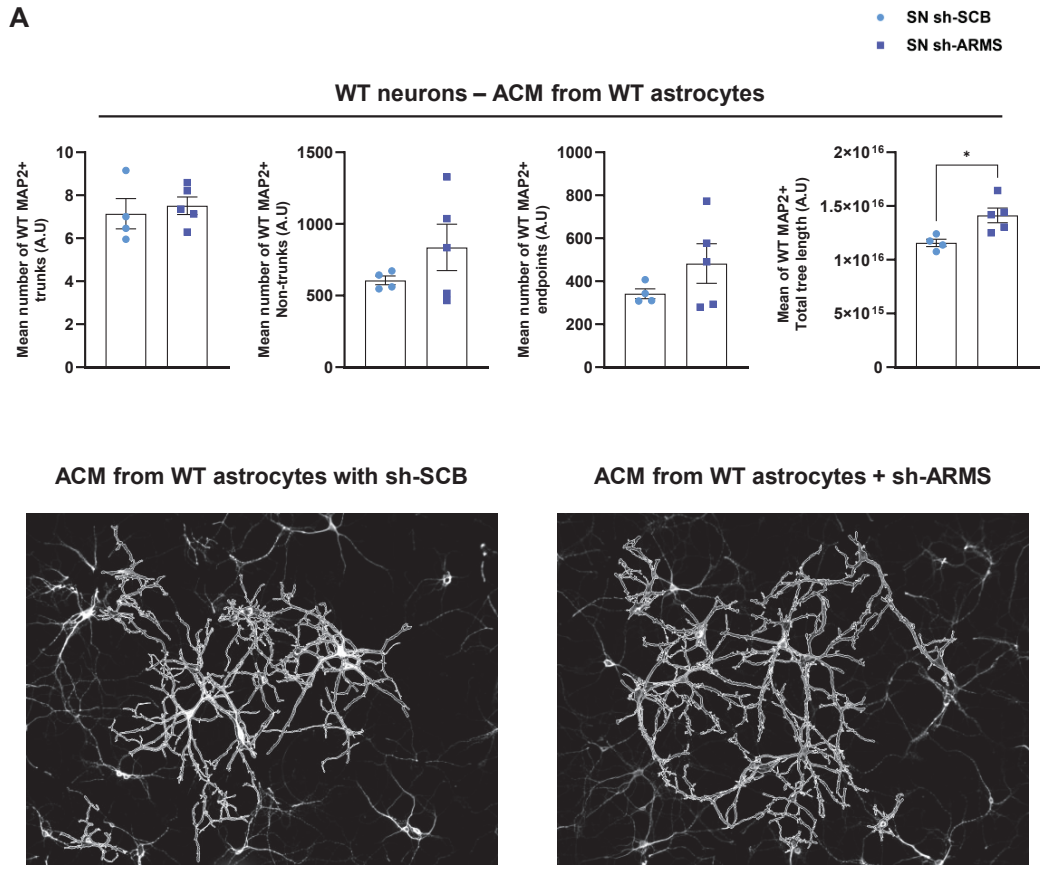


Figure 39A. Knocking down ARMS in WT primary striatal astrocytes improves WT MAP2+ neuronal morphology. Histograms represent the mean number of WT MAP2+ trunks, non-trunks, endpoints, and total tree length. *P<0.05: compared to WT sh-SCB by Student's *t*-test. All data represent the mean ± SEM (n=4-5 embryos/group and 2 technical replicates/embryo). Each point represents the mean of all the MAP2+ cells in 10 pictures of randomized fields. Representative images obtained by immunofluorescence against MAP2 (red) and DAPI (blue) showing increased dendritic arborization in those neurons receiving the supernatant from WT astrocytes treated with sh-ARMS.

Regarding R6/1 neurons, our findings demonstrated a significant improvement in morphology, as evidenced by a significantly higher number of non-trunks and endpoints upon challenge with R6/1 sh-ARMS ACM, with no differences in the number of trunks or the total tree length (Figure 39B). These results suggest that the secretomes obtained from R6/1 astrocytes with reduced ARMS expression have the potential to enhance the morphology of R6/1 neurons.

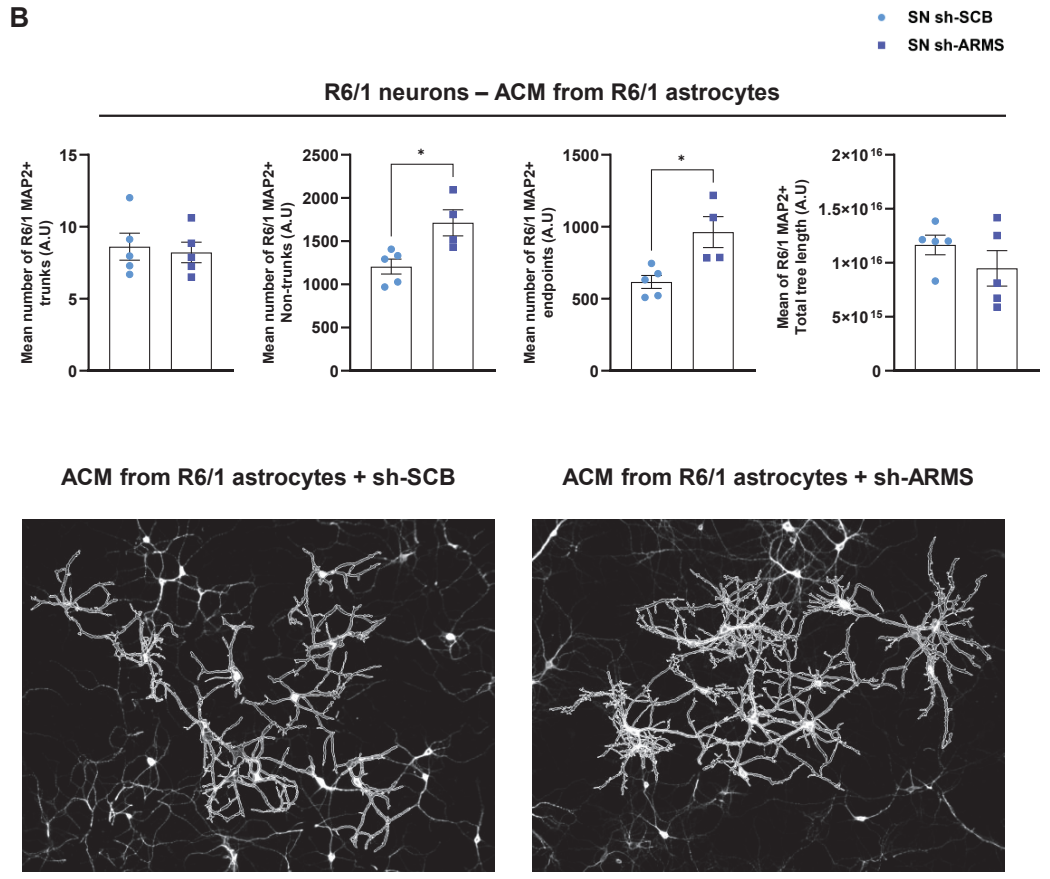


Figure 39B. Knocking down ARMS in R6/1 primary striatal astrocytes improves R6/1 MAP2+ neuronal morphology. Histograms represent the mean number of R6/1 MAP2+ trunks, non-trunks, endpoints, and total tree length. *P<0.05: compared to R6/1 sh-SCB by Student's *t*-test. All data represent the mean ± SEM (n=4-5 embryos/group and 2 technical replicates/embryo). Each point represents the mean of all the MAP2+ cells in 10 pictures of randomized fields. Representative images obtained by immunofluorescence against MAP2 (red) and DAPI (blue) showing increased dendritic arborization in those neurons receiving the supernatant from R6/1 astrocytes treated with sh-ARMS.

DISCUSSION

Huntington's disease lacks effective disease-modifying therapies and is currently managed by focusing on alleviating its symptoms. The most commonly used drug for HD approved by the FDA dates back to 2008 and is tetrabenazine, a synaptic vesicular amine transporter inhibitor for treating chorea (Marshall, 2006). Still, clinical trials have moderately increased in number over time, with an exponential rise in the average number of participants. However, the vast majority of studies have not shown efficacy so far (Bonelli & Hofmann, 2007; Mestre & Ferreira, 2012). Ongoing clinical trials aim to alter the underlying disease pathogenesis though are also focused on symptomatic relief. For instance, gene therapies intend to prevent all downstream aberrant pathways by correcting or muting the underlying abnormal trinucleotide expansion at the DNA level. Similarly, therapies aimed at the RNA level try to intervene early in the pathogenic process to prevent activation of downstream mHTT toxicity. Nevertheless, these treatments have therapeutic limitations, such as invasive administration and potential off-target effects (Pan & Feigin, 2021). As we wait for a cure for HD, gaining a better understanding of the molecular mechanisms behind its motor, cognitive, and psychiatric symptoms may lead to the development of new treatments that can alleviate these issues. Surprisingly, although the genetic mutation that causes HD is well known since 1993 (MacDonald et al., 1993), the pathogenesis from mutant gene to neurotoxicity, aberrant aggregation, and dysfunction is complex and not fully understood. Although the striatum is the main brain region affected by HD (J. P. Vonsattel et al., 1985), the disease also affects other areas such as the hippocampus and cortex (Walker, 2007), leading to cognitive and psychiatric symptoms that often appear before the onset of motor symptoms and cause the most distress to HD patients (McColgan & Tabrizi, 2018). While altered cellular mechanisms contributing to motor and cognitive deficits have been identified in HD models (Anglada-Huguet et al., 2014; Giralt et al., 2013; Giralt, Saavedra, et al., 2011; Miguez et al., 2015; Pupak et al., 2022; Suelves et al., 2017, 2019), none of them have been successfully translated into clinical treatments. Therefore, this Thesis aims to study the ER stress response and the astrocyte-neuron crosstalk to explore new therapeutic strategies for addressing both cognitive and motor symptomatology in HD. Firstly, we conducted a comprehensive study on ER stress caused by mHtt toxicity and tested the

potential therapeutic impact of a GSK compound that acts as a PERK inhibitor on cognitive dysfunction.

The first part of the Thesis focused on the hippocampus, while the second one concentrated on the striatum and aimed to examine the role of astrocytic pathology in the vulnerability of MSNs. Our goal for this second part was to assess the contribution of the protein ARMS to the astrocytic secretome alterations and explore its potential as a therapeutic target in HD.

Now, we redirect the attention to the first part of our research, which involves studying ER stress in an HD mouse model and evaluating the modulation of this process using a genetic approach to downregulate GRP78 and a PERK inhibitor.

Emerging role of the GRP78-PERK axis in Huntington's disease cognitive impairments.

ER stress is a common pathological hallmark shared by many neurodegenerative diseases including HD that leads to the activation of the UPR signaling cascade to maintain correct proteostasis and cellular health (Hetz & Saxena, 2017; Riccardi et al., 2015). Specifically, in HD it has been reported that mHtt might cause ER stress by sequestering and depleting the cytosolic chaperone p97/VCP and its cofactors Npl4 and Ufd1, both essential for the ERAD pathway enhancing protein accumulation in the ER (Duennwald & Lindquist, 2008; Leitman et al., 2013). The resultant ER stress triggers chronic upregulation of UPR markers in HD cellular and animal models as well as in *postmortem* samples from HD patients (Carnemolla et al., 2009; Duennwald & Lindquist, 2008; Leitman et al., 2013, 2014; Reijonen et al., 2008; Shacham et al., 2019). However, how the different UPR branches are affected depending on the brain region, and the potential link with cognition remain unexplored in the context of HD. To date, most of the studies have been focused on the connection between ER stress and striatal cell death or dysfunction with no studies addressing the possible relationship between HD memory impairment and ER stress. Indeed, defects in

memory function following induction of ER stress have been previously reported in the context of AD while recent evidence has underlined the importance of ER stress in synaptic function and memory formation under healthy conditions (Lin et al., 2018; N. Sen, 2019). Here, we have defined the emerging role of the GRP78/PERK axis, a major transducer of ER stress signaling to HD hippocampal and striatal neuropathology and memory dysfunction.

We demonstrated that early in the disease process, in stages before motor coordination and cognitive deficits are present (Mangiarini et al., 1996) there is a sustained ER stress activation, manifested as increased levels of GRP78 and CHOP in the hippocampus of R6/1 mice without any major significant alteration in the striatum or the cortex. These results are in accordance with previous studies showing no significant chamges in GRP78 expression in the striatum of symptomatic R6/1 mice (Fernandez-Fernandez et al., 2011) nor in GRP78 and CHOP levels in Knock-in^{Q111} striatal cells following ER stress induction by tunicamycin (Leitman et al., 2014). In contrast, at late disease stages (18 months of age) other HD mouse models exhibit increased levels of GRP78 and CHOP in the striatum and cortex (H. Lee et al., 2012) which may indicate that the ER stress response propagates in a specific brain region manner along HD disease progression.

To investigate the specific contribution of GRP78 to HD neuropathology and early memory disturbances, we generated a new double-mutant mouse line by crossbreeding R6/1 mice with heterozygous GRP78$^{+/-}$ mice. The resultant mice exhibited reduced levels of GRP78 compared to WT mice and normalized CHOP expression alleviating the overall ER stress response in the hippocampus. Besides, when the cognitive behavior was assessed, we found that spatial and recognition memories were preserved, suggesting that ER stress activation in the HD hippocampus negatively affects cognitive function. Similar to these results, in ApoE4 transgenic mice used as a model of AD, the reduction of GRP78 levels by curcumin treatment improved spatial memory deficits in the Morris water maze test (Kou et al., 2021). In line with these results, cognitive impairment induced in rats by microcystin-LR was prevented by treatment with the ER stress blocker tauroursodeoxycholic acid (TUDCA) along with a reduction in the ER stress response (Cai et al., 2015). Notably, TUDCA treatment also prevented spine density loss and synaptic activity dysfunction in APP/PS1 mice

(Ramalho et al., 2013) suggesting that the detrimental effect of ER stress on hippocampal function is related to changes in structural synaptic plasticity. According to this hypothesis, we have demonstrated that the reduction of GRP78 expression in R6/1 mice prevented the loss of dendritic spines in CA1 hippocampal neurons without gross hippocampal volume changes.

In this context, it is worth noting that while the decrease in GRP78 did not prevent the impairment of motor learning and spontaneous locomotor activity in R6/1 mice, there was a moderate improvement in motor coordination and the prevention of striatal atrophy. These findings align with prior research demonstrating that administration of TUDCA to R6/2 mice at middle-advanced disease stages reduces striatal atrophy and mitigates motor coordination deficits evaluated through the fixed rotarod task (Keene et al., 2002). These results may reflect a different role of ER stress, particularly GRP78 according to the HD disease stage and the affected brain region.

The observed behavioral improvements in R6/1 mice were accompanied by an increase in dendritic spine density in both the CA1 hippocampal region and striatum, indicating a potential role of GRP78 in structural synapses. Different studies have suggested that the UPR activation process is necessary for dendrite morphogenesis (Wei et al., 2015). Notably, the ER is not only present in the soma but also in dendrites, where it can facilitate local protein synthesis (Ju et al., 2004; Torre & Steward, 1996). Additionally, dendrites contain the translational machinery and components involved in protein folding and glycosylation, such as eIF2α and GRP78 (Gardiol et al., 1999), and are equipped with the ER-Golgi trafficking pathway (Horton & Ehlers, 2003). In accordance, a study from 2007 found in primary cultures of hippocampal neurons that in response to ER stress, GRP78 and P-eIF2α were also up-regulated in dendrites (Murakami et al., 2007).

The genetic reduction of GRP78 expression in R6/1 mice also appears to decrease the formation of striatal and hippocampal intranuclear mHtt inclusions. This observation raises questions about the role of GRP78 in protein folding and quality control. GRP78 is a chaperone protein that binds to nascent polypeptides and facilitates their proper folding (Casas, 2017). Therefore, it seems paradoxical that reducing GRP78 levels would have a positive effect on the number of mHtt aggregates. However, it is possible that this reduction may activate compensatory homeostatic alternative pathways, such

as autophagy, which could contribute to the reduction of mHtt aggregates. In fact, it has been reported that GRP78 knockdown in mouse arginine vasopressin (AVP) neurons leads to enhanced autophagy clearance (Kawaguchi et al., 2020).

An example of the complexity of ER stress activation in neurodegenerative diseases is the fact that in Parkinson´s Disease (PD), some studies have reported the benefits of GRP78 knockdown against the toxicity induced by α-synuclein (Salganik et al., 2015) or rotenone (M. Jiang et al., 2016) while others have demonstrated that upregulation of GRP78 suppresses α-synuclein aggregation and toxicity in PD rat models (Gorbatyuk et al., 2012). Therefore, understanding the precise involvement of GRP78 activation in neuronal dysfunction and neurodegeneration in specific pathogenic conditions is critical when designing new therapies targeting ER stress. In this scenario, studying the UPR branches and their signaling pathways in HD may help optimize these therapeutic interventions.

Several pieces of evidence have suggested that sustained activation of PERK impairs cognitive function due to prolonged inhibition of essential new protein synthesis involved in neuronal plasticity and memory formation (M. Ohno, 2018; Taalab et al., 2018). For this reason, we focused on this branch of the UPR pathways as a potential mechanism underlying hippocampal-dependent cognitive dysfunction in HD. According to the detrimental role of chronic PERK signaling in memory, the hippocampus of R6/1 mice showed increased PERK phosphorylation without any significant change in IRE1α phosphorylation or ATF6 levels. To further investigate the role of PERK hyperactivation in HD-related memory impairment, R6/1 mice were intraventricularly administered GSK2606414, a well-known PERK inhibitor (Axten et al., 2012) at the onset of cognitive symptoms. Remarkably, pharmacological inhibition of PERK prevented spatial and recognition memory deficits in R6/1 mice, which is in line with previous studies that have demonstrated the beneficial effects of PERK inhibition in various neuropathological conditions such as AD or PD (Devi & Ohno, 2014; Ma et al., 2013; Mercado et al., 2018). Besides, it is noteworthy that PERK inhibition also positively impacted the spontaneous locomotor activity as evidenced by an increase in the mean speed in treated R6/1 mice in the open field. These results, however, are not consistent with previous research by another group, showing that PERK activation improved motor function in R6/2 mice (Ganz et al., 2020). This apparent controversy

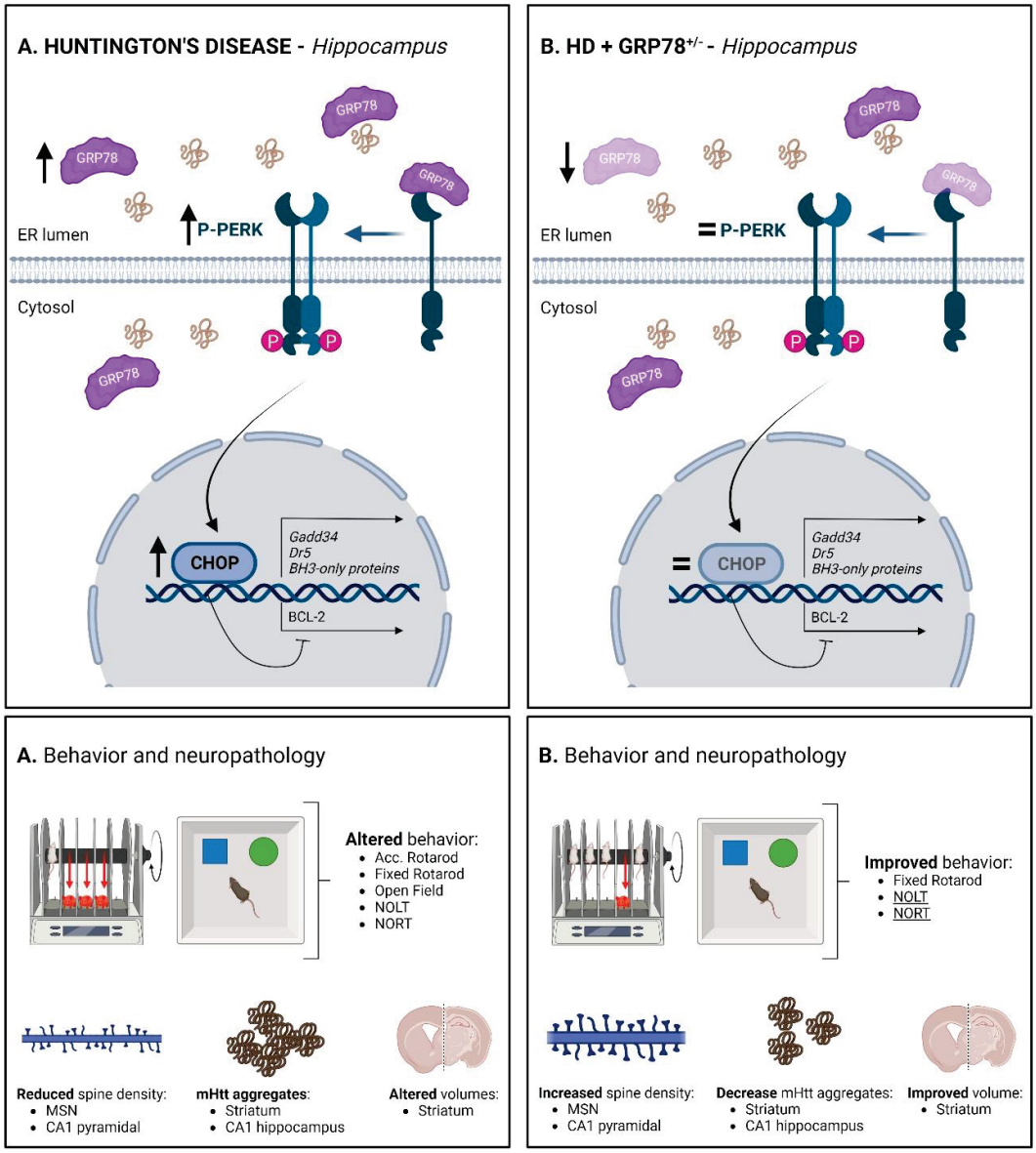
may rely on the fact that (1) they used subcutaneous mini-pumps for continuous delivery of the drug, and (2) the motor assessment was performed using the rotarod task which tests coordination instead of spontaneous locomotor activity as we did in the open field.

Different mechanisms may explain how PERK activation is involved in the observed HD memory impairments. The PERK-eIF2α axis has been considered a key regulator for memory disturbances in neurodegenerative diseases and neurological conditions since phosphorylation of eIF2α inhibits general translation and stimulates ATF4 expression, repressing CREB-mediated long-term potentiation and memory (Hetz & Saxena, 2017; Hughes & Mallucci, 2019; M. Ohno, 2018; Zhu, Henninger, et al., 2016). Thus, genetic, or pharmacological inhibition of eIF2α phosphorylation has been shown to facilitate memory (H. Q. Jiang et al., 2014; Rubovitch et al., 2015). Accordingly, elevated levels of P-eIF2α have been detected in the brains of AD patients and mouse models, while genetic deletion of PERK prevented synaptic plasticity and memory deficits in AD mice by reducing eIF2α phosphorylation (Ma et al., 2013). In contrast, here we have reported that inhibition of PERK is not associated with reduced phosphorylation of eIF2α even though PERK inhibition does rescue cognitive impairments in R6/1 mice. These results suggest that other molecular pathways downstream of PERK and independently of eIF2α may contribute to HD memory deficits. Thus, the prevention of spatial and recognition memory deficits in R6/1 mice treated with the PERK inhibitor was accompanied by the rescue of dendritic spine loss in CA1 pyramidal neurons suggesting that sustained PERK activation may induce a negative effect on dendritic spine formation or stability. Similar results were found in mouse models of traumatic brain injury where treatment with the PERK inhibitor GSK2656157 normalized dendritic spine density in cortical neurons preventing PERK phosphorylation but not phosphorylation of eIF2α (T. Sen et al., 2017). Similarly, in cortical primary cultures, genetic or pharmacological inhibition of PERK rescued the loss of dendritic spines reducing the phosphorylation of PERK but not of eIF2α (T. Sen et al., 2017).

In addition, our findings suggest that PERK phosphorylation may also play a role in regulating the levels of important immediate early genes (IEGs), such as Arc and c-Fos, which are rapidly and selectively upregulated during the formation of long-term memory (Minatohara et al., 2016). However, increased PERK phosphorylation does not

affect CREB phosphorylation at Ser133 even though an association between PERK-eIF2α signaling activation and CREB dysfunction has been previously described (Costa-Mattioli et al., 2005; Devi & Ohno, 2014). In line with our results, specific knockdown of PERK in the hippocampal CA1 region of middle-aged mice enhances memory without altering P-CREB levels or other synaptic proteins such as PSD95. Although we cannot completely rule out a contribution of PERK-induced CREB dysfunction in R6/1 memory problems, other PERK-related mechanisms might be involved. For instance, PERK may be contributing to the homeostasis of neuronal properties by modulating neuronal excitability, likely by regulation of neuronal calcium dynamics (Sharma et al., 2018; Zhu, Henninger, et al., 2016; Zhu, McGrath, et al., 2016). Since calcium dyshomeostasis can alter the activation of Ca^{+2}-dependent kinases that regulate the production of IEGs, we can hypothesize that PERK activation may contribute to memory disturbances by mechanisms related to aberrant neuronal excitability and calcium homeostasis leading to the dysregulation of synaptic proteins involved in structural and synaptic plasticity.

In sum, in our first study, we discovered a novel mechanism that contributes to cognitive deficits in HD, which involves the dysregulation of the GRP78/PERK axis (Figure 40). These findings offer promising evidence that PERK inhibition could be a valuable therapeutic strategy for preventing memory impairments in HD.


A. HUNTINGTON'S DISEASE - Hippocampus
GRP78
ER lumen
P-PERK
Cytosol
CHOP
Gadd34
Dr5
BH3-only proteins
BCL-2
B. HD + GRP78+/- - Hippocampus
A. Behavior and neuropathology
Altered behavior:
Acc. Rotarod
Fixed Rotarod
Open Field
NOLT
NORT
Reduced spine density:
MSN
CA1 pyramidal
mHtt aggregates:
Striatum
CA1 hippocampus
Altered volumes:
Striatum
B. Behavior and neuropathology
Improved behavior:
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CA1 hippocampus
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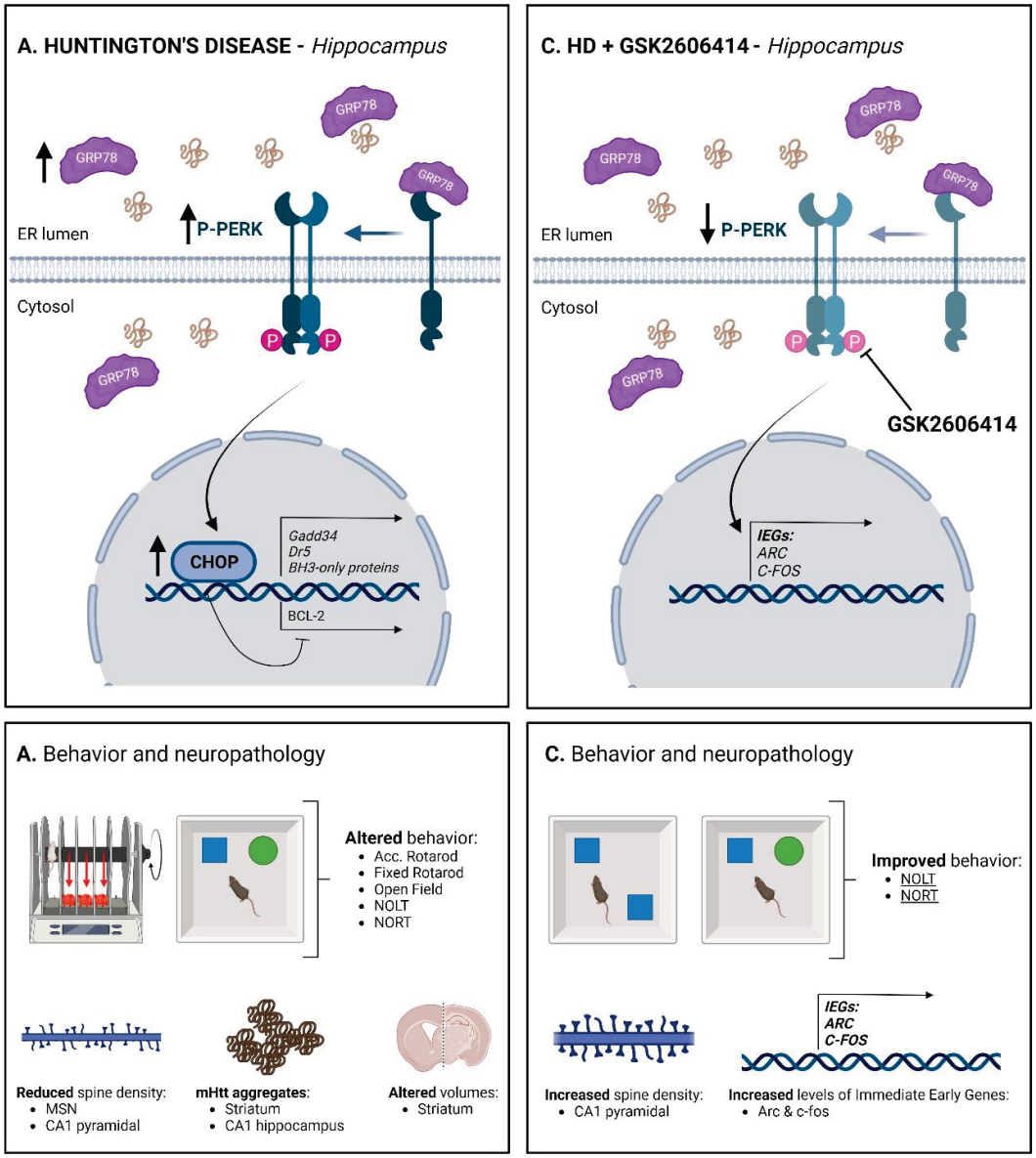


Figure 40. Role of the GRP78-PERK axis in Huntington's Disease neuropathology and cognitive impairments. **A** At 12 weeks of age, before motor coordination and cognitive deficits appear there is a sustained ER stress activation, manifested as increased levels of GRP78, PERK, and CHOP in the hippocampus of R6/1 mice without any significant alteration in the striatum or the cortex. **B** DM mice have preserved spatial and recognition memories and moderately improved motor coordination. This goes together with an increase in the dendritic spine density of CA1 pyramidal neurons and MSN, a decrease in the number of mHtt aggregates in the CA1 and striatum, and the prevention of striatal atrophy. **C** Pharmacological inhibition of PERK by GSK2606414 prevented spatial and recognition memory deficits in R6/1 mice. This was accompanied by the rescue of dendritic spine loss in CA1 pyramidal neurons and the upregulation of important immediate early genes (IEGs), such as Arc and c-Fos.

Astrocytes at the hub of striatal neuronal dysfunction in HD: dissecting the role of ARMS on the astrocytic secretome.

The proper function of the nervous system largely depends on the communication between neurons and astrocytes and involves the release of various factors that can impact both cell types and regulate synaptic transmission and neuronal function (Robertson, 2018; Schiera et al., 2020). However, in pathological contexts like HD, alterations in the HD astrocyte-secretome could lead to neuronal vulnerability and dysfunction by deficient secretion of neurotrophic factors like BDNF, as well as an abnormal astrocytic inflammatory response. As a common mechanism involved in the altered astrocytic secretion in HD, we have focused on ARMS, a multi-functional scaffolding protein highly expressed in the nervous system (Scholz-Starke & Cesca, 2016) discovered in 2000 (Iglesias et al., 2000) that modulates neuronal processes of survival, differentiation, and synaptic plasticity (Neubrand et al., 2012). Thus, different studies conducted on ARMS knockout mice have demonstrated that alterations in this protein can negatively impact neuronal survival and development. For instance, the complete removal of the protein causes embryonic death and extensive apoptosis in the central and peripheral nervous systems (Cesca et al., 2011, 2012). Even a partial reduction of ARMS levels induces defects in spine turnover and synaptic plasticity, ultimately affecting higher-order functions such as learning and memory in adult mice (Arévalo et al., 2010; Duffy et al., 2011; S. H. Wu et al., 2009, 2010). Together, these findings suggest that alterations to the *KIDINS220* gene and/or the ARMS protein could be associated with various human pathologies. However, research in this area is still very limited. For instance, ARMS has been found to behave as an oncogene in melanoma, a type of cancer of neural crest origin (Liao et al., 2007, 2011), and neuroblastoma, which affects the peripheral nervous system (Rogers & Schor, 2013a, 2013b). Increased ARMS levels have also been observed in human AD samples, where it accumulates with hyperphosphorylated Tau protein, leading to defective neurotrophic signaling due to increased resistance of ARMS to calpain cleavage

associated with ARMS hyperphosphorylation (López-menéndez et al., 2013; Scharfman & Chao, 2013). In addition, genetic studies have found alterations in *KIDINS220* gene expression in PD and Autism Spectrum Disorder patients, with decreased and increased levels, respectively (S. W. Kong et al., 2012; Simunovic et al., 2009). More recently, *KIDINS220* missense polymorphisms and a novel gene variant have been identified in some schizophrenic patients (Kranz et al., 2015). Hence, several studies have identified ARMS as a critical player in neuronal regulation, thereby offering a promising avenue for therapeutic intervention in neurodegenerative diseases and other neurological disorders. In the case of HD, a recent study revealed that the protein ARMS can produce two main isoforms through alternative terminal exon splicing, resulting in the Kidins220-C32 (full-length) and Kidins220-C33 (spliced) variants (Schmieg et al., 2015; Sebastián-Serrano et al., 2020). Kidins220-C33 is selectively downregulated in the striatum of HD patients and in R6/1 mice from early symptomatic stages and may play a crucial role in the development of HD. However, these changes are specific to the C33 variant only and are dependent on the activation of the protease calpain, while the Kidins220-C32 variant remains unaffected (Sebastián-Serrano et al., 2020). Interestingly, these results slightly differ from our studies published in 2018, which found increased levels of ARMS in the hippocampus and cortex of human HD brains, and only in the hippocampus of HD mouse models (López-Benito et al., 2018). The reason for the differences observed could be due to variations in the patient samples, including differences in the *postmortem* interval, storage, or methods of preparing brain lysates. Additionally, discrepancies could be attributed to the HD mouse model used in the two studies and the age or stage of the disease of the animals that were analyzed.

Although the role of ARMS in neurons has been extensively studied *in vitro* and *in vivo*, its function in astroglial cells remains largely unexplored and is now starting to take center stage. A recent study has shed light on this topic by examining the role of ARMS in astrocytes using *Kidins220*$^{-/-}$ embryos. The findings revealed that, in addition to its established role in neurons, ARMS also plays a significant role in astrocytes by modulating intracellular Ca^{2+} dynamics, survival, and death pathways, as well as facilitating communication between astrocytes and neurons (Jaudon et al., 2020,

2021). For this reason, in this second project, we aimed to analyze the role of astrocytic ARMS in the context of HD's striatal vulnerability. Thus, we first evaluated the levels of ARMS in astrocytes and neurons and discovered that ARMS is highly more abundant in HD primary striatal astrocytes than in striatal neurons. Next, to study the contribution of ARMS to HD motor deficits, we generated AAVs expressing shARMS under the GFAP promoter to selectively knock down ARMS expression in astrocytes. Interestingly, knocking down astrocytic ARMS in R6/1 mice led to improved motor learning, coordination, fine motor behavior, and balance with no effect on spontaneous locomotor activity. These results go in accordance with a recent study that investigated the potential therapeutic effects of a bioflavonoid named morin hydrate (MH) on a 3-NP model of HD (Mohamed et al., 2023). In this study, the balance beam walking deficits observed in mice treated with 3-NP were accompanied by an increase in the cortical levels of ARMS, NF-κB, TNFα, and IL-1β, along with a decrease in BDNF levels. Notably, the treatment with MH reversed motor deficits and the aberrant cortical increase in ARMS levels, which was associated with a depletion of BDNF and its receptor TrkB, consistent with our published findings in HD (López-Benito et al., 2018).

Based on these data, we next focused on BDNF as a potential mechanism underlying the beneficial effects of normalizing astrocytic ARMS expression in R6/1 motor deficits. Prior studies have well established a connection between reduced levels of BDNF and the development of neuropathology in HD (B. Lu et al., 2005; Zuccato & Cattaneo, 2007). Furthermore, it has been demonstrated that augmenting BDNF levels can alleviate neuropathology and symptoms of HD in animal models (Giralt, Carretán, et al., 2011; Zuccato et al., 2005). Different studies have also shown that the depletion of BDNF in neurons is induced by mHtt, which affects the transcriptional expression of BDNF (Zuccato et al., 2001) and its transportation from cortical neurons to the striatum (Gauthier et al., 2004), thereby contributing to HD pathogenesis. However, it is worth noting that astrocytes are also an essential source of BDNF (Miyamoto et al., 2015) and release it via exocytosis to sustain nearby neurons and modulate their functions (Parpura & Zorec, 2010; X. Wu et al., 2008). Considering that 70% of cells in the striatum are astrocytes, their role in this brain region is likely going to be critical. Accordingly, it has been shown that overexpressing mHtt in cultured astrocytes leads to

reduced levels of BDNF by trapping BDNF transcriptional factors into mHtt aggregates (L. Wang, Lin, Wang, Wu, Han, Zhu, Difiglia, et al., 2012). In addition, back in 2016, Hong et al. conducted a study using primary cultures and brain slices from HD mice to examine the impact of mHtt on BDNF secretion. Their results revealed that endogenous levels of mHtt impaired the secretion of BDNF from HD-cultured astrocytes and brain slices, likely due to the specific binding of mHtt to Rab3a in astrocytes (Hong et al., 2016).

On the other hand, several studies have shown that ARMS plays a crucial role in the tightly regulated secretion of BDNF in cultured DRG neurons in response to NGF (López-Benito et al., 2016). Moreover, our group demonstrated that increased expression of ARMS was associated with deficient BDNF-regulated secretion in HD mice (López-Benito et al., 2018). Recent research in 2023 has also emphasized the significance of ARMS in regulating nociception by controlling BDNF secretion (Sánchez-Sánchez et al., 2023). Therefore, given the aberrantly elevated levels of ARMS in striatal astrocytes, we aimed to study the role of astrocytic ARMS in the release of BDNF. To this aim, we stimulated primary striatal astrocytes with KCl, and BDNF release was measured in the medium using ELISA analysis. We found similar BDNF release between WT and R6/1 sh-SCB primary astrocytes. However, treatment with AAV2/5-GFAP-shARMS-GFP increased the release of BDNF in R6/1 astrocytes, indicating that the aberrantly increased ARMS expression in R6/1 astrocytes hinders the release of BDNF, which may account for the behavioral improvements observed. Besides BDNF, activated astrocytes can also secrete cytokines and chemokines that can have either neuroprotective or neurotoxic effects, thereby contributing to the pathogenesis of HD (Hsiao et al., 2013; Linnerbauer et al., 2020; Rocha et al., 2016; Stephenson et al., 2018). For instance, elevated production of IL-6, MMP-9, VEGF, and TGF-β1 have been detected in the plasma of HD patients correlating with HD progression (K. H. Chang et al., 2015; Dalrymple et al., 2007; Wild et al., 2011). In addition, other studies have also found signs of immune activation in CSF from HD patients with upregulation of IL-6, IL-8, and TNFα (Björkqvist et al., 2008). Moreover, HD *postmortem* tissue exhibits elevated levels of TNFα and IL-1β in the striatum, and upregulation of MMP-9, IL-6, and IL-8 in the cortex and cerebellum (Silvestroni et al., 2009a). Therefore, the next logical

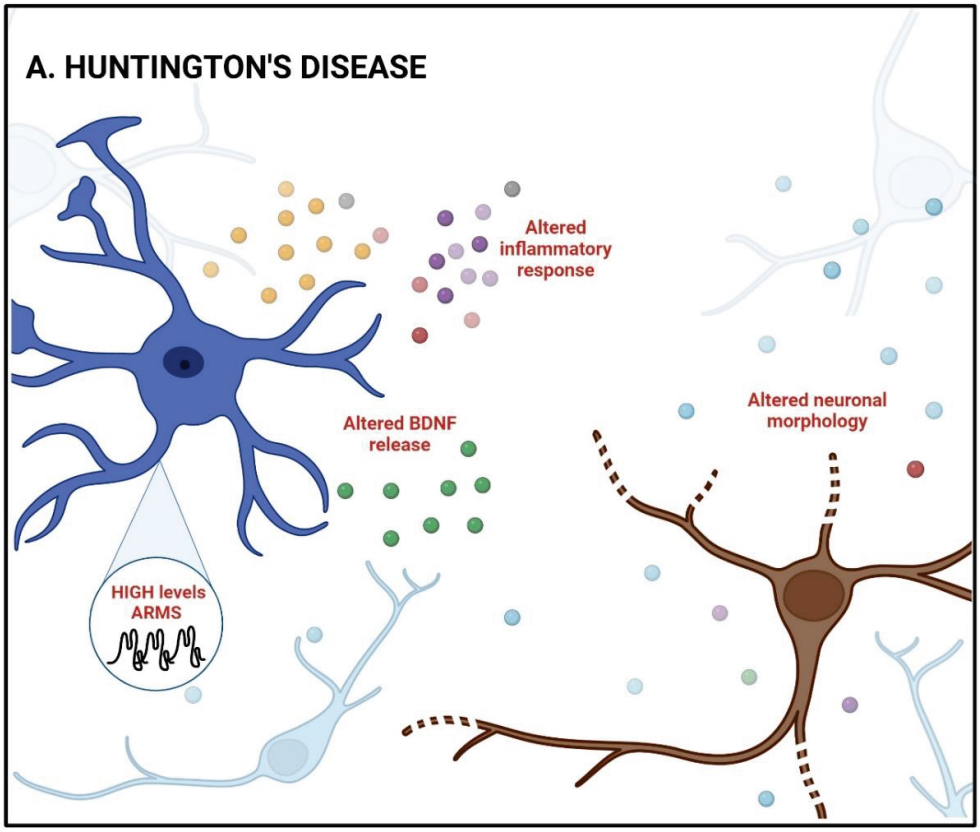
step was to examine the impact of astrocytic ARMS on cytokine release. Our study successfully detected CXCL12, INF-Y, IL-1α, IL-16, TIMP-1, and TNFα in the secretomes obtained from primary striatal astrocytes. Regardless of the genotype, primary striatal astrocytes mainly released CXCL12, followed by TIMP-1, TNFα, and to a lesser extent IL-1α, INF-Y, and IL-16. Notably, the most striking observation was the significant increase in CXCL12 after the knockdown of ARMS, irrespective of the genotype. CXCL12 has a direct effect on different types of brain cells, including neurons, astrocytes, and microglia, which express the CXCR4 receptor (Banisadr et al., 2002; Baudouin et al., 2006; de Haas et al., 2007; Meucci et al., 1998; Reaux-Le Goazigo et al., 2012; van der Meer et al., 2000). This chemokine was initially believed to contribute to CNS inflammation by attracting T- and B-lymphocytes and inflammatory monocytes (Calderon et al., 2006; Krumbholz et al., 2006; McCandless et al., 2008). However, it was later discovered that CXCL12 could also guide neuronal progenitor cells to damaged areas of the brain, promoting recovery in contexts such as ischemia, MS, and AD (M. Li et al., 2012; Robin et al., 2006). Several studies have also shown that CXCL12 has neuroprotective effects by reducing apoptotic cell death, promoting pro-survival signaling, regulating cell-cycle proteins in various types of neurons (Khan et al., 2003, 2005, 2008), and promoting astrocyte and microglial cell activity (Khan et al., 2004). In the context of ischemia, CXCL12 reduces brain damage and behavioral deficits in animal models (Y. Li et al., 2014; Shyu et al., 2008; Yoo et al., 2012), and can be secreted by astrocytes in the ischemic penumbra (Hill et al., 2004; J. T. Miller et al., 2005; Ohtaki et al., 2006; Stumm et al., 2002). Moreover, previous studies have also found that CXCL12 has a protective effect on AD by reducing amyloid protein deposition and promoting phagocytosis by microglia (Janssens et al., 2018; Q. Wang et al., 2012). CXCL12 may also be a potential biomarker for certain neurodegenerative diseases. In the case of PD, α-synuclein can stimulate the secretion of CXCL12 by microglia (Y. Li et al., 2019), which can be detected in peripheral blood and serve as a biomarker for the disease (Bagheri et al., 2019). In addition, CXCL12 can also be detected in CSF in sporadic ALS (Andrés-Benito et al., 2020) and MS (Magliozzi et al., 2019). Finally, while its expression is impaired in progressive supranuclear palsy (PSP), it remains unaltered in frontotemporal dementia (FTD) (Bonham et al., 2018). Therefore, the discovery that ARMS plays a crucial role in regulating the release of

CXCL12 by astrocytes paves the way for further investigations into the function of this chemokine in HD and its potential as a neuroprotective therapeutic target for the disease.

In addition, we found that TNFα and TIMP-1 levels were significantly decreased in both WT and R6/1 astrocytes when ARMS protein levels were knocked down. In the brain, TNFα is a pro-inflammatory cytokine produced by neurons and glial cells with a crucial role in initiating inflammatory cytokine signaling cascades (Clark & Vissel, 2018; Parameswaran & Patial, 2010). TNFα regulates acute phase inflammation and has various physiological functions, such as fetal development, cardiovascular health, and immune system components (Mizrahi & Askenasy, 2014; Urschel & Cicha, 2015). However, uncontrolled TNFα expression leads to chronic inflammation, high gliosis, synaptic loss, and glutamatergic toxicity, which are associated with several neurodegenerative disorders, such as MS, HIV-associated dementia, PD, ischemia, and AD (Decourt et al., 2016; Jung et al., 2019; Ramos-Cejudo et al., 2018). Therefore, TNFα may be a principal target for treatment approaches to reduce the onset of neurodegenerative diseases with an inflammatory component (Hsiao et al., 2014). In the case of HD, postmortem analysis of human HD tissue has revealed elevated levels of inflammatory molecules such as TNFα and IL-1β in the striatum (Möller, 2010; Silvestroni et al., 2009b). Besides, reactive astrocytes from R6/1 mice have been associated with the upregulation of NF-κB signaling and the increased production of pro-inflammatory mediators such as TNFα and iNOS (Miguez et al., 2015). These results are consistent with previous findings that suggested a positive correlation between TNFα levels and disease progression in HD patients (Björkqvist et al., 2008). Thus, targeting the upregulation of NF-κB and TNFα has also been identified as an effective therapeutic approach in R6/2 mice (Hsiao et al., 2013, 2014). Finally, in our study INF-Y levels remained unchanged in both genotypes and treatment conditions, while the levels of pro-inflammatory cytokines like IL-16 and IL-1α significantly increased by treatment with sh-ARMS only in WT astrocytes. Altogether, our research so far has identified ARMS as a crucial regulator of the astrocyte secretome, hindering BDNF release and contributing to an atypical astrocytic inflammatory profile. The next question was to investigate the potential impact of these changes on neuronal

vulnerability and dysfunction. To this aim, primary striatal neurons were treated with ACM obtained from ARMS knockdown astrocytes previously activated with KCl. Our findings indicated that neuronal viability was not affected by the astrocytic secretome regardless of ARMS levels, since there were no differences in the percentage of viable and condensed nuclei between WT and R6/1 neurons treated with ACM from sh-SCB or sh-ARMS astrocytes. However, when we examined the impact on neuronal morphological features, our data suggest that secretomes obtained from WT and R6/1 astrocytes with reduced ARMS expression have the potential to improve some morphological parameters of striatal neurons, both WT and R6/1. This positive effect may be attributed to the increase in the release of BDNF and CXCL12 and the decrease in the secretion of TNFα.

In sum, our results suggest that aberrant expression of ARMS in striatal astrocytes contributes to HD motor behavioral deficits and that knocking it down offers a promising new therapeutic target in HD treatment. It is worth noting that although the improvements observed were moderate, they were obtained solely by knocking down ARMS expression in astrocytes, indicating that this approach could be a valuable addition to HD therapeutics.



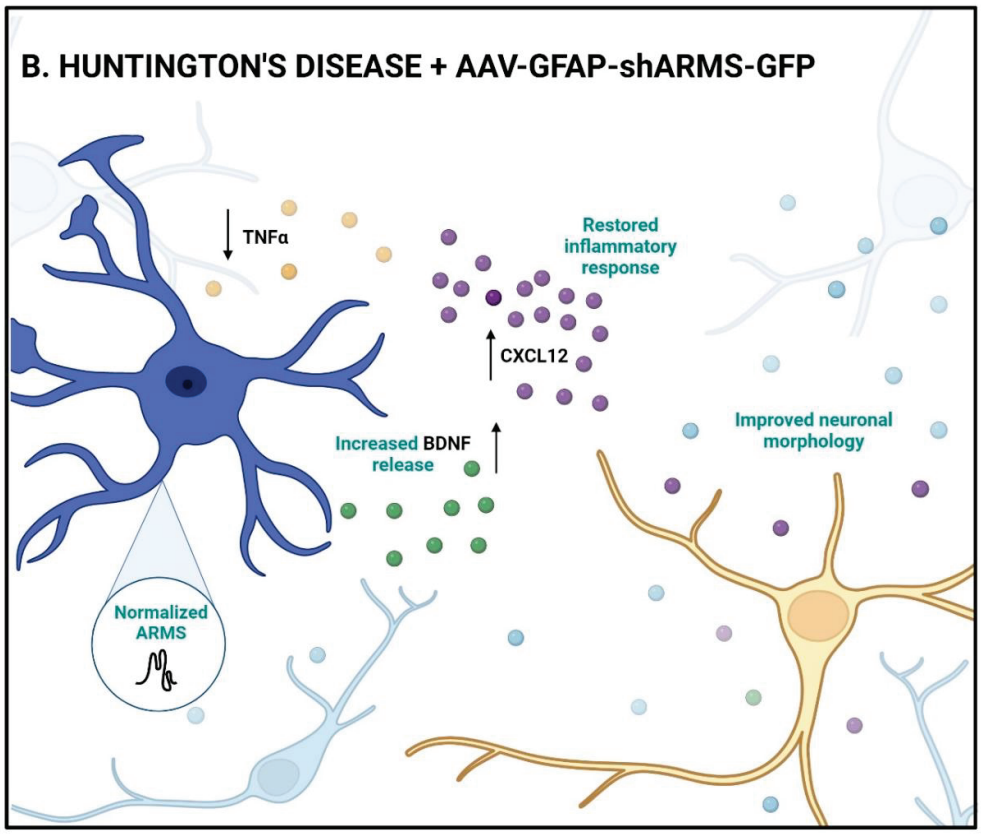


Figure 41. The role of ARMS on the astrocytic secretome alterations in HD. A In pathological contexts like HD, ARMS is aberrantly increased in striatal astrocytes. This leads to deficient secretion of neurotrophic factors like BDNF, as well as an abnormal astrocytic inflammatory response, which contributes to neuronal vulnerability and dysfunction. **B** We used AAVs expressing shARMS under the GFAP promoter to selectively knock down ARMS expression in astrocytes which led to improved motor learning, coordination, fine motor behavior, and balance with no effect on spontaneous locomotor activity in R6/1 mice. This positive effect may be attributed to the increase in the release of BDNF and CXCL12 and the decrease in the secretion of TNFα.

DISCUSSION

As 30 years have passed with no cure for HD, the goal of this Thesis was to explore cellular mechanisms involved in HD pathology to identify new therapeutic opportunities for managing HD symptoms. The first part of the Thesis focused on the hippocampus and neuronal dysfunction, analyzing the role of ER stress in HD cognitive deficits, and investigating the potential of GRP78 and PERK as therapeutic targets to address memory impairments. A novel mechanism was discovered, which showed that dysregulation of the GRP78/PERK axis contributes to cognitive deficits in HD. These findings suggest that inhibiting GRP78 and PERK could be an effective therapeutic strategy for preventing memory impairments in HD. The second part of this Thesis centered on the striatum and the role of astrocytes in striatal vulnerability. We investigated the role of ARMS in the astrocytic-secretome alterations in HD and explored its potential as a therapeutic target. Our findings indicated that abnormal ARMS expression in striatal astrocytes contributes to motor behavioral deficits in HD, likely by impeding the release of BDNF and impacting the release of CXCL12 and TNFα. Thus, knocking down astrocytic ARMS represents a promising new therapeutic strategy for treating motor symptoms in HD. Given that ARMS is highly expressed in the hippocampus compared to other brain regions and our previous research in 2018 identified elevated levels of ARMS in the hippocampus of individuals with HD, investigating the potential involvement of astrocytic ARMS in this brain region could be a logical next step in this project.

In summary, these investigations emphasize the need to understand the molecular and cellular mechanisms behind HD, not just from a neuronal perspective, but also considering the role of astrocytes. Our findings highlight the importance of the communication between neurons and astrocytes and suggest that a combination of different therapeutic targets may be necessary for effectively treating HD. I hope that this Thesis will help raise awareness of these key points and inspire further research into finding a cure for HD.

CONCLUSIONS

The main conclusions extracted from the results presented in this Thesis are the following:

1. There is an early and specific ER stress response in the hippocampus of R6/1 mice at pre-symptomatic disease stages characterized by an increase in GRP78 and CHOP.
2. Genetic reduction of GRP78 expression prevents the appearance of hippocampal-dependent memory disturbances in R6/1 mice by acting on dendritic spines and mutant huntingtin aggregate formation.
3. Genetic reduction of GRP78 expression moderately prevents motor coordination deficits and striatal neuropathological features in R6/1 mice manifested as improved dendritic spine density and mutant huntingtin aggregate formation.
4. PERK signaling is the downstream UPR sensor responsible for cognitive impairments in HD as chronic inhibition by GSK2606414 in R6/1 mice at pre-symptomatic disease stages prevents long-term memory deficits.
5. Deregulation of the GRP78/PERK axis in the HD hippocampus results in synaptic structural alterations manifested as loss of dendritic spines and protein expression of immediate early genes.
6. ARMS levels are significantly increased in R6/1 striatal primary astrocytes.
7. Knocking down ARMS in striatal astrocytes prevents motor learning, coordination, and balance deficits in R6/1 mice.
8. knocking down ARMS in striatal astrocytes increases the release of BDNF and modulates the secretion of cytokines and chemokines ultimately affecting neuronal morphology.

Collectively, our findings offer additional understanding regarding the role of ER stress in cognition and the control of the HD astrocytic secretome by ARMS in HD pathogenesis. Our results also underline the potential of GRP78 and PERK as promising targets for therapy to address the memory deficits observed in HD and the potential of astrocytic ARMS as a promising target to alleviate the motor impairments in HD.

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ANNEX

Publications derived from this Thesis

Espina M, Brañas-Navarro M, Costas-Insua C, Di Franco N, Guzmán M, Ginés S. The GRP78-PERK axis contributes to memory and synaptic impairments in Huntington's disease R6/1 mice. Neurobiology of disease. **2023**. *Manuscript sent, reviewed & waiting for feedback*.

Espina M, López-Molina L, Pupak A, Di Franco N, Brito V, Arévalo JC, Ginés S. Astrocytes at the hub of striatal neuronal dysfunction in Huntington's Disease: Dissecting the role of ARMS on the astrocytic secretome. **2023**. *Manuscript under preparation*.

Pupak A, Singh A, Sancho-Balsells A, Alcalá-Vida R, **Espina M**, Giralt A, Martí E, Ørom UAV, Ginés S, Brito V. Altered m6A RNA methylation contributes to hippocampal memory deficits in Huntington's disease mice. Cell Mol Life Sci. **2022** Jul 11;79(8):416. doi: 10.1007/s00018-022-04444-6. PMID: 35819730; PMCID: PMC9276730. ***Impact factor: 9.207***

Costas-Insua C, Moreno E, Maroto IB, Ruiz-Calvo A, Bajo-Grañeras R, Martín-Gutiérrez D, Diez-Alarcia R, Vilaró MT, Cortés R, García-Font N, Martín R, **Espina M**, Botta J, Ginés S, McCormick PJ, Sánchez-Prieto J, Galve-Roperh I, Mengod G, Urigüen L, Marsicano G, Bellocchio L, Canela EI, Casadó V, Rodríguez-Crespo I, Guzmán M. Identification of BiP as a CB1 Receptor-Interacting Protein That Fine-Tunes Cannabinoid Signaling in the Mouse Brain. J Neurosci. **2021** Sep 22;41(38):7924-7941. doi: 10.1523/JNEUROSCI.0821-21.2021. Epub 2021 Aug 5. PMID: 34353897; PMCID: PMC8460140. ***Impact factor: 6.167***

Brito V, Giralt A, Masana M, Royes A, **Espina M**, Sieiro E, Alberch J, Castañé A, Girault JA, Ginés S. Cyclin-Dependent Kinase 5 Dysfunction Contributes to Depressive-like Behaviors in Huntington's Disease by Altering the DARPP-32 Phosphorylation Status in the Nucleus Accumbens. Biol Psychiatry. **2019** Aug 1;86(3):196-207. doi: 10.1016/j.biopsych.2019.03.001. Epub 2019 Mar 13. PMID: 31060804. ***Impact factor: 12.095***