Study of the neurodegenerative process in Mucopolysaccharidosis type VII and its correction by gene therapy

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ACADEMIC DISSERTATION

To obtain the Degree of PhD in Biochemistry, Biomolecular Biology and Biomedicine by the Universitat Autònoma de Barcelona

Directed by:

Assumpció Bosch Merino and Ángela Sánchez Osuna

2023

This thesis has been conducted at the Institut de Neurociènces (INc) in the Group of Gene Therapy for Neurometabolic Disorders, part of the Research Group in Demyelinating and Autoimmune Diseases and the Department of Biochemistry and Molecular Biology, and has been directed by Drs. Assumpció Bosch Merino and Ángela Sánchez Osuna.

All the research described has been financially supported by the FI-AGAUR fellowship (2018FI_B_00458) and the ISC III (PI 15/1271).

AGRADECIMIENTOS

En primer lugar, me gustaría agradecer a mis directoras, las Dras. Assumpció Bosch y Ángela Sánchez, por darme la oportunidad de realizar esta tesis doctoral, por confiar en mí y motivarme cuando las cosas no salían como esperaba. Gracias también por todo lo que me habéis enseñado y por vuestra dedicación sin la cual esta tesis no sería la misma.

Al Dr. Miguel Chillón por sus sugerencias y aportes durante los *lab meetings* que me han ayudado a mejorar a nivel científico y técnico.

A las Dra. Silvia Ginés y Verónica Brito y al Dr. Alfredo Miñano, sin los cuales no hubiese sido posible la realización del amplio abanico de técnicas utilizado en esta tesis.

También quiero dar gracias a todos los compañeros que han pasado por la 5ª planta del CBATEG durante todos estos años.

A Txell y a Javi por su inestimable ayuda en todo lo que he necesitado, y a la UPV, que siempre han sido una gran fuente de conocimiento en todo lo relacionado con el *cloning*, el cultivo celular y la producción de vectores virales y no han dudado nunca en compartirlo conmigo. Gracias por hacerme hueco en las cabinas del P2 siempre que lo he necesitado, aunque tuviésemos que trabajar todos un poco más apretados. Y sobre todo gracias a Susana, cuya dedicación y energía inagotable me ha proporcionado el empuje que necesitaba a veces. Las mañanas, o debería decir madrugadas en el laboratorio no hubiesen sido iguales sin ti y sin Ángela para hacerme compañía y ayudarme a cazar ratones en ocasiones especiales.

También agradecer a Isa, que siempre ha tenido una sonrisa para mí independientemente de la hora a la que yo llegase y por enseñarme que se puede continuar trabajando duro incluso cuando las circunstancias no son las mejores.

A Alejandro, por su gran contribución al progreso de esta tesis. Junto con Rubén, su presencia en el laboratorio ha hecho más entretenidos los largos ratos de trabajo.

A Laia, por infundir esa alegría tan y tan necesaria cuando las cosas no salen bien, por esas charlas llenas de cotilleos en las que se notaba que por mucho que ella hubiese llegado al CBATEG más tarde que yo siempre está mucho más enterada de todo lo que pasa.

A Sergi, por ser un ejemplo de dedicación y esfuerzo… y por ponerme música cada vez que se lo pedía, aunque la respuesta a "¿Qué quieres que ponga?" siempre fuese "No sé, lo que quieras" y le tocase a él decidirlo. Gracias por ser una gran persona tanto dentro como fuera del lab y estar siempre dispuesto a proponer cosas para hacer en grupo y que nos sigamos viendo.

A Joan, por su ayuda siempre que lo he necesitado en el lab y todas esas charlas con el café que me volvían a cargar las pilas para seguir trabajando. Compartir todas estas experiencias, sobre todo en la etapa final del doctorado lo han hecho mucho más llevadero y me han hecho sentir mucho más acompañada.

A Marc, por esas largas y largas, pero muy largas horas inyectando animales. Tuyos, míos, de otros… daba igual. Por venir a buscarme con la moto para llevarme a trabajar incluso cuando iba con muletas, y compartir conmigo la afición por el deporte, aunque eso nos llevase a momentos incómodos en alguna ocasión. Biorad, nada más que decir.

En estos años habéis pasado de ser compañeros de trabajo a ser amigos que me han hecho disfrutar de esta etapa y me habéis dado el soporte que necesitaba en los momentos más duros.

A mi gran y unida familia, por apoyarme durante esta etapa y animarme a conseguir lo que quería. En especial a mis padres, por todo su apoyo y su confianza en que puedo lograr todo aquello que me proponga. También a Ana, con quien una vez más y para sorpresa de nadie he compartido todo este proceso y a Sandra, que nos ha tenido que aguantar a ambas durante este tiempo y que ha intentado a la vez que trabajásemos duro y que descansásemos lo suficiente. También a mis gatos Kovu y Zira, sin los cuales la redacción de esta tesis hubiese sido un proceso mucho más rápido, pero mucho más solitario, frío y aburrido.

Me gustaría acabar agradeciendo a toda la gente que ha estado conmigo a lo largo de esta etapa de mi vida. No importa si habéis estado desde el principio o si sólo habéis llegado para ver el final. Vuestro soporte y cariño han sido inestimables para mí. Gracias.

Contents

1. Abbreviations

AAP	assembly-activating protein
AAV	adeno-associated virus
AD	alzheimer's disease
AEC	amniotic epithelial cell
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
AV	adenovirus
BBB	blood brain barrier
Bcl-2	B-cell lymphoma protein 2
b-gluc	b-glucuronidase
bp	base pairs
BSA	bovine serum albumin
CB	cerebellum
CED	convection-enhanced delivery
CLN1	infantile neuronal ceroid lipofuscinosis
CMA	chaperone-mediated autophagy
CNS	central nervous system
CPF	prefrontal cortex
CS	chondroitin sulfate
CSF	cerebrospinal fluid
CX	cortex
CYP	cytochrome P450
DMEM	Dulbecco's modified eagle medium
DRG	dorsal root ganglia
DS	dermatan sulfate
dsDNA	double-stranded DNA
ECM	extracellular matrix
ECx	entorhinal cortex
ER	endoplasmic reticulum
ERT	enzyme replacement therapy
FADD	fas-associated protein with death domain
FBS	fetal bovine serum
FTD	frontotemporal dementia
GABA	gamma-aminobutyric acid
GA	golgi apparatus
GAG	glycosaminoglycan
GECI	genetically-encoded calcium indicator
GFAP	glial fibrillary acidic protein
GluR1	glutamate receptor 1
gRNA	guide RNA

GSTM1	glutathione S-transferase-micro 1
HA	hyaluronan
HC	hippocampus
HD	Huntington's disease
HOPS	homotypic fusion and protein sorting
HRP	horseradish peroxidase
HS	heparan sulfate
HSC	hematopoyetic stemm cell
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
IA	intra-arterial
IC	intracranial
ICM	intra-cisterna magna
ICV	intra-cerebroventricular
IDS	iduronate 2-sulfatase
IF	immunofluorescence
iPSC	induced pluripotent stem cell
IT	intrathecal
ITR	inverted terminal repeat
IV	intravenous
kDa	kiloDalton
LAMP-1	lysosome-associated protein 1
LINCL	late infantile neuronal ceroid lipofuscinosis
LPLD	lipoprotein lipase deficiency
LSD	Lysosomal storage disorders
LV	lentivirus
M6P	mannose -6-phosphate
MAC	membrane attack complex
mg	milligram
MHC	major histocompatibility complex
µl	microliter
MLKL	mixed lineage kinase like
mo	Month old
MPS	mucopolysaccharidosis
MSC	mesenchymal stem cell
NCAM	neural cell adhesión molecule
NHP	non-human primate
NPC	niemann-Pick disease type C
OMIM	online mendelian inheritance in man
PBS	phosphate-buffered saline

PD	parkinson’s disease
PFA	paraformaldehyde
PFC	Prefrontal cortex
PI3P	phosphatidylinositol-3-phosphate
PIK3C3	phosphatidylinositol 3-kinase class III
PNS	peripheral nervous system
PSD	postsynaptic density
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RV	retrovirus
s	second
SC	spinal cord
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNARE	N-ethylmaleimide sensitive factor attachment protein receptor
SPF	specific pathogen free
SPM	presynaptic membrane
SRT	substrate reduction therapy
TBS	tris buffered solution
TBS	tris-buffered saline
TEMED	N, N, N', N', tetrametil-etilen-diamine
TFEB	Transcription factor EB
TNF-R	tumor necrosis factor receptor
tRNA	transfer RNA
TUJ	β-tubulin III
UPR	unfolded protein response
WB	western blot
WT	wild type

2. Introduction

2.1. Lysosomal storage disorders

The term lysosomal storage diseases encompasses a family of more than 50 monogenic inherited diseases caused by a deficiency in one enzyme essential for the correct functioning of the lysosome(Kingma et al., 2015).

Lysosomes are membrane-bound organelles present in all cell types, and their function is essential for a correct cell homeostasis. Lysosomes are spherical vesicles with an acidic interior optimal for the activity of the hydrolytic enzymes they contain. Besides the large number of enzymes that can be found in the lysosomes, they also contain membrane-bound proteins and proton pumps(Saftig & Klumperman, 2009).

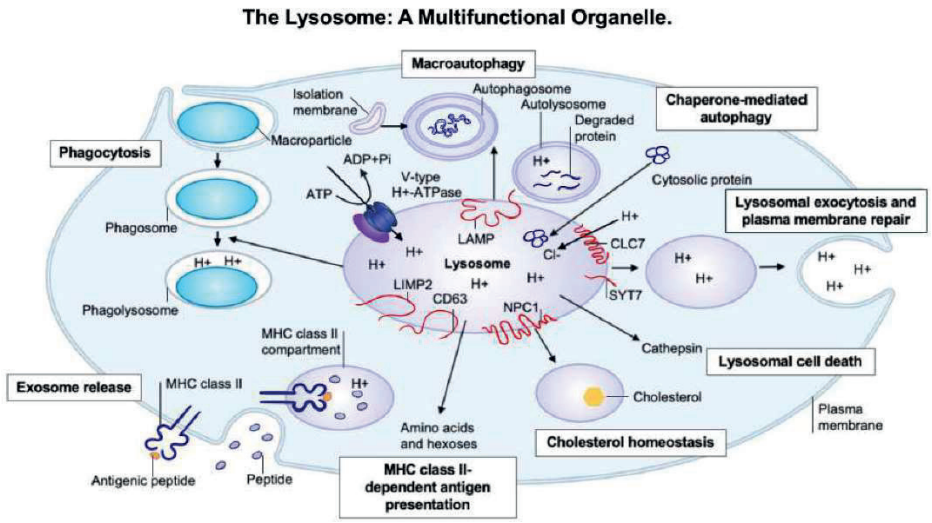

Figure 1: The lysosome and its main functions (Stepien et al., 2022).

They are the primary catabolic compartment of eukaryotic cell, breaking down excess or worn-down macromolecules and generating simple compounds (amino acids, fatty acids, nucleic acids…), which the cell can reuse. The degraded material can be intracellular components digested through autophagy, or extracellular material internalized by endocytosis(Saftig & Haas, 2016).

The catabolic capacity of the lysosome is constituted by the multitude of lysosomal hydrolases that target specific substrates for their degradation. Approximately 50 soluble lysosomal hydrolases have been described including phosphatases, nucleases, proteases, glycosidases, lipases and sulphatases. Most lysosomal enzyme precursors are biosynthesized in the endoplasmic reticulum (ER) and modified in the Golgi apparatus (GA).

As lysososmal hydrolases pass through the *cis*-Golgi network, a mannose -6-phosphate (M6P) group is added to their N-linked oligosaccharides generating a unique marker that is recognized by two independent transmembrane M6P receptors located in the *trans*-Golgi network (Coutinho et al., 2012). Binding of the M6P tagged enzymes to the receptors leads to their packing into clathrin-coated vesicles that will deliver their cargo to the early endosomes. The acidic intraluminal pH of the endosomes induces the uncoupling of the M6P receptors and the hydrolases.

The M6P receptors will return to the *trans*-Golgi network and the enzymes will remain in the fluid phase through the maturation process of the lysosome and start digesting the endocytic material (Trivedi et al., 2020). Nonetheless, some newly synthesized lysosomal enzymes do not bind to M6P receptors and are secreted to the extracellular medium. These hydrolases can be taken up by the M6P receptors present at the plasma membrane through clathrin-mediated endocytosis and delivered to the late endosome.

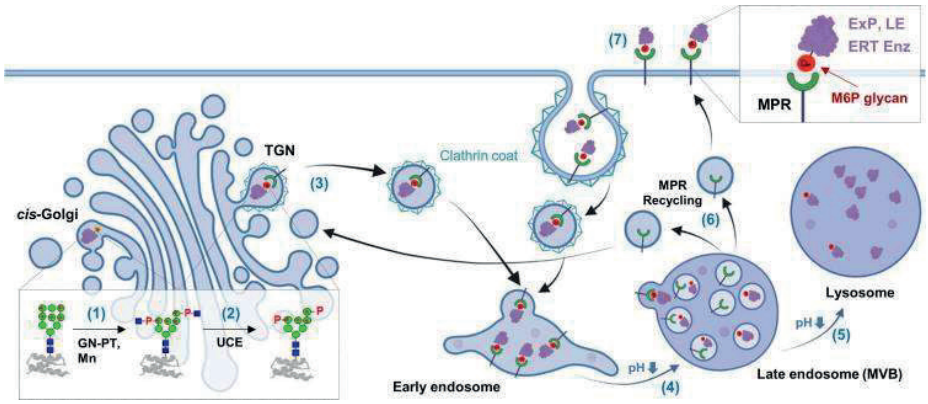

Figure 2: Intracellular trafficking and intercellular trafficking processes associated to mannose 6-phosphate receptors. Schematic representation of lysosomal targeting of enzymes by M6P (Seo & Oh, 2022).

Recently, the lysosome has been established as a signaling hub with a critical role in several cellular activities such as metabolic adaptation, regulation of inflammation, quality control of proteins and nutrient sensing(Perera & Zoncu, 2016; Yang & Wang, 2021). Furthermore, they are also involved in destroying invading viruses and bacteria(Luzio et al., 2007; Randow & Youle, 2014).

2.1.1. Features of LSDs

The most common cause of LSD are mutations generating defective enzymes by altering their amino acid sequence. However, mutations in other non-enzymatic proteins can also lead to this specific type of disorders. Examples of these proteins could be proteins essential for the posttranslational processing of the lysosomal enzymes and transport proteins such as CLN3 in the case of Batten disease(Huizing & Gahl, 2020).

It is not yet well understood how the defects in macromolecule storage develop into the pathological symptoms of each disorder(Parenti et al., 2021; Scerra et al., 2022). The oversimplified idea is that accumulation of a specific component due to the respective defective enzyme impairs cell homeostasis, activating signaling pathways within the cell that could result in cell death or at least cell malfunction. However, LSDs are not characterized by the accumulation of a single product but the buildup of a myriad of secondary products. The reason for that could be an alteration in autophagy which could be over-induced to compensate a reduced efficiency(Lieberman et al., 2012; Shimada & Klionsky, 2012).

Although rare when taken singly, their global prevalence of LSD is quite significant being the combined incidence between 1 in 5000 to 1 in 8000 (Schultz et al., 2011). Most lysosomal storage disorders follow an autosomal recessive pattern of inheritance, but there are a few with an X-linked inheritance patter.

Lysosomal storage disorders present elevated levels of heterogeneity in their pathology due to the high variety in defects that can cause this type of disease. Firstly, a wide range of mutations in the same protein can lead to a completely defective protein or can generate enzymes with a residual activity, enough to ameliorate the pathology of the disease.

Furthermore, depending on the pathway and exact reaction affected by the mutation, the global affectation in the cell can vary and therefore, the symptoms could be more or less severe(Platt et al., 2019).

Some of the most shared pathological symptoms amongst LSD are cardiomyopathy, hepatosplenomegaly, facial dysmorphism, upper airway obstruction, hydrops fetalis and neurological impairment. All lysosomal storage disorders are progressive and lead to a premature death even though the onset and clinical course of each disease and patient is different. Infant phenotypes are usually more severe than those with a later onset.

2.1.2. Neurological implications of LSDs

Neurological impairment is a common feature among LSD affecting about two thirds of the pattients, and is characterized by both neurodegeneration and neuroinflammation. The specific pathology is higly variable with pattients presenting developmental delay, seizures or behavioral/psychiatric problems among others (Para et al., 2020).

Neurodegeneration has been described to occur in a region-specific manner with varying affectation depending on the neuronal subtype through the central nervous system (CNS). Several hypotesis have been proposed as to why this happens. It could be that the proportion of macromolecule production varies in the different neuronal populations leading to more or less buildup, that the specific metabolite being accumulated exerts differential effects depending of the neuronal substype or that some neurons are more vulnerable than others to macromolecule storage (Pandya & Patani, 2021). Besides neurodegeneration, neuroinflammation is also known to play a role in the development of cognitive manifestations in LSDs. Both micro- and astrogliosis have been described in several lysosomal storage disorders as factors exacerbating the pathology development.

However, the number of factors involved in the neurological impairment has been increasing steadily as result of extensive research on the mechanisms linking the metabolite acumulation to the pathological manifestations.

Dysfunction of intracellular protein degradation and autophagy, altered axonal transport, mitochondrial dysfunction and altered calcium metabolism

are several of the events associated to the progresion of the neuropathology in LSDs(Bellettato & Scarpa, 2010).

2.1.3. Classification of LSDs

The overlap in symptoms and principal compound being stored hinders a clear classification of the different diseases englobed in LSD. Therefore, several classifications exist.

On one hand, LSD can be classified depending on the stored compound. The broad categories would then be Sphingolipidosis, oligosaccharidosis, mucopolysaccharidosis, neuronal ceroid lipofuscinosis, salicylic acid disorders, mucolipidosis. However, a miscellaneous group needs to be created for the Lysosomal Acid lipase deficiency, Pompe and Danon disease, which store glycogen, and cystinosis(Rajkumar & Dumpa, 2022).

On the other hand, the classification can be based on the molecular defect affecting the lysosomal system. Under this classification, the main subtypes are defects in post-translational processing of lysosomal enzymes, lysosomal membrane and transport defects and defects in the lysosomal enzymes (Table 1).

Table 1: Classification of lysosomal storage disorders based on the molecular deffect.

Disease	Mutated Protein (Gene)	Storage material	Organs affected
LYSOSOMAL ENZYME DEFECTS			
Sphingolipidoses including sphingolipid activator defects			
Gaucher disease types 1, 2 and 3	**β-Glucosidase** (GBA)	glucosylceramide	Spleen, Liver, Lung,Skeleton, Bone marrow, CNS
Fabry disease	**α-Galactosidase** (GLA)	galactosylated glycolipids	Kidney, Heart
GM1-gangliosidosis types I, II & III	**β -Galactosidase** (GLB1)	GM1-ganglioside, KS, oligosaccharides, glycolipids	Heart, Skeleton, CNS
Tay-Sachs disease (GM2-gangliosidosis)	**β -Hexosaminidase A** (HEXA)	GM2-ganglioside, oligosaccharides, globoside, glycolipids	CNS
Krabbe disease (globoid cell leukodystrophy)	**β -Galactocerebrosidase** (GALC)	galactosylceramide	PNS, CNS
Metachromatic leukodystrophies	**Arylsulfatase A** (ARSA), **Saposin B** (PSAP)	sulfatides	PNS, CNS
Niemann-Pick types A & B	**Sphingomyelinase** (SPMD1)	sphingomyelin	Spleen, Liver. Lung and skeleton in type B. CNS in type A
Mucopolysaccharidoses (detailed in table 2)			
Glycoproteinoses			
α-Mannosidosis	**α-D-Mannosidase** (MAN2B1)	oligosaccharides	Skeleton, CNS, connective tissue
β- Mannosidosis	**β-D-Mannosidase** (MANBA)	oligosaccharides	Skeleton, CNS, connective tissue

Aspartylglucosaminuria	**aspartylglucosaminidase** (AGA)	glycosyl-asparagines	Skeleton, CNS, connective tissue
Other enzyme defects			
Pompe (glycogen storage disease type II)	**α- Glucosidase** (GAA)	Glycogen, oligosaccharides	Skeletal muscle
DEFECTS IN POST-TRANSLATIONAL PROCESSING OF LYSOSOMAL ENZYMES			
Multiple sulfatase deficiency (mucosulfatidosis)	**Formylglycine generating enzyme** (SUMF1)	sulfatides, GAGs, glycolipids	CNS, skeleton, Liver
Mucolipidosis II (I-cell disease)	**N-acetylglucosamine-1-phosphotransferase α /β subunit** (GNPTAB)	Oligosaccharides, GAGs, lipids	Spleen, Liver, Heart, Skeleton, CNS
Mucolipidosis IIIA (pseudo-Hurler polydystrophy)	**N-acetylglucosamine-1-phosphotransferase α /β subunit** (GNPTAB)	Oligosaccharides, GAGs, lipids	Heart, Skeleton
LYSOSOMAL MEMBRANE AND TRANSPORT DEFECTS			
Cystinosis	**Cystinosin** (CTNS)	cysteine	Kidney
Danon disease	**Lysosome-associated membrane protein 2** (LAMP2)	cytoplasmic debris, glycogen	Heart, Skeletal muscle
Niemann-Pick type C1	**Niemann-Pick type C1 protein** (NPC1)	Glycolipids, Cholesterol	Liver, CNS
Niemann-Pick type C2	**Niemann-Pick type C2 protein** (NPC2)	Glycolipids, Cholesterol	Liver, CNS
NEURONAL CEROID LIPOFUSCINOSES			
Group of diseases characterized by the accumulation of lipopigments (lipofuscin) caused by mutations in genes related to the endosomal-lysososmal system. Shared clinical symptoms include decline of mental and motor capacity, epilepsy and vision loss.			
DEFECTS IN LYSOSOME AND LYSOSOME-RELATED ORGANELLE BIOGENESIS			
Group of disorders caused by mutations in genes involved in the biogenesis of lysosome-related organelles. The organelles most commonly affected are the melanosomes and platelet dense granules. The pathology is highly variable among the disorders, but common symptoms include variable oculocutaneous albinism and excessive bleeding and bruising.			

2.2. Mucopolysaccharidoses

2.2.1. Glycosaminoglycans

Mucopolysaccharidoses are a group of rare, inherited lysosomal storage disorders characterized by the cellular accumulation of glycosaminoglycans (GAG). They are caused by mutations leading to lack of function in the enzymes involved in glycosaminoglycan degradation.

The long unbranched polysaccharide chains that characterize the glycosaminoglycans, also known as mucopolysaccharides are formed by covalently linked repeating disaccharide units. These units exhibit an amino sugar and a uronic acid. The GAGs present in eukaryotic cells are chondroitin sulfate (CS), heparan sulfate (HS), dermatan sulfate (DS) and hyaluronan (HA).

The most common location for GAGs is the extracellular matrix (ECM), but they are also present within the plasma membrane in the cell surface and in secretory vesicles (secretory granules).

Most GAGs are usually found bound to a polypeptide backbone and therefore are called proteoglycans. The GAG component of proteoglycans interacts with numerous biologically significant components such as growth factors, chemokines, survival factors, etc. All these interactions are often crucial to basic processes of cellular differentiation and proliferation(Schwartz & Domowicz, 2023). Because of this, GAGs play an important role in multiple biological and pathological processes such as angiogenesis, bacterial and viral infections, inflammation, axonal growth, and cancer progression(Afratis et al., 2012; Morla, 2019).

In the degradation of GAGs there are 4 different catabolic pathways involved. The specific GAG accumulated in the cell depends on the pathway blocked, although multiple GAGs can be accumulated due to a single deficient enzyme. This is because some of the enzymes are involved in several of the catabolic pathways.

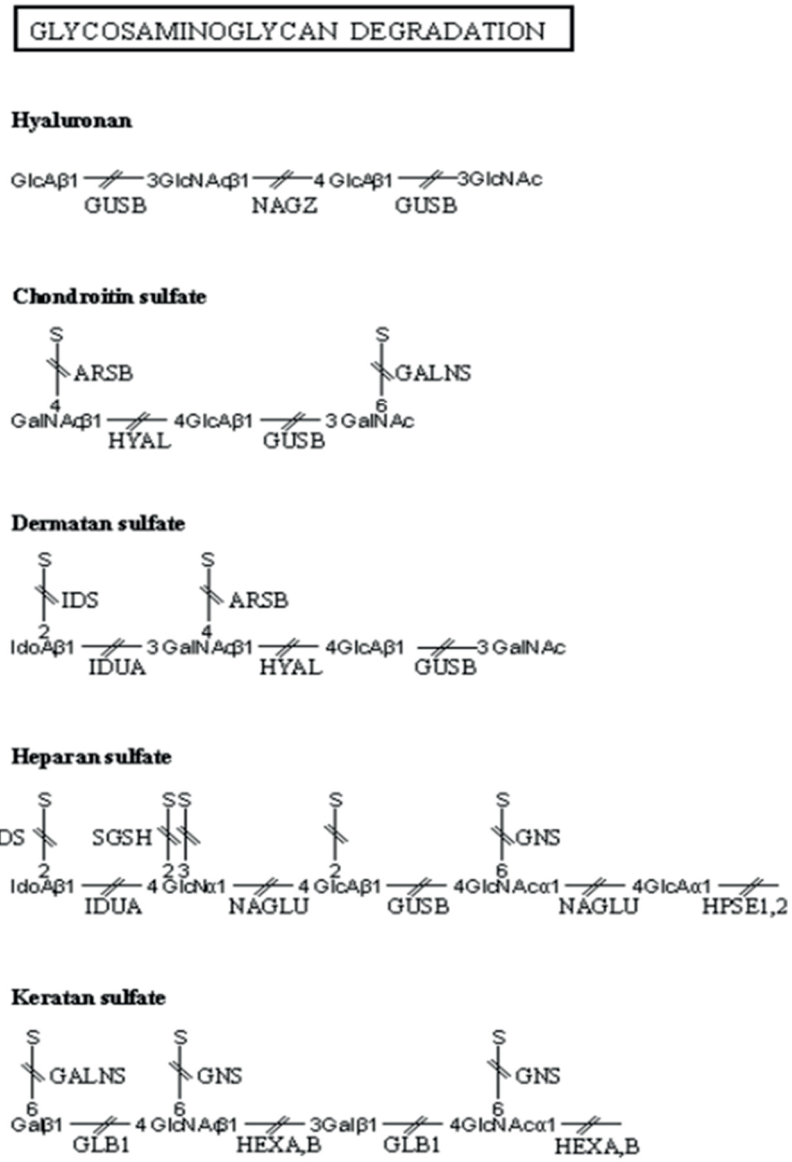

Figure 3: Glycosaminoglycan degradation pathways. Obtained from https://www.genome.jp/pathway/map00531

Inside the cell, the undegraded GAG accumulate within the lysosomes, causing an impairment of the lysosomal function which results in cellular damage in multiple organs. Elevated levels of GAGs can also be detected in blood, urine and the cerebrospinal fluid, serving as a diagnostic biomarker for this type of disorders.

2.2.2. Clinical features and Classification

Mucopolysaccharidosis are described as chronic and progressive diseases with multisystemic affectation. All MPS diseases, except MPS II, which is inherited in an X-linked manner, follow an autosomal recessive pattern of inheritance (Zhou et al., 2020). Even though the prevalence of each mucopolysaccharidosis is different, they are all considered rare diseases accounting for less than 0,1% of all genetic diseases(Gaffke et al., 2021).

Table 2: Classification of Mucopolysaccharidoses.

Disease (Syndrome)		**Prevalence**	**Mutated protein** (gene)	**Locus**	**Stored GAG**
MPS I (Hurler, HurlerScheie, Scheie)		1:100,000	**α-L-Iduronidase** (IDUA)	4p16.3	DS, HS
There are three variants differing widely in their severity. **Hurler:** most severe phenotype with early diagnosis and childhood demise. Symptoms include skeletal abnormalities, heart disease, respiratory problems, hepatosplenomegaly and cognitive impairment. **Hurler-Scheie:** Intermediate phenotype characterized by delayed motor development and skeletal deformities. Other symptoms include corneal clouding, cardiomyopathy and neurosensorial hearing loss. **Scheie:** milder phenotype with joint stiffness, coarse face, aortic valve disease and corneal clouding.					
MPS II (Hunter)		0.6:100,000	**Iduronate-2-Sulfatase** (IDS)	Xq28	DS, HS
The phenotype can be severe or mild with some of the symptoms including dysostosis multiplex and cardiomyopathy. Cognitive deficiencies are only present in the severe forms of the disease.					
MPS III (Sanfilippo)	A	1:100,000	**Heparan-N-Sulfatase** (SGSH)	17q25.3	HS
	B	0.6:100,000	**α-N-Acetylglucosaminidase** (NAGLU)	17q21.2	HS

	C	0.1:100,000	**α-Glucosamine N-Acetyl transferase** (HGSNAT)	8p11.21	HS
	D	01:100,000	**N-Acetylglucosamine-6-Sulfatase** (GNS)	12q14.3	HS
Characterized by severe and rapid intellectual deterioration with mild somatic symptoms. Other symptoms include developmental delay and hearing loss.					
MPS IV (Morquio)	A	0.75:100,000	**N-Acetylgalactosamine 6 -Sulfatase** (GALNS)	16q24.3	KS, CS
	B	0.07: 100,000	**b-Galactosidase** (GLB1)	3p22.3	KS
The main feature is skeletal pathology, but patients also present respiratory impairment, corneal clouding, hearing loss and hepatomegaly.					
MPS VI (Maroteaux - Lamy)		0.16:100,000	**N-Acetylgalactosamine 4-Sulfatase** (ARSB)	5q14.1	CS, DS, HS
Wide spectrum of symptoms from slowly to rapidly progressing forms. Most characteristic symptoms include joint problems, reduced pulmonary function, corneal clouding, dysostosis multiplex and hepatomegaly.					
MPS VII (Sly)		0.01:100,000	**β-Glucuronidase** (GUSB)	7q11.21	CS, DS, HS, hyaluronan
Detailed clinical phenotype is provided in the 2.3.2 section					
MPS IX		4 cases reported since 1996	**Hyaluronidase** (HYAL1)	3p21.31	Hyaluronan
Only four cases described. The main clinical manifestation is diffuse joint involvement with auricular swelling. Patients also presented short stature and dysmorphic craniofacial features.					

Despite their molecular heterogeneity, the different mucopolysaccharidosis share key clinical manifestations. Some of the most commonly shared traits are short stature coupled with skeletal dysplasia and joint abnormalities, coarse facial features, hepato- and splenomegaly, corneal clouding, frequent upper respiratory tract infections, heart valve disease, shortened lifespan and cognitive impairment of variable severity(Galimberti et al., 2018).

The neurocognitive effects found in the different MPS range from mild attention problems to a progressive and degenerative neuronal affectation (Shapiro & Eisengart, 2021). The neuroimaging manifestations, although different for any specific MPS, include white matter injury, hydrocephalus, brain atrophy, enlargement of the perivascular and subarachnoid spaces among others (Nicolas-Jilwan & AlSayed, 2018; Reichert et al., 2016).

Even within each MPS, the high variety of mutations in the same enzyme result in a wide range of severity. Depending on the residual enzymatic activity of each patient, the clinical manifestations can range from very severe to mild. On one side, the most severe forms show pathological features in the first months of life and exhibit a fast progression of the disease needing urgent medical intervention to preserve quality of life(Muenzer, 2011). On the other side, the milder forms of the disease have a later onset, with patients showing normal cognitive abilities and attenuated somatic pathology that are often underestimated and result in a delayed diagnosis of the disease (Galimberti et al., 2018).

Another relevant difference between severe and mild forms of this group of syndromes is life expectancy. Patients with the more severe types usually die during childhood or early adulthood, with some cases of stillborn patients. Although most patients have an average lifespan of one or two decades, individuals presenting milder forms can live well into adulthood (Zhou et al., 2020).

The small prevalence of these disorders coupled with the genetic and clinical variability usually leads to a delayed diagnosis, which can be even exacerbated in the attenuated forms. However, early diagnosis is crucial in the outcome of this patients.

2.2.3. Diagnosis of MPSs

To diagnose an MPS disorder the measurement of GAG levels in urine is a useful screening method. Positive results are very suggestive of an MPS. However, negative results do not rule-out the disorder, because false-negative results are quite common when using this method of detection(Mabe et al., 2004; Mahalingam et al., 2004).

The basic diagnostic flow then continues with specific enzyme assays and ends with the identification of the specific mutation causing the disorder. The measurement of specific enzymes activity is commonly based on cultured fibroblast, leucocytes, plasma or serum. Furthermore, in high-risk families, prenatal diagnosis can be obtained through activity assays of cultivated chorionic villus(Young, 1992) or amniocytes, mass spectrometry of the amniotic fluid(Aboulnasr et al., 2022). In the last years, mass spectrometry(Mashima et al., 2020), multiplex activity assays using blood droplets(Sista et al., 2013) and gene panel testing have also been proposed as alternative diagnostic options(Gheldof et al., 2019). However, this technology is not available everywhere(Filocamo & Morrone, 2011).

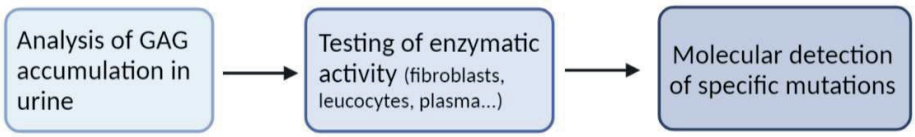

Figure 4: Diagnostic flow chart for mucopolysaccharidoses.

The attenuated forms of MPS are even more difficult to diagnose and face significant diagnostic delay. Patients with these forms of the disease usually present affectation in the osteoarticular system, heart valve disease, hearing loss, ocular problems and/or respiratory problems(Rigoldi et al., 2018). They are usually referred to a specialist for these symptoms, and most likely will not be correctly diagnosed. Some of the most common erroneous diagnoses are other neurological diseases or rheumatology and orthopedic disorders(Wisniewska et al., 2022).

The fast progression and urgent need for intervention make delayed diagnosis catastrophic because irreversible organ damage may be already present in most cases.

Furthermore, if therapy is available, accurate diagnosis of the MPS disorders is critical to start an early treatment when the somatic affectation is still incipient.

2.2.4. Treatment strategies for MPSs

Currently, there is no cure for mucopolysaccharidoses, but several therapies have been developed to ameliorate or even reverse some of the clinical symptoms. The first treatments that have been available for MPS are bone marrow/hematopoietic stem cell transplantation (HSCT)(Kubaski et al., 2017) and enzyme replacement therapy (ERT)(Jurecka et al., 2012; Rowan et al., 2012).

One of the key factors in treating MPS is the cross-correction of the enzyme-deficient cells by extracellular enzyme supply. As stated in the 2.1 section, lysosomal hydrolases can be internalized by interaction to M6P receptors, therefore external supplies of the enzyme can be endocytosed to the lysosomes leading to the correction of the deficit in the cell. This is the mechanism by which the recombinant enzyme administered in ERT exerts its therapeutic effect. Furthermore, since a portion of lysosomal enzymes are secreted into the extracellular medium instead of transported through the Golgi apparatus, the presence of corrected cells either as a result of gene therapy or HSCT can lead to the cross-correction of adjacent cells increasing the therapeutic effect of the treatment.

2.2.4.1. Enzyme Replacement Therapy

Enzyme replacement therapy is currently available for various mucopolysaccharidoses (Table 3) but is only able to successfully resolve the pathology in Gaucher disease, another type of lysosomal storage disorder(Lachmann, 2020; Valayannopoulos, 2013).

One of the main problems of ERT is the almost negligible effect on the neuropathology due to the blood-brain barrier, which prevents the administered enzyme from accessing the brain. Other weaknesses of this

therapeutic strategy are its elevated cost and the lifelong necessity for the patients to undergo repeated administrations. Furthermore, it has been demonstrated that interruption and reintroduction of ERT can lead to deterioration of function in some organs(Schneider et al., 2016). To solve at least some of these issues, nanotechnology-driven ERT strategies have been developed. These treatments are based in the encapsulation of the administered enzyme in different types of particles such as liposomes, micelles or polymeric nanoparticles(Del Grosso et al., 2022).

Table 3: Enzyme replacement treatments available for Mucopolysaccharidoses.

Disease	Drug	Limitations
MPS I	Aldurazyme™ (Iaronidase)	Unable to treat the neurological manifestations
MPS II	Elaprase™ (idursulfase)	Does not improve pulmonary function
MPS IVA	Vimizim™ (elosulfase alfa)	The only significant improvement was detected in physical endurance
MPS VI	Naglazyme™ (galsulfase)	Does not seem to improve pulmonary and skeletal function
MPS VII	MEPSEVII (glucuronidase)	Unable to treat the neurological symptoms

To obtain greater correction of the neuropathology present in some of the mucopolysaccharidosis, other completely different therapeutic strategies have also been developed. One of these strategies is substrate reduction therapy (SRT). The aim of SRT is to prevent the buildup of undegraded material by reducing the levels of biosynthesis(Coutinho et al., 2016).

2.2.4.2. Gene Therapy

A completely different therapeutic approach to treat MPS disorders is the use of hematopoietic stem cell (HSC), mesenchymal stem cell (MSC), and induced pluripotent stem cell (iPSC)-related gene therapy. Furthermore, the combined use of several treatments has showed promising results in ameliorating the symptoms of the disease(Kose et al., 2021).

Although HSC treatments have been studied for more than 30 years to treat mucopolysaccharidoses (Taylor et al., 2019), there are several clinical trials based in the use on hematopoietic stem cells ongoing (www.clinicaltrials.gov: NCT04201405, NCT03488394)

Another gene therapy approach entails the use of viral (retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAV)) and non-viral (Sleeping Beauty-mediated transposon)(Aronovich et al., 2007) vectors to deliver the corrected gene to the cells. Pre-clinical data firmly supports the use of gene therapy-based therapies for MPS and currently, a considerable number of clinical trials are taking place.

Adeno-associated vectors are the most used vector in gene therapy clinical trials for mucopolysaccharidoses at the moment (www.clinicaltrials.gov: NCT03580083, NCT03612869, NCT03173521, NCT02716246, NCT03173521) with only one clinical trial using a retroviral vector (www.clinicaltrials.gov: NCT00004454).

The results of the first gene therapy clinical trial using AAV vectors for mucopolysaccharidoses, specifically MPS IIIA were published in 2014 and reported mixed success in stabilizing brain atrophy in these patients, with the youngest patient being the most likely to display neurocognitive benefit (Tardieu et al., 2014).

Additionally, two clinical trials employing AAVs have already been completed for Sanfilippo syndrome types A and B, although results have only been published for MPS IIIB (www.clinicaltrials.gov: NCT01474343, NCT03300453). Intracerebral gene therapy with a recombinant AAV2/5 vector in children with MPS IIIB resulted in improved neurocognitive progression in all patients, although longer follow-up is required to assess safety and the persistence of the benefits (Tardieu et al., 2017).

Furthermore, genome editing is surging as a novel alternative therapy. However, the clinical trial performed using this approach in MPS II patients did not yield good results (www.clinicaltrials.gov: NCT03041324).

2.3. Mucopolysaccharidosis type VII

Mucopolysaccharidosis (MPS) type VII, also known as Sly's syndrome, is an autosomal recessive disorder caused by mutations in the gene GUSB1 which encodes for β-glucuronidase, a lysosomal enzyme essential for a correct GAG degradation.

This disorder was described for the first time in 1973 when a patient presenting an MPS-like phenotype was found to have a deficiency of the lysosomal hydrolase β-glucuronidase(Sly et al., 1973).

A β-glucuronidase deficient cell is not able to metabolize GAGs. This leads to a partial degradation of heparan sulfate, dermatan sulfate, hyaluronan and chondroitin sulfate, which then accumulate in the lysosomes.

2.3.1. Genetics of MPS VII

The GUSB 1 gene is located on chromosome 7, more precisely in the 7q21.11 region. The gene is composed of 12 exons and 11 introns encompassing approximately 21 kb and is highly conserved between species(Miller et al., 1990).

MPS VII is a rare disease with an estimated prevalence of 1 out of 250,000 births. Therefore, epidemiologic data are scarce.

However, through exhaustive analysis of all available cases up to 49 GUSB different mutations have been identified(Tomatsu et al., 2009). This heterogeneity in mutations greatly contributes to the immense clinical variability among patients. The most common mutations in the GUSB gene are missense mutations which account for more than 75% of the total. Besides, nonsense mutations, deletions and splice-site mutations have also been described. Even considering all the variability specific exonic point mutations were found with higher frequency.

Mutational spectrum of MPS VII is still increasing with new cases presenting mutations that had not been yet described in other patients (Lee et al., 2022; Poyatos-Andujar et al., 2021).

Furthermore, in silico analysis is being used to further understand the effects of each specific mutation on the structure and function of the β-glucuronidase(Khan et al., 2016).

The product encoded by the GUSB gene is a globular β-glucuronidase composed by 651 amino acids (Jain et al., 1996). The become a functionally active protein, this enzyme needs to through a proteolytic process, be glycosylated and form an homotetramere (Shipley et al., 1993). The active site of the enzyme is proposed to be formed from a large cleft between to monomers, with the specific residues Glu 451 and Glu 540 being especially relevant for the catalytic activity.

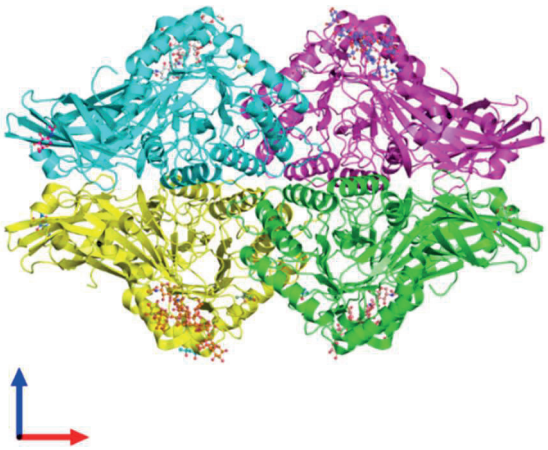

Figure 5: Front view of the β-Glucuronidase homotetramer with differently colored chains. Obtained from http://www.ebi.ac.uk/pdbe/entry/pdb/3HN3.

Residual levels of enzymatic activity can be enough to prevent the development of the disease, or at least to diminish the symptoms. This is another reason as to the wide range of severity presented by the MPS VII patients.

2.3.2. Clinical Symptoms

Mucopolysaccharidosis type VII is a chronic and progressive disease with multisystemic affectation. Antenatal forms usually result in death in utero. Besides that, the more severe forms of MPS VII present multiple somatic pathologies already in the first months of age and show a severely reduced life expectancy, dying during infancy(Montano et al., 2016).

Milder forms present a latter onset of the disease with fewer pathological features leading to a longer lifespan which may reach adulthood(Pelloso et al., 2021). Even though the pathology of MPS VII is highly heterogeneous, there are some symptoms that appear in most of the patients.

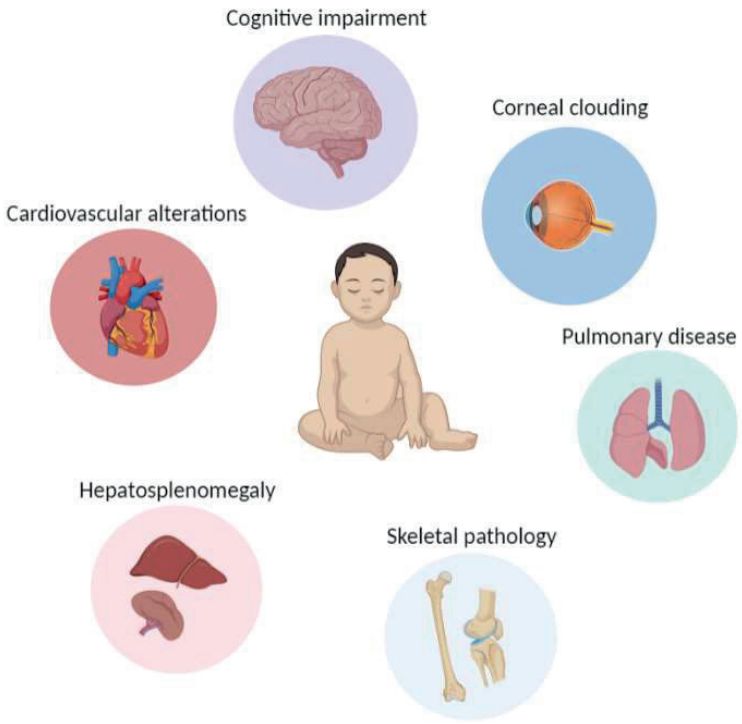

Figure 6: Main clinical features of mucopolysaccharidosis type VII.

Skeletal defects are one of the most common and visible defects present in this disease. Growth failure tends to become apparent between 6 months and 3.5 years of age. Bones of MPS VII are shorter and thicker, leading to short stature. Furthermore, the progression of the skeletal pathology is one of the main needs for medical consultations and usually needs orthopedic intervention to try and maintain some independence in movement(Macsai et al., 2012). It has been proposed that accumulation of chondroitin sulfate (specifically chondroitin-4-sulfat C4S) is the cause of reduced chondrocyte proliferation leading to MPS VII phenotype(Jiang et al., 2020; Metcalf et al., 2009). A related manifestation of the disease is the development of lumbar spine disease which can lead to spinal cord compression(Smith et al., 2012).

Hydrops fetalis is a frequently seen feature but it is only characteristic of the severe forms of MPS VII(Hizem et al., 2021; Holtz et al., 2020). Cardiovascular alterations are also recurrent involving aortic dilatation, thickening of several arteries and ventricular hypertrophy(Baldo et al., 2011; Gniadek et al., 2015). Other common complications of Sly syndrome include pulmonary disease, corneal clouding and hepatosplenomegaly (Oldham et al., 2022).

Furthermore, developmental delay and a progressive intellectual disability are also common and incapacitating symptoms of MPS VII(Gawri et al., 2023; Wallace et al., 1990). The impaired cognitive abilities are usually present paired with difficulties in language and speech and/or behavioral problems.(Shapiro et al., 2017). Neuroimaging has been pivotal in finding brain abnormalities in neurological pathologies finding white matter abnormalities in several mucopolysaccharidosis including MPS VII(Zafeiriou & Batzios, 2013)

2.3.3. Treatment strategies for MPS VII

No cure has been discovered for MPS VII, but several therapeutic approaches have been intensively studied. The first disease-specific treatment available for this syndrome is enzyme replacement therapy (ERT) with intravenous administration of vestronidase alfa (Mepsevii™), a recombinant form of human β-glucuronidase (McCafferty & Scott, 2019). Treatment with vestronidase alfa shows sustained clinical benefits including reduced urinary GAG levels (Cadaoas et al., 2020; Qi et al., 2019). Long-term treatment showed a tolerability profile with most adverse reactions ranging from mild to moderate. Even patients who exhibited positive results for neutralizing antibodies continued to show reduced levels of GAGs in urine(R. Y. Wang et al., 2020). In pediatric patients, treatment was able to maintain growth and improve hepatosplenomegaly(Lau et al., 2022).

However, ERT is not able to ameliorate the neurological signs of the disease because it cannot cross the blood brain barrier. Therefore, alternative strategies have been studied to solve this issue.

One alternative is the use of hematopoietic stem cells which has shown promising results in combination with ERT(Dubot et al., 2019). Interestingly, brain transplantation of genetically modified bone marrow stromal cells was

also able to correct the neuropathology in an animal model of MPS VII(Sakurai et al., 2004).

Nonetheless, the most studied approach is the use of gene therapy using vectors to deliver a corrected copy of the GUSB gene. Several types of vectors have been used including non-pathogenic variants of different viruses. Using an HIV-based vector and either a single intrastriatal injection or multiple injections at different brain regions, a significant correction of the pathology was observed throughout the brain of MPS VII mice, even in areas in which a enzyme could not be detected (Bosch et al., 2000). Localized injection of the nonpathogenic HSV-1 resulted in the reversion of the pathogenic storage lesions in most regions of MPS VII mice brain and lead to a reduction in the anxiogenic behaviors that are associated to the pathology (W. Liu et al., 2015).

A helper-dependent canine adenovirus (Ariza et al., 2014) has also been tested for treating the CNS pathology of mucopolysaccharidosis type VII. Intracranial administration of this vector led to long-term expression of a functional β-Glucuronidase and correction of the neuropathology in both the injection site and distal areas. Furthermore, treated animals showed significant cognitive improvement but a full correction was not obtained.

Other vectors that have been used to treat MPS VII are lentiviral vectors which have been proved to provide sustained expression of the enzyme, and partially improved exploratory behavior and spatial learning (Bielicki et al., 2010; Bosch et al., 2000; Derrick-Roberts et al., 2016; Derrick-Roberts et al., 2014). Moreover, systemic lentiviral-mediated gene therapy led to improvement in parameters of bone mass and architecture, but the neurological beneficial effects were diminished with this administration route(Macsai et al., 2012).

Even so, the most used gene therapy vectors are without doubt the adeno associated viruses. Different serotypes and administration routes have been tested obtaining good results in animal models.

One of the studies targeted the progressive retinal degeneration present in the mouse model of MPS VII by performing intraocular injection of a recombinant AAV vector. This strategy led to improvement of retinal function although the efficacy of the treatment was highly variable depending on methodological parameters (Hennig et al., 2004). Intrahepatic

injection has also been tested as therapeutic strategy to treat MPS VII. Administration of a recombinant AAV2 in adult MPS VII mice obtained stable long-term hepatic expression of the functional enzyme and reduced lysosomal levels in peripheral tissues and even in some regions of the brain (Sferra et al., 2004).

Since one of the main challenges when treating Mucopolysaccharidosis type VII is the correction of the CNS, the effect of a bilateral intrastriatal injection of AAV5 was analyzed in the mouse model. Although the treatment improved spatial memory and learning a complete correction could not be achieved (Liu et al., 2007).

Moreover, AAV vectors have not only been used in the mouse model but also in dogs. A study comparing several administration routes of either AAV9 or AAVrh10 vectors was performed in the canine model of MPS VII. The results of this study show that although intravenous injection can lead to β-Glucuronidase activity in the cerebrospinal fluid, it is not enough to correct the GAG accumulation in the brain. Nonetheless, intrathecal injection resulted in normal activity of the enzyme in the brain and reduced GAG accumulation when compared to untreated mice suggesting an increased effectiveness of this administration to treat the CNS pathology (Gurda et al., 2016).

In one of the most recent studies, Pages et al. demonstrated that intrathecal administration of an AAV encoding the human β-Glucuronidase resulted in systemic and CNS histopathological correction but could not completely revert the behavioral deficits of MPS VII animals (Pages et al., 2019).

2.3.4. Animal models for MPS VII

Mucopolysaccharidosis type VII is a disease naturally occurring in canine, feline and murine species. Since it has been said that MPS VII in human patients is caused by alleles presenting deletions or missense mutations in over 95% of the cases, all animal models are developed from this specific type of genetic alteration to replicate the human disorder in a liable way (Tomatsu et al., 2009).

The feline model has been described in the past(Fyfe et al., 1999; Gitzelmann et al., 1994), with newer cases surging in recent years(Schultheiss et al., 2000; Wang et al., 2015) but has not been widely used to study the disease.

The dog model for the disease was first described in 1984 (Haskins et al., 1984) and has been used to further understand the mechanisms underlaying MPS VII and to test several therapeutic strategies(Gawri et al., 2023). It has been mostly used to study the skeletal abnormalities characteristic of Sly syndrome(Gawri et al., 2023; Jiang et al., 2021; Peck et al., 2021; Simonaro et al., 2005; Smith et al., 2010).

Furthermore, a *Drosophila* model exhibiting the typical features of MPS VII has been generated. These flies show accumulation of storage materials in the brain, reduced lifespan, progressive decline in locomotor activity and neuropathology (Bar et al., 2018).

Among the murine models of the disease there are some found naturally due to spontaneous mutations. However, other models have been generated in the laboratory to mimic more precisely the human patients and to implement useful characteristics.

In 1989 the first natural mutant mouse lacking β-Glucuronidase activity was characterized exhibiting most of the features of MPS VII patients (Birkenmeier et al., 1989).

To mimic the heterogeneity in genotypes presented in the human patients of MPS VII several murine models with missense mutations have been created using targeted mutation. This allows for animal models presenting varying levels of enzymatic activity (Tomatsu et al., 2002).

A doubly tolerant murine model (for both mouse and human forms of GUSB) was generated to evaluate enzyme and/or gene therapies in mouse models using the human enzyme. This prevents immune responses against the foreign enzyme from interfering with the results of the studies. It was then demonstrated that this murine model maintained all biochemical, morphological and histopathological characteristics of the original MPS VII mouse model(Tomatsu et al., 2003).

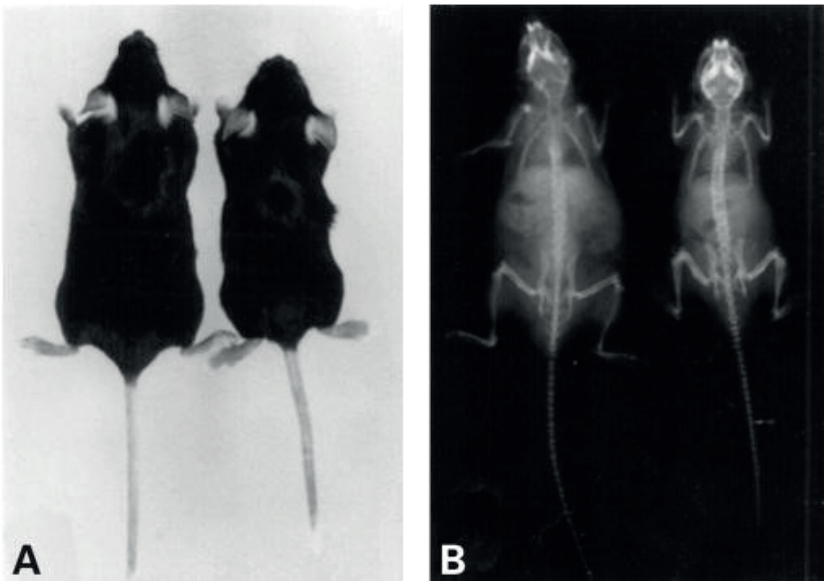

Figure 7: Phenotype comparison between a 4-month-old wild type and a β-Glucuronidase deficient mouse. A) Overall morphology B) Skeleton (Tomatsu et al., 2003).

The MPS VII doubly tolerant mice presents a distinct phenotype since birth. The show distinctive dysmorphic facies with a stunted muzzle and are slightly smaller in size. As they grow, they exhibit a significant growth retardation and obvious skeletal abnormalities including shorter stubby limps and dysplasia. When they reach adulthood, they start becoming less active and reduce their food and water intake. This leads to dehydration and drastic weight loss which results in premature death when compared to wild type (WT) mice with the same genetic background(Vogler et al., 1990).

The reduced mobility and skeletal abnormalities correlate with accumulation of GAGs in the articular and cartilaginous tissue. Furthermore, the deficient hydrolase causes enlargement of the lysosomes due to overaccumulation of fibrillar and granular storage material (Vogler et al., 1990). This abnormalities with the consequent vacuolar accumulation can be found in most organs, for example: spleen, kidney, liver, heart, bone, eye and brain among others(Birkenmeier et al., 1989).

It has been described that MPS VII mice exhibit cognitive deficits with progressive behavioral abnormalities(Chang et al., 1993). These animals show impairment of muscle and lower motor neuron functions, and poorer coordination and prehensility, and lower muscular strength when compared to WT littermates. Classical unconditioned tests like the T maze, open-field

and corner test have indicated reduced horizontal and vertical locomotor activities in MPS VII mice. Reduced exploratory behavior is also decreased in GUSB animals indicating severe problems to interact with the environment. Furthermore, they exhibit deficits in learning acquisition as demonstrated by a lower baseline performance in the last day of water maze training with a visible platform. Moreover, the same test proved that GUSB animals present both short- and long-term memory deficits. All these findings demonstrate the decreased cognitive ability of this animal model. This deficits were found to be exacerbated as the difficulty of the tasks increased as showcased by the even worse results of MPS VII animals in place learning, a more difficult task in the water maze because the platform is hidden and its location changed(Ariza et al., 2014). Importantly, MPS VII mice are unable to breed. This can be partially attributed to their reduced mobility, but it has also been attributed to a disrupted mating behavior.

2.4. Mechanisms involved in neurodegeneration

Many research efforts have been dedicated to the study neurodegeneration but the specific processes leading to neurological impairment are still not well understood as it is a complicated process modulated by a high number of factors with distinct roles and impacts depending on the specific disorder.

2.4.1. Neuronal death

One of the starting points of study in neurodegenerative disorders is the analysis of neuronal death, which would be the easiest reason to explain the phenotype. Neuronal loss has been studied through the years using mostly imaging techniques while labeling neurons with specific markers.

The mechanisms leading to neuronal death are numerous, highly variable and not yet fully understood. However, two distinct types of cellular death have been stablished: Necrosis and apoptosis. Furthermore, the term necroptosis has also been used for cases combining both types of death.

2.4.1.1. Apoptosis

Apoptosis is described as a programed and organized caspase 8-dependent cell death. As such, it is considered an essential regulatory mechanism through all life stages: development, aging and disease.

Apoptosis is characterized by cellular shrinking, condensation of the chromatin, nuclear fragmentation, and involution of the cell membrane. Cells then fragment producing apoptotic bodies which can be then cleared from the brain by phagocytic cells. Rapid elimination of cellular fragments is critical to maintain tissue homeostasis. Failure to eliminate the apoptotic cells can activate the innate immune system(Poon et al., 2014).

This type of cellular death is a highly conserved process with a tight regulation, essential for maintaining tissue homeostasis. Dysregulation of apoptosis has been proposed to be involved in a myriad of pathologies(Fulda et al., 2010). The factors that can trigger apoptosis are numerous and heterogeneous comprising both intrinsic and extrinsic signals. Some signals that can lead to apoptosis include reactive oxygen species (ROS), DNA damage, viral infection, and hypoxia. Furthermore, exposure to chemotherapeutic drugs can also lead to cell apoptosis(Orrenius et al., 2011).

Most apoptotic pathways result in the activation of cysteine-dependent aspartate-specific proteases (caspases), which exist inside the cell as inactive pro-caspases until cleaved. Besides apoptosis it has been described that caspases play an important role in other processes like inflammation , autophagy and tumor progression(Shalini et al., 2015). The apoptotic pathway consists of upstream initiators like caspase 8 and downstream effector caspases, for example caspase 3.

Activation of the pathway leads to the cleavage of a multitude of substrates including proteins involved in cell cycle regulation, DNA repair and structural proteins. Furthermore, cleavage of specific substrates can lead to disruption of the electron transport chain in the mitochondria and to an abnormal reorganization of the cytoskeleton(Green & Llambi, 2015).

There are several signaling pathways known to activate apoptosis. One of them is the mitochondrial pathway which is regulated by proteins belonging to the B-cell-lymphoma protein 2 (Bcl-2) family(Leibowitz & Yu, 2010).

Another pathway is the death receptor family, in which apoptosis is triggered through receptors of the tumor necrosis factor receptor (TNF-R) super-family(Tanzer, 2022). Furthermore, endoplasmic reticulum stress and unfolded protein response (UPR) can also lead to apoptosis activation(Wang & Kaufman, 2016).

2.4.1.2. Necrosis and Necroptosis

Necrosis in contrast is a non-programed haphazard cellular death, commonly result of ischemic or traumatic injury. Necrotic death happens quickly after an extreme physiochemical stress and is characterized by condensation of the nucleus and swelling of the endoplasmic reticulum and the mitochondria. A wide range of intrinsic and extrinsic factors can trigger necrosis, but it is known that acute energy depletion is one of the most potent triggers in neurons. This is because low energy levels in neurons cause a collapse of the resting potential leading to a depolarization and the massive release of neurotransmitters. Other toxic substances as well as ROS have been demonstrated to elicit necrosis(Nicotera & Lipton, 1999).

Necroptosis is a caspase 8-independent cell death. Necroptosis activation will lead to the formation of the necrosome, a protein complex formed by RIPK1, RIPK3 and Fas-associated protein with death domain (FADD) (Seifert & Miller, 2017). This complex will promote the activation of the pseudo kinase mixed lineage kinase like (MLKL) by phosphorylation. This activation will rapidly be followed by plasma membrane rupture, release of cytokines resulting in an increased inflammatory response and swift cell death(Callizot et al., 2019) .

However, these processes are not exclusive and easy to differentiate as they share some of the signals that trigger them as well as some of the intracellular effectors involved in the process. In the case of neuronal injury caused by excitotoxicity, the intensity of the exposure to the triggering agent seems to play an important role in the type of cell death that follows. Furthermore, dying cells can present distinctive features of both death mechanisms(Syntichaki & Tavernarakis, 2003).

2.4.1.3. Neuronal death in MPS VII and other LSD

As cognitive impairment is a shared characteristic of most mucopolysaccharidoses, neuronal loss has been a recurring parameter studied with varying results. For MPS I, IIIA and IIIB the mouse models did not show decreased cerebral cortical thickness nor reduced number of Nissl positive cells when compared to WT littermates(Wilkinson et al., 2012). However, a previous study of the murine MPS I model had detected progressive neuronal loss within the cerebellum(Russell et al., 1998).

A murine model of MPS IIIC exhibited normal stratification of the brain cortex but when quantifying NeuN-stained cells neuronal loss was detected starting at 10 months of age in the somatosensory cortex, the cerebellar cortex. Furthermore, Purkinje cell loss was also substantial (Pshezhetsky, 2015). Besides, decreased density of GABAergic interneurons in the cerebral cortex has also been described for MPS IIB (Hamano et al., 2008). Increased caspase 3 was found in both a murine neural stem cell model and a patient of Hunter disease suggesting increased apoptosis of neuronal cells (Fusar Poli et al., 2013).

In the case of MPS VII neuronal loss has not been established as a cause for the neuropathology although a postmortem analysis showed neuronal loss in the substantia nigra and the anterior columns of the spinal cord(Vogler et al., 1994). Interestingly, studies have shown that several pro-apoptotic genes are activated in the mouse brain although apoptotic cells could not be detected in those same tissues(Heuer et al., 2002; Richard et al., 2008). The use of a fly model of MPS VII uncovered a neurodegeneration mechanism based in the loss of dopaminergic neurons, even if the translatability of this findings to the murine and human model have not been studied yet(Bar et al., 2018).

All these sometimes-contradictory results demonstrate the difficulty faced when studying the neurodegenerative process taking place in the mucopolysaccharidosis. It has been stablished that neuropathology is a dynamic process taking place at different paces and involving different mechanisms in different regions of the CNS.

Other lysosomal disorders also present neuronal death. Gaucher disease is characterized by profound neurological impairment and neuronal cell death,

but the extend of the cell loss is dependent on the severity of the disease(Farfel-Becker et al., 2011; Wong et al., 2004).

The loss of neurons is also a hallmark of Niemann-Pick disease type C (NPC) with neurons dying by necroptosis, a form of inflammatory cell death(Cougnoux et al., 2016; Erickson & Bernard, 2002). Furthermore, Tay-Sachs and Sandhoff mouse models and patients also present increased apoptosis in brain and spinal cord(Huang et al., 1997; Lecommandeur et al., 2020). These results establish neuronal death as a common characteristic for several sphingolipidoses.

2.4.2. Synaptic and Neuronal function

However, not all neurodegenerative disorders show significant neuronal death. This is increasingly important in the first steps of the disease in which incipient cognitive deficits are present without overall neuronal death.

Synaptic pathology has also been proposed as a possible cause underlying cognitive impairment in diseases without apparent neuronal loss. Even though the study of the synapse is not a recent event, technological advancements have made possible to study neurons in a more detailed and completed manner.

2.4.2.1. Generalities about synapse

Functional synapses are essential in neurotransmission and network dynamics between neurons. Therefore, alterations in synapse function and even more importantly synaptic loss might be a major factor causing the neurological symptoms in disorders with neurological affectation.

From a structural point of view, synapses are the point of contact between two neurons. Most commonly neurons communicate through chemical synapses by releasing and uptaking neurotransmitters. However, electrical synapses in which the communication between cells is based in an ionic exchange are also relevant for cognitive function (Pereda & Miller, 2021; Vaughn & Haas, 2022).

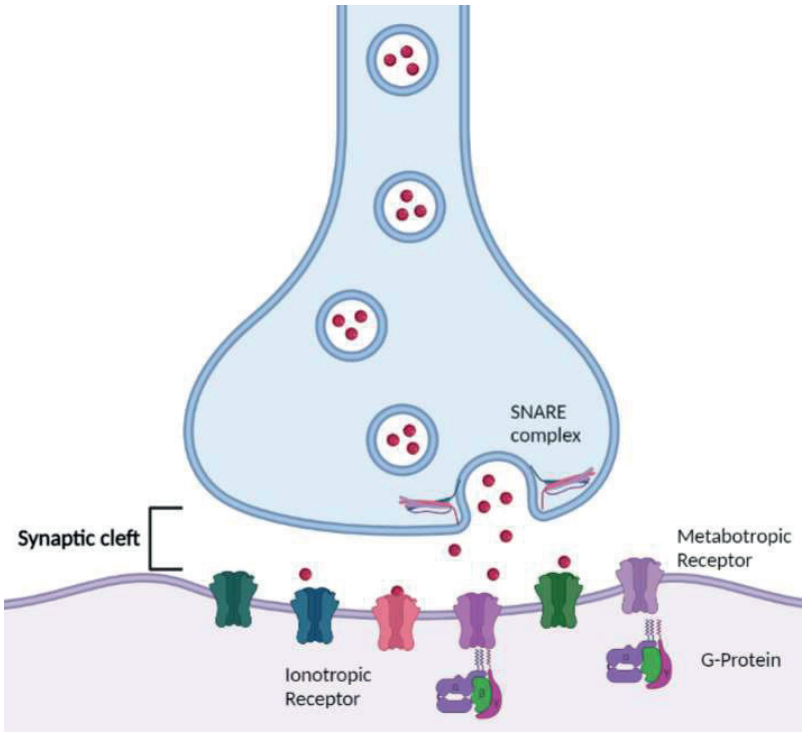

Figure 8: Synapse structure.

Synapse can have excitatory or inhibitory effects depending on the chemical signal they release and are situated in different regions of the neuron. While excitatory synapses tend to be in the dendritic spines, inhibitory synapses are commonly form directly onto the dendritic branch.

Synapse plasticity, which has been suggested to play an essential role in memory acquisition and removal, allows for each synapse to strengthen or even disappear in response to activity level(Trachtenberg et al., 2002).

2.4.2.2. Presynaptic region

In the presynaptic bouton neurotransmitters are synthesized and stored until an action potential signals their release. The neurotransmitters are then released by calcium-triggered synaptic vesicle exocytosis(Maritzen & Haucke, 2018; Sudhof, 2012).

This complex mechanism in which synaptic vesicles fuse with the presynaptic membrane to release the neurotransmitters is dependent on several proteins from the N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) family(Sauvola & Littleton, 2021; Stewart et al., 2000).

The small space between the presynaptic bouton and the postsynaptic membrane where the neurotransmitters are released is called synaptic cleft. Neuroligins and neurexins are the proteins in charge of maintaining this cleft and have been implicated in several neurological disorders (Sudhof, 2008).

2.4.2.3. Postsynaptic region

Finally, neurotransmitters reach the postsynaptic membrane where they interact with specific receptors. These receptors can be classified in two groups: ionotropic receptors and metabotropic receptors.

Ionotropic receptors are also known as ligan-gated ion channels and are responsible for quick depolarization of the neuron. Structurally, these receptors present an extracellular domain that binds to the neurotransmitter and a membrane domain that conforms an ion channel. The membrane domain is made up of four or five individual subunits forming a pore in the membrane(Burada et al., 2022; Wyllie & Bowie, 2022).

Metabotropic receptors are more slow acting. This is because they are not directly linked to an ionic channel. Instead, interaction between the neurotransmitter and the receptor leads to the activation of intermediate molecules called G-proteins.

These proteins open or close ionic channels through a series of enzymatic reactions after dissociating from the receptor. This type of receptors are monomeric proteins with a neurotransmitter binding site in their extracellular domain and an intracellular domain which interacts with the G-proteins(Sherman, 2014).

Interestingly, SNARE proteins have also been found in the postsynaptic region regulating neurotransmitter receptor trafficking(Madrigal et al., 2019).

2.4.2.4. Synaptic alterations in MPS

Defects in synapse structure or function have been extensively linked to neurological disorders. In mucopolysaccharidosis type VII lower expression of several proteins related to synaptic function has been described using iPSC-derived neurospheroids (Bayo-Puxan et al., 2018).

In MPS IIIA changes in the postsynaptic region of cortical neurons occur before other neurodegeneration signs become apparent(Dwyer et al., 2017). Furthermore, it has been described in the same disorder that impaired lysosomal activity leads to aberrant presynaptic SNARE phenotype leading to defective neurotransmitter trafficking(Sambri et al., 2017). Reduced expression and mislocalization of pre and postsynaptic proteins have been described in murine models of MPS I, MPS IIA and MPS IIIB(Wilkinson et al., 2012).

In other lysosomal disorder impaired synaptic function has also been studied. In a fly model of Gaucher disease, lysosomal defects have been associated with synaptic loss and neurodegeneration(Kinghorn et al., 2017). Dysregulation of developmental NMDA receptor has been described in the mouse model of infantile neuronal ceroid lipofuscinosis (CLN1), and primary cultures exhibited dysregulation of calcium flux(Koster et al., 2019).

Niemann-Pick type C disease, which had already been demonstrated to develop neuronal loss, also exhibited impaired induction and maintenance of long-term synaptic potentiation in the mouse model(D'Arcangelo et al., 2016).

2.4.3. Inflammation and complement system

2.4.3.1. Neuroinflammation

The central nervous system has been considered for a long time to be “immune privileged” without any involvement in inflammation. However, it is now clearly known that CNS exhibits features of inflammation and upon injury or disease can induce an inflammatory response.

Inflammation can be defined as a complex biological response to injury or infection. The main objectives of inflammation are tissue protection and the maintenance of tissue homeostasis. Inflammation is mediated by the release of cytokines, chemokines, reactive oxygen species and secondary messengers that trigger the recruitment of specific immune cell populations and coordinate the response(Fung et al., 2012). These mediators are mainly produced by resident CNS microglia and astrocytes although they can also be produced at low levels by other cellular types such as endothelial cells.

The degree of inflammation is highly variable and depends on the context, duration and course of the stimulus that triggered it. Inflammation occurs at numerous time points in physiological conditions.

While brief and controlled inflammatory responses can be beneficial in the brain in several situations such as combating infections or inducing axonal regrowth after injury (Benowitz & Popovich, 2011) , chronic inflammation has been increasingly associated with brain pathology.

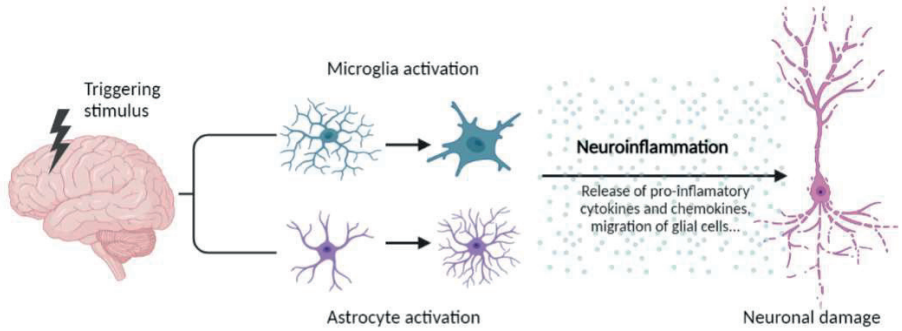

Figure 9: Events leading to neuronal damage through inflammation.

Among the cell types involved in neuroinflammation astrocytes and microglia are the principal immune effector cells of the brain. Astrocytes are the most abundant cell type In the CNS and are involved in multitude of physiological functions such as maintenance of the blood-brain barrier and regulation of axonal outgrowth and myelination(Kiray et al., 2016). Besides, they are also involved in intracellular communication and signaling via Ca^{2+} transport(Kimelberg & Nedergaard, 2010). Microglia perform a constant surveillance of the environment and become activated in response to injury or disease. One activated they start a rapid production and release of cytokines and chemokines and undergo morphological changes that altern the pattern of receptor expression in the cell surface.

All these changes allow microglia to migrate through the brain towards the site of injury. Microglia are also responsible for the phagocytosis of cellular debris and presenting antigens to T cells.

Activated microglia and astrocytes have been traditionally categorized in two functional types: neurotoxic (M1 or A1) and neuroprotective (M2 or A2). However, this classification does not realistically reflect al phenotype variability of microglia in the brain. Furthermore, the activation status of microglia has been described as a dynamic process changing based on the progression of the disease (Kwon & Koh, 2020).

Homeostatic microglia participate in essential functions through development. They act as regulators of cell death and neurogenesis and play a critical role in synaptic pruning through development by actively engulfing synaptic material (Paolicelli et al., 2011). This synaptic pruning, while necessary for a correct postnatal development of the brain has been found dysregulated in neurological disorders (Cardozo et al., 2019). This complex process depends on neural activity and the complement pathway, a microglia-specific signaling pathway(Skaper et al., 2019).

2.4.3.2. The complement system

The complement system is a major effector of the innate immune system. This protein cascade involves over 40 different plasmatic or membrane-associated proteins. This cluster of proteins is organized in proteolytic cascades that start with the detection of a pathogenic surface, is followed by the generation of proinflammatory molecules, coating of the pathogenic surface with diverse complement opsonins and is completed by targeted endocytosis(Dunkelberger & Song, 2010). Therefore, its activation is critical for detection and prosecution of pathogens, and clearance of apoptotic cells and cellular debris.

The complement system is traditionally described to be activated through three recognition pathways: classical, alternative and lectin.

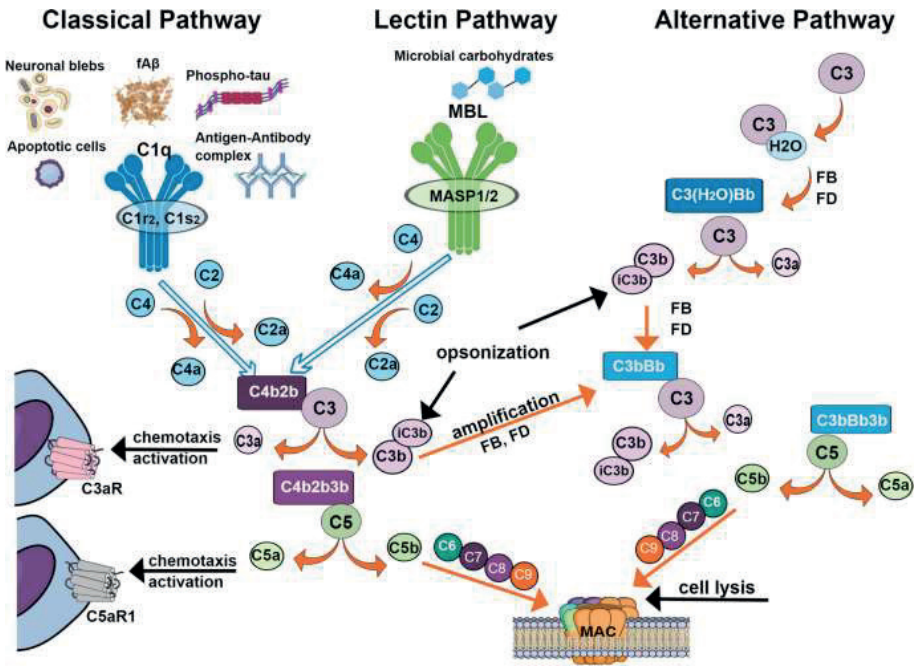

Figure 10: Complement activation pathways(Schartz & Tenner, 2020).

The events leading to the activation are an important difference between pathways, but they end up converging in the C3 convertase enzyme. This leads to cleavage of C3 creating C3a and C3b. Complement C3b can bind to the surface of pathogen to promote phagocytosis or lead to the cleavage of C5 into C5a and C5b. Complement C3a and C5a have anaphylatoxin activity triggering different mechanisms through G-protein coupling and downstream signaling. Some of the triggered mechanisms are chemotaxis, degranulation and cytokine production. Furthermore, C5b is the initiator of the membrane attack pathway which results in the formation of the membrane attack complex (MAC), the cytolytic effector of the complement cascade. MAC forms a transmembrane channel which causes osmotic lysis of the target cell or pathogen(Schartz & Tenner, 2020).

Recently, two additional mechanisms involving properdin and coagulation proteins have been proposed for activation of the complement pathway (Markiewski et al., 2007; Spitzer et al., 2007).

Besides its role in inflammation, recent studies have revealed a variety of unrelated functions of complement pathway. During CNS development, the complement pathway regulates neuronal migration and neural progenitor cell proliferation (Coulthard et al., 2018; Gorelik et al., 2017).

Involvement of the complement system in differentiation of neural progenitor cells has been proven *in vitro* (Shinjyo et al., 2009). Moreover, it can promote neurogenesis in adult mouse brain after injury (Rahpeymai et al., 2006).

However, inflammation and complement system are also involved in pathogenic processes through the body. Moreover, aberrant activation of both has been described in the injured, aged or disease CNS.

2.4.3.3. Inflammation in MPSs

Metabolic inflammation has been described as a secondary effect of glycosaminoglycan accumulation in mucopolysaccharidoses. This inflammation has systemic effects all through the body playing a significant role in neurological pathology.

MPS VII, like most lysosomal storage disorders is characterized by chronical inflammation which has been suggested as a mechanism leading to the cognitive impairment found in this disorder. Several chemokines such as CCL2, CCL3 and IL-1β, and inflammatory receptors like CCR2 and TLR-4 have been described to be overexpressed in the brain of the MPS VII mouse model, with increased astrocyte activation determined by higher GFAP levels. Moreover, increased expression of TNF-R1 have also been detected suggesting possible increased apoptosis as a result of neuroinflammation(Richard et al., 2008).

Similar proinflammatory phenotype has been described for other mucopolysaccharidoses. Upregulation of numerous immune-related genes of adaptative and innate response, increased lymphocyte infiltration and expression of proinflammatory cytokines have been described in a mouse model of MPS IIIB(DiRosario et al., 2009; Killedar et al., 2010). Despite the fact that increased inflammation is a shared phenotype among mucopolysaccharidoses the specific process and affectation levels are variable. It has been demonstrated that MPS I exhibit less microglial activation and proinflammatory cytokine release than both MPS IIA and MPS IIIB (Wilkinson et al., 2012).

The complement pathway seems to be dysregulated in MPS VII mouse models and has been suggested to play a role in the pathogenesis of aortic dilatation (Baldo et al., 2011). Inflammation and complement pathway have been proposed as major mechanisms involved in the skeletal pathology of MPS diseases. However, a study failed to show improved skeletal pathology in mice carrying homozygous mutations of either TLR4 and/or C3.

The involvement of the complement cascade in the CNS has not been much studied in mucopolysaccharidosis. Nonetheless, in cortex mouse models of MPS I and IIIB overexpression of complement proteins C4 and C1 were found to be elevated showing potential involvement of this system in the neuropathology of mucopolysaccharidosis. (Ohmi et al., 2003).

2.4.4. Oxidative stress

Oxidative stress is described as an imbalance between reactive oxygen species (ROS) production and accumulation and the ability of the cell to detoxify these reactive products and counteract their action by antioxidant defences. Disturbances in the redox state of a cell can be caused by increased production of ROS or from decreased endogenous antioxidant activity.

Reactive oxygen species can be simply defined as oxygen-containing molecules that easily react with other molecules. Among all ROS some can include free radicals like O_2^-, OH^- or be non-free radical oxygenated molecules like H_2O_2 and O_2. The term free radical refers to molecules or atoms that present one or more unpaired electron occupying their external orbit.

ROS have several physiological functions and are generated as by-products of normal cellular processes like detoxification, mitochondrial respiration and peroxisomal beta-oxidation. Even so, environmental stressors and xenobiotics cans increase ROS production leading to oxidative stress. Besides ROS, reactive nitrogen species (RNS) are also present in the cells(Li et al., 2016).

The main source of endogenous production of ROS is the production of oxidative ATP in the mitochondria. In this process, molecular oxygen is reduced in the electron transport chain. However, enzymatic reactions able to generate ROS are also found in other mechanisms such as phagocytosis(Canton et al., 2021; Dupre-Crochet et al., 2013).

Superoxide radical (O_2^-) can be generated by NAPDH oxidase, xanthine oxidase and several peroxidases. In turn, superoxide radical can generate other ROS. Hydrogen peroxide (H_2O_2) is produced by various sources being enzyme-catalyzed superoxide dismutation the main one. It can also result from NADPH oxidases, normal protein folding, peroxisomal enzymes and the cytochrome P450 (CYP) catalytic cycle(Andres et al., 2022). Another relevant ROS is the hydroxyl radical (OH^-) which constitutes the chemically most active reactive species(Chen & Schopfer, 1999). Hydroxyl radical is commonly formed non-enzymatically by the Fenton reaction between H_2O_2 and Fe^{2+} and as a result of water radiolysis. Besides, alkoxyl, hydroperoxyl and peroxyl radicals are other ROS found in the cells.

High ROS amount have been associated to a plethora of diseases. However, physiological levels of ROS production play a central role in redox signaling through posttranslational modifications. ROS are involved in pivotal cell processes such as cell proliferation, gene expression and apoptosis, and even participate in phagocytosis-mediated defense against pathogens.

Redox signaling is known to affect protein function resulting in abnormal signaling outputs, enzymatic activity, gene transcription and gene and membrane integrity. The number of protein targets of redox signaling is immense including transporters, receptors, transcriptional factors, cytoskeletal elements, scaffold proteins and heat shock proteins amongst others(Go & Jones, 2013).

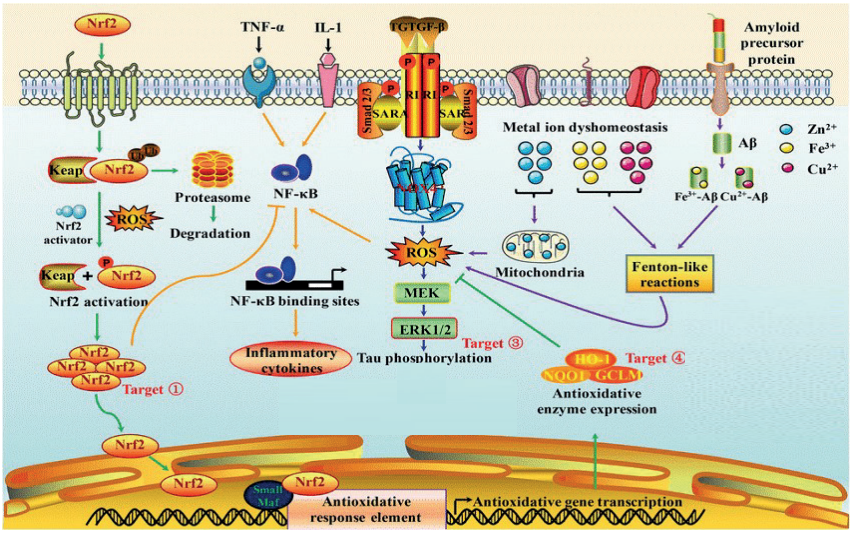

Figure 11: Mechanisms of oxidative stress (Chen et al., 2020).

As the cause of pathology is not ROS production per se but an imbalance in this process, cells have developed complex regulatory systems to regulate the production and neutralization of these molecules. The process of ROS neutralization is complicated and includes the participation of antioxidant enzymes like glutathione peroxidase and superoxide dismutase, and non-enzymatic compounds such as glutathione, ferritin, transferrin and albumin and the low molecular weight scavengers coenzyme Q and lipoic acid (Pisoschi & Pop, 2015; Snezhkina et al., 2019).

Some of the pathways essential maintaining oxidative homeostasis in the cells responding to oxidant challenge are the NRF2-KEAP1 system and the NF-κB pathway. KEAP1 acts as an NRF2 inhibitor that upon oxidation changes its conformation and prevents NRF2 ubiquitylation. This increases NRF2 stability and allows its translocation to the nucleus where it will act as a transcriptional factor(Yamamoto et al., 2018). NF-κB pathway is also under redox regulation in which H_2O_2 can have both inhibitory and excitatory roles and plays a major role in inflammation(Oliveira-Marques et al., 2009).

However, the targets of redox signaling also involve response to hypoxia, regulation of stress sensors, metabolic adaptation, regulation of mitochondrial energetics and regulation of conductance through ion channels. Furthermore, oxidative stress and ROS overproduction have also been linked to endoplasmic reticulum stress and the activation of unfolded protein response (UPR) (Cao & Kaufman, 2014).

Because the main endogenous source of ROS production is the mitochondria, oxidative stress has been proposed to be linked with mitochondrial dysfunction. This is especially relevant for neuronal cells whose correct function and survival is dependent on a continuous supply of ATP from the mitochondria.

Mitochondria are often referred to as the powerhouse of the cell and are highly dynamic organelles that regulate their number, size and morphology through cycles of fission and fusion(Tilokani et al., 2018). It has been described that ROS can induce oxidative damage to the mitochondrial membrane and the proteins conforming the electron-transport chain leading to decreased ATP production (Ghezzi & Zeviani, 2012; Stewart & Heales, 2003; Wei et al., 2001).

Mitochondrial dysfunction has been described in a number of lysosomal storage disorders. Nonetheless, the mechanisms leading to it are still uncertain.

A partial impairment of the mitochondrial oxidative phosphorylation system has been described in the brain of MPS IIIC with reduced activity of specific enzymes and decreased coenzyme Q10 suggesting compromised energy metabolism(Martins et al., 2015). Moreover, progressive accumulation of pleomorphic swollen mitochondria has been found in the same animal model starting at 5 months of age (Pshezhetsky, 2016).

In the brain of a murine model of NPC1 the mitochondrial production of ATP was found to be reduced due to decreased ATP synthase activity. They also described increased levels of cholesterol within the mitochondrial membrane the reduction of which led to the restoration of ATP synthase activity, suggesting altered cholesterol metabolism as a possible cause underlaying the neuronal impairment(Yu et al., 2005). Oxidative stress parameters have also been found increased in NPC1 mouse models proposing a combined therapy with antioxidants to increase clinical benefits(Hammerschmidt et al., 2022).

Inhibition of the glucocerebrosidase 1, the gene mutated in Gaucher disease, has been proven to cause mitochondrial disfunction through increased ROS production, reduced mitochondrial membrane potential and decreased ATP production(Cleeter et al., 2013).

2.4.5. Autophagy and cellular signaling

Autophagy is one of the first and most affected mechanisms in mucopolysaccharidosis type 7 as well as in all other lysosomal storage disorders. Furthermore, the central role of autophagy and its connection to other fundamental cellular process heighten the need for a clear understanding of this process both in physiological and pathological conditions.

Autophagy is the process by which old, damaged or abnormal molecules and subcellular elements are eliminated via lysosome degradation. There are three types of autophagy: macro-autophagy, micro-autophagy and chaperone-mediated autophagy (CMA).

In macro-autophagy, the cytoplasmic material to degrade is internalized in the phagosome, a double membrane-bound vesicle, and delivered to the lysosome by a fusion process resulting in the formation of the autolysosome. Micro-autophagy in contrast, is characterized by the direct invagination of the lysosomal membrane to capture the cytoplasmic components without phagosome involvement. In chaperone-mediated autophagy, specific proteins are translocated through the lysosomal membrane forming a complex with chaperone proteins that are recognized by a lysosomal membrane receptor. When the term autophagy is used, it mostly refers to macro-autophagy due to its increasingly relevant involvement in disease.

The autophagic process always involves a series of sequential steps: initiation, elongation, maturation, fusion and degradation.

Initiation of autophagy is typically the result of stressful conditions such as hypoxia, oxidative stress or protein aggregation among others (Dikic & Elazar, 2018). The ULK1 complex is a chore machinery in the initiation of autophagy and is tightly regulated by mTOR and AMPK.

Activation of this complex stimulates the class III phosphatidylinositol 3-kinase (PIK3C3) complex containing BECN1, triggering the nucleation step of autophagy. A complex cascade of protein interactions leads to the production of phosphatidylinositol-3-phosphate (PI3P), recruitment of WIPI proteins and elongation of the phagophore. An important part of the lipidic

bilayer used for the elongation is delivered by ATG9-containing vesicles, but other membranes are suggested to be involved.

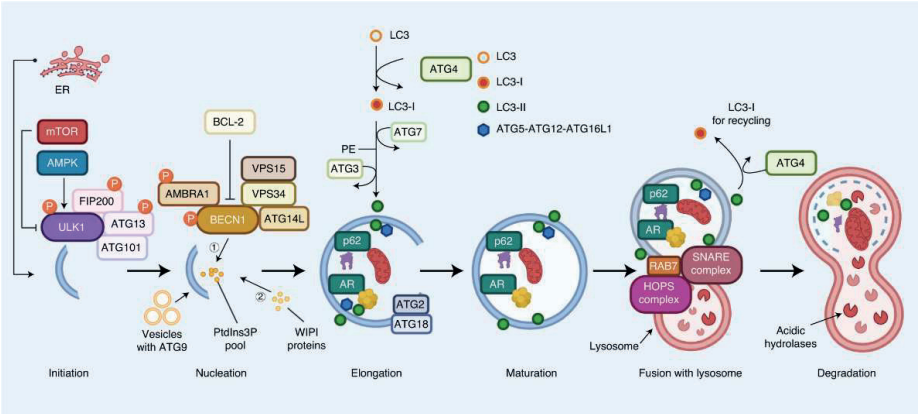

Figure 12: Autophagy process (Aman et al., 2021).

LC3 processing and insertion into the extending phagophore membrane is a key process leading to autophagosome maturation. LC3 binds to autophagy receptors such as p62/SQSTM1 leading to the degradation of polyubiquitinated protein aggregates bound to p62. Interestingly, transcription of p62 is modulated by several mechanisms being oxidate stress one of them. Furthermore, p62 participates in a wide range of cellular functions beside autophagy by interacting with several protein kinases (MEK5, ERK1, etc.) (Liu et al., 2016).

Fusion of autophagosomes with lysosomes is mediated by SNARE proteins, Rab proteins and a homotypic fusion and protein sorting (HOPS) complex. At this point, LC3 is recycled by ATG4 cleaving and lysosomal hydrolases start degrading the cytoplasmic components initially found in the phagosome.

The transcription factor EB (TFEB) is a master regulator of lysosomal biogenesis modulating the expression of autophagy and lysosomal genes. It has been demonstrated that overexpression of this factor leads to higher number of autophagosomes in the cell and results in the generation of new lysosomes increasing autophagic activity (Settembre et al., 2011).

Moreover, TFEB does not only play a role in the induction of autophagy but is involved in multiple critical steps through the autophagic process such as: autophagosome formation, autophagosome-lysosome fusion and degradation of the components inside the phagosome (Settembre &

Ballabio, 2011). Under aberrant lysosomal storage conditions, like those observed in mucopolysaccharidoses, TFEB is activated by its translocation from the cytoplasm to the nucleus increasing the degradation of complex molecules (Sardiello et al., 2009).

Even though autophagy initially acts as a survival mechanism reclining cellular components to maintain cell homeostasis, its dysregulation has been increasingly linked to disease. In recent years it has been demonstrated that decreased autophagy is an important feature of aging, both through reduced expression of autophagy-related proteins and decreased translocation of cytoplasmic cargo to the lysosomes (Kaushik et al., 2012; Lipinski et al., 2010; Ott et al., 2016). Besides, the connection of autophagy to other critical cellular processes like oxidative stress and inflammation further showcases its involvement in pathological processes.

Mucopolysaccharidosis type VII, like all lysosomal storage disorders is characterized by the macromolecule accumulation within the lysosomes which leads to impaired autophagy and cell accumulation of dysfunctional mitochondria and polyubiquitinated proteins. One of the proposed mechanisms by which impaired autophagy could be contributing to neuropathology in lysosomal storage disorders is through decreased autophagosome-lysosome fusion due to deficient SNARE protein levels in the lysosomes (Tanaka et al., 2022).

2.5. Gene Therapy

The advances in medicine, technology and the increased knowledge of how and why diseases develop have generated a raise in the number of therapeutic options to treat disease. This allows to select the most beneficial strategy for each disorder based on the underlying cause and progression of the symptoms.

Gene therapy arises as a solution for the main complication that conventional drug administration faces in treating diseases with a neurological pathology. The blood-brain barrier prevents the therapeutic molecule from reaching the CNS and even the blood-nerve barrier does the

same for the peripheral nervous system (PNS). Furthermore, the short-term availability of this drug in the body limits the duration of the therapy. This entails repeated administration of the therapeutic drug at higher concentrations. Consequently, adverse side effects and economic and time burdens for the patients are increased. Long—term effect and great specificity are some of the benefits provided by gene therapy.

Gene therapy is described as a therapeutic approach based in the correction of the genetic problem underlaying a specific disorder through the administration of genetic material.

At the beginning, gene therapy was proposed only for the treatment of monogenic disorders, but it has also been proved useful in treating multifactorial diseases (Khatib et al., 2021; Takahashi et al., 2018). Depending on the specific cause of the pathology gene therapy can work through several mechanisms.

Using gene therapy, it is possible to replace a mutated gene with a healthy version of the same gene, silence the gene that is causing the disease and introduce a new or modified gene to help fight the disease.

To efficiently deliver the genetic material into the target cells several types of vectors can be used. These vectors can be classified in two main groups: non-viral and viral vectors.

Non-viral vectors which are basically comprised of plasmid DNA offer low immunogenicity and cytotoxicity as their main benefits. Other motives supporting the use of non-viral vectors are the ease of production and their low cost. However, non-viral vectors present several drawbacks that have limited their application (Ramamoorth & Narvekar, 2015). A critical problem of this type of vectors is their reduced efficiency to transfect cells. A multitude of delivery systems have been proposed over the years to overcome this issue with variable success(Dobson, 2006; Jin et al., 2014; Tasset et al., 2022).

Viral vectors on the other hand have no problem in cell transduction due to the natural ability of the virus to infect cells. This type of vectors is designed replacing all pathogenic genes in the viral genome while maintaining the ones responsible for capsid formation. Viral vectors can be classified in 5 main groups: retroviruses (RV), lentiviruses (LV), herpes simplex viruses (HSV), adenoviruses (AV) and adeno-associated viruses (AAV).

Just like non-viral vectors, the different types of viral vectors face several complications that hinder their application. For one, they are costly and harder to produce in mass scale. Besides, they present higher immunogenicity and introduce an oncogenic risk through the integration of the genetic material in the genome of the target cell(Thomas et al., 2003). Furthermore, since viral vectors maintain the same tropism as the parental virus targeting a specific cell population after a systemic administration represents a challenge (High & Roncarolo, 2019).

Although no perfect delivery vector for gene therapy has been developed yet, multitude of clinical trials mostly using viral vectors are taking place with some gene therapy products already approved for use(Ghosh et al., 2020).

2.5.1. AAV vectors

Nowadays, the most widely used viral vectors in gene therapy are the adeno-associated virus (AAV) vectors. There have been over 153 clinical trials using this vector and the number of trials starting per year is still increasing. Most of the trials can be classified in five therapeutic areas: Blood disorders, CNS disorders, eye disorders, lysosomal disorders and neuromuscular disorders. However, there are also clinical trials for HIV, cancer and diseases affecting the heart, lung and liver.

Despite the high number of clinical trials ongoing, 75% are still at early stages and only 2 products are being commercialized (Au et al., 2021). The first AAV-based gene therapy approved in Europe was Glybera in 2012 for the treatment of hereditary lipoprotein lipase deficiency (LPLD). However, in 2017 it was withdrawn from the market. The same year Luxturna was approved by the FDA for the treatment of Leber's congenital amaurosis. Zolgensma was approved in 2019 as treatment for spinal muscular atrophy with more than 2500 patients injected so far around the world. Finally, in 202 UPSTAZA (PTC) has been approved for AADC deficiency, Hemgenix for Hemophilia B and ROCTAVIAN for Hemophilia A.

2.5.1.1. Generalities about AAV vectors

Adeno-associated viruses are small, non-enveloped viruses with a single-stranded DNA of approximately 4.7kb surrounded by an icosahedral capsid. AAV viruses belong to the *Parvovididae* family and depends on co-infection with a helper virus, commonly an adenovirus, to be able to replicate. The inability of AAVs to replicate by themselves make them a safe option for long term transgene expression after a single administration(Bulcha et al., 2021).

The single-stranded genome of AAV virus contains three genes: *Rep* (Replication), *Cap* (Capsid) and *aap (*Assembly) flanked by two palindromic inverted terminal repeats (ITR). Over nine products result from these three genes through the use of three promoters, alternative splicing and alternative translation sites. *Rep* encodes four proteins (Rep78, Rep68, Rep52, Rep40) necessary for the replication and packaging of the genome (Samulski & Muzyczka, 2014) . *Cap* gene encodes the viral capsid proteins (VP1, VP2 and VP3 in an estimated molar ratio of 1:1:10) and *aap,* in an alternate reading frame overlapping *cap,* encodes the assembly-activating protein (AAP) which has been suggested to perform a scaffolding function for capsid assembly(Naumer et al., 2012).

AAV enters the target cell via endocytosis after interaction to a primary receptor, which differs among the AAV serotypes. From there, intracellular trafficking delivers the AAV first to the cytoplasm and later on to the nucleus, although the specific mechanisms are not well known (Riyad & Weber, 2021). In the nucleus AAV uncoats releasing its genome which is then turned from ssDNA to double-stranded DNA (dsDNA). If Rep proteins are present, the AAV genome may integrate in the 19q13 locus of the host genome or maintained in a circular episomal state.

2.5.1.2. AAV serotypes and their tropism

Numerous AAV serotypes with variable tropism (Table 4) have been identified after being isolated as contaminants of adenovirus preparations from primates and other species.

Table 4: AAV 1-9 vector serotype, origin, receptor usage and in vivo tropism.

Serotype	Isolation Origin	Primary Receptor	Co-receptor	In vivo tissue tropism
AAV1	Monkey	Sialic acid	-	**Skeletal Muscle,** Heart, CNS, Eye, Liver
AAV2	Human	Heparin	FGFR-1, HGFR, Laminin receptor, Integrin	Skeletal Muscle, CNS, Liver, Eye
AAV3	Human	Heparin	FGFR-1, HGFR, Laminin receptor	Skeletal Muscle, Liver
AAV4	Monkey	Sialic acid	Unknown	Eye, CNS, Kidney, Lung
AAV5	Human	Sialic acid	PDGFR	Skeletal Muscle, **CNS,** Lung, Eye
AAV6	Human	Heparin, Sialic acid	EGFR	**Skeletal Muscle**, CNS, Heart, Lung
AAV7	Monkey	Unknown	Unknown	Skeletal Muscle, CNS, Eye, Liver
AAV8	Monkey	Unknown	Laminin receptor	**Liver, Skeletal Muscle,** Pancreas, CNS, Heart, Eye, Lung
AAV9	Human	Galactose	Laminin receptor	**Skeletal Muscle, CNS**, Lung, Retina, Heart, Liver
AAVrh10	Monkey	Unknown	Laminin receptor	Skeletal Muscle, **CNS**, Lung, Retina, Heart, **Liver**

FGFR fibroblast growth factor receptor, HGFR hepatocyte growth factor receptor, PDGFR Platelet derived growth factor receptor, EGFR epidermal growth factor receptor, CNS Central nervous system. **Bold** – most used target tissue *in vivo*

Genome size and organization is conserved across serotypes although the transcription profile is slightly variable (Qiu & Pintel, 2008). The capsid of AAV serotypes 1-9 shows almost a 45% of overall amino acid identity, and capsid comparison between serotypes has revealed 12 hypervariable surface regions which accumulate most of the variability (Gao et al., 2003).

Although these variable domains have not been found directly responsible of the differences in expression levels and duration seen in *in vivo* studies (Zincarelli et al., 2008), the variation in the surface topology of the capsid has been suggested to determine the specific receptor usage and cell surface attachment of the different serotypes (Weitzman & Linden, 2011).

The knowledge obtained from the natural AAV variants and the need to optimize AAV vectors for gene therapy application have led to the engineering of new optimized AAV variants through directed evolution and rational design of the capsid to improve specific properties. Some of the engineered AAV capsids are currently under pre-clinical and even clinical evaluation and the improved properties include increased transduction efficiencies, alternate tropisms with less sequestration in non-target organs (mostly the liver), and importantly, reduced immunogenicity (Kotterman & Schaffer, 2014; Li & Samulski, 2020).

2.5.1.3. Administration Routes

The efficiency of transduction in the different tissues and organs does not only depend on the tropism of the AAV vector used but also on the administration route used. Depending on the pathology of the disease being treated, delivery of the AAV vector can be done either directly into the specific organ affected or via systemic administration to obtain a widespread transduction in several organs.

In diseases with CNS pathology, the route of administration needs to be carefully selected because different AAVs administration routes affect the transduction rate and the cellular types transduced in the brain.

The easier and less invasive approach is the systemic administration of AAV vectors via intravascular (IV) administration which lead to a broad distribution of the AAV, although specific organ transduction depends mainly

on AAV serotype. This type of delivery allows the simultaneous transduction of various organs and tissues with only one administration, which would be beneficial in multi-organ disorders. However, an important handicap of IV administration is the difficulty to treat diseases with CNS affectation because the blood-brain barrier (BBB) severely limits the pass of the AAV vector from the blood to the brain.

Several AAV serotypes including AAV9 (Saraiva et al., 2016) and AArh10 (Tanguy et al., 2015) have been described to cross the BBB after intravascular administration, although the level of transduction was much lower when compared to direct CNS administration routes (Fischell & Fishman, 2021). Moreover, intra-arterial (IA) administration has been suggested as an alternative with increased brain delivery of the vector compared to the liver. It is interesting to notice that the benefits from IA administration are higher for engineered vectors with high efficiency to cross the BBB such as AAV PHP.B (Liguore et al., 2019) when compared to other vectors like AAV9 that can cross the BBB but are less efficient in doing it.

Although it has not reached the clinic yet, physical methods for transient BBB disruption offer another approach to increase CNS transduction after IV or IA administration. The two more studied physical methods that allow for a transient disruption of the BB are focused ultrasound (Fishman & Frenkel, 2017; Noroozian et al., 2019; Stavarache et al., 2018) and intra-arterial delivery of mannitol (Chu et al., 2018; Foley et al., 2014).

Another drawback of a systemic administration is the requirement of a much higher dose compared to directed administration. This higher dose could not only lead to an exorbitant production cost but also to increased immune response- related toxicity. Therefore, for neurodegenerative disorders, direct administration to the CNS could be a better therapeutic approach. This can be done by either direct intracranial injection of the AAV into the brain parenchyma or by cerebrospinal fluid (CSF) administration.

Direct intraparenchymal injection is performed using a stereotaxic instrument to precisely deliver the AAV to a selected set of coordinates in the brain. The advantages of this administration are the smaller dose requirement and reduced immune response when compared to IV administration. Nonetheless, this strategy presents a completely different set of drawbacks.

One limitation of this route is the inadequate distribution volume of the therapeutic vector. In general, AAV can only diffuse a relatively short distance from the injection site and therefore, multiple injection in several sites might be required to reach the desired brain coverage.

Some engineered AAV serotypes can spread by anterograde and retrograde transport improving its distal distribution and providing new strategies for targeting neural circuits efficiently (Green et al., 2016; Salegio et al., 2013). Furthermore convection-enhanced delivery (CED), a technology that uses pressure gradients to increase the diffusion area of the therapeutic vector is being evaluated (Mehta et al., 2017; Sudhakar & Richardson, 2019).

The major drawback of direct intraparenchymal injection is its invasiveness, which can cause brain injury. One example of this is the increased rick of intracranial hemorrhage and headache described in a clinical trial after intraluminal injection (Christine et al., 2009).

The other direct administration to the CNS is the delivery of the vector to the cerebrospinal fluid either in cerebral ventricles, the cisterna magna or the lumbar vertebral region (intrathecal).

The cerebrospinal fluid is produced by the cells in the choroid plexus lining the cerebral ventricles and circulates through all the subarachnoid space, around the brain and along the spinal cord before being drained to the venous circulation and the lymphatic system. The CSF provides mechanical and relative immunological protection of neural structures and is also involved in the trafficking of metabolites (Telano & Baker, 2022). Therefore, delivery to the CSF produces a wide distribution of the AAV vector to most of the central nervous system

Intracerebroventricular (ICV) injection has demonstrated transduction throughout the whole brain in several animal models (Chakrabarty et al., 2013; Haurigot et al., 2013). The specific cellular populations transfected depends on the tropism of the specific AAV serotype used, with AAV1, AAV4 and AAV5 showing mainly transduction of the ependymal cells (Davidson et al., 2000) while AAV8, AAV9 and AAVrh10 transduced mostly neurons but also astrocytes and oligodendrocytes (Wang et al., 2014). The widespread distribution resulting from this approach makes it a valuable tool for CNS treatment. However, it requires a cranial perforation which increases its invasiveness compared to the other CSF administration routes.

Injection into the cisterna magna is achieved through puncture between the occipital bone and the atlas. This administration route has been proved to obtain widespread transduction through the whole brain and part of the spinal cord in multiple animal models including large mammals (Haurigot et al., 2013; Zhou et al., 2022). Moreover, newly engineered variants like the AAV-PhP.eB showed even higher transduction after intracisternal injection in rats (Chatterjee et al., 2022). However, the main limitation of the administration in the cisterna magna is the methodology. The insertion of the needle so close to vital brain centers and to the medulla poses a risk of injury that could result in permanent disability or death (Samaranch et al., 2016; Taghian et al., 2020). This is increasingly important for pediatric pathologies with skeletal deformities a common characteristic of several lysosomal storage disorders including MPS VII.

Intrathecal (IT) injection in the lumbar region is the less invasive method, being routinely performed in outpatient settings by puncture between two lumbar vertebrae. Various AAV serotypes have proved able to transduce neurons in the dorsal root ganglia (DRG), motor neurons in the spinal cord and neurons in different areas of the brain (Homs et al., 2014; Schuster et al., 2014; Towne et al., 2009). Furthermore, IT administration could reach the same transduction levels than ICV and ICM in pigs and non-humane primates respectively (Bevan et al., 2011; Gray et al., 2013). However in a recent comparison with the injection into the cisterna magna in mice, lumbar IT administration showed slightly reduced brain transduction but increased levels of vector expression in the lower spinal cord and peripheral organs which makes it a fitting strategy to treat diseases with both CNS pathology and multisystemic affectation (Bailey et al., 2020).

Interestingly, intranasal administration has been suggested as non-invasive route targeting the brain. Although transduction has only been observed in the nasal epithelium and the olfactory bulb, if the therapeutic product can diffuse from the transfected neurons global effects can be achieved through the brain (Belur et al., 2017).

As of now intrathecal administration of the therapeutic vector seems to offer the best results for multisystemic disorders such as MPS VII because it avoids the BBB obtaining better transduction levels than IV administration and provides a more global and less invasive delivery than intracranial administration.

In fact, recent studies from our group have demonstrated that single IT administration in the lumbar region of MPS VII allows global and stable transduction generating supraphysiological levels of β-Glucuronidase throughout the brain (Figure 13) (Pages et al., 2019).

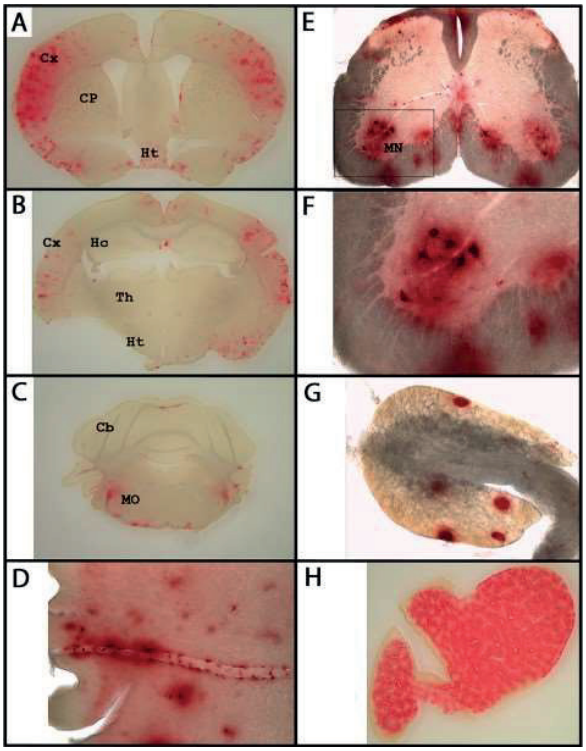

Figure 13: In toto ß-Glucuronidase staining in AAV-treated MPS VII mice. (A–C) Brain coronal sections of Cx, cortex; CP, caudatoputamen; Ht, hypothalamus; Hc, hippocampus; Th, thalamus; Cb, cerebellum; MO, medulla oblongata. (D) Magnification of a cortex area. (E) Lumbar spinal cord section (F) Magnification of a lumbar spinal cord section. (G) Lumbar dorsal root ganglion. (H) Liver section (Pages et al., 2019).

Moreover, drainage of the AAV vector to the bloodstream results in the transduction of the liver and is sufficient to achieve enzymatic activity in serum comparable to the wild type animals. This therapeutic approach was efficient in correcting the biochemical and histological hallmarks of MPS VII at short and long term in the central nervous system and other somatic organs, including the skeleton in which the progression of the pathology was reduced. Nonetheless, the biochemical correction of the enzyme deficiency led to a significant improvement in the cognitive abilities of the GUSB mice, but it did not result in a complete correction of the neurological phenotype.

3. Objectives

Mucopolysaccharidosis type VII, like most lysosomal storage disorders is characterized by multisystemic affectation, with chronic and progressive symptoms that are highly variable among patients, but intellectual disability is a prominent feature in almost all of them.

The mechanisms by which the glycosaminoglycan accumulation leads to cognitive impairment remain unknown. Other MPS disorders like Hurler (MPS I) Hunter (MPS II) and Sanfilippo (MPS IIIA, B and C) syndromes, which also present heparan sulfate as the main GAG accumulated show noticeably different neurological symptoms. This suggest that HS accumulation is not the only mechanism involved in the neuronal dysfunction and there may be specific alterations leading to neurodegeneration for each mucopolysaccharidosis.

Preclinical studies have stablished gene therapy as potential strategy to treat MPS VII, reversing different pathological features, but the lack of knowledge of the cause of the intellectual disability has impaired the detailed analysis of neuropathological correction of this disease.

This study is focused on understanding how glycosaminoglycan accumulation in the lysosomes has an impact on the functionality and viability of the neurons in MPS VII and if gene therapy can correct these effects

The following objectives have been stablished to address our aims:

1. To analyze neuronal death as a possible cause behind the neuropathology.

2. To characterize the mechanisms involved in the central nervous system pathology using the animal and cellular models.

3. To evaluate the potential of our gene therapy strategy in treating the neurological features of MPS VII.

4. Methodology

4.1. Animal care and experimental design

All mice used in this work were bred at the SPF animal facility of the CBATEG in the Universitat Autònoma de Barcelona, fed ad libitum with a standard diet and kept under a light-dark cycle of 12 hours. Easier access to food was provided for MPS VII animals at 4-5 months of age due to decreased mobility.

We used a MPS VII mouse model tolerant to human β-glucuronidase, with a C57BL/6 genetic background (Sly et al., 2001). Heterozygous mice were bred due to the inability of KO mice to breed. To genotype the animals, we obtained tail clips of weaned mice and quantified β-Glucuronidase activity. Starting at 5 months of age animals were weighted weekly to ensure continued health and were euthanized if they lost 20% of body weight.

Animal care and experimental procedures were performed in accordance with 86/609/EEC regarding the care and use of animals for experimental procedures and were approved by the Biosafety and the Ethical Committees of the Universitat Autònoma de Barcelona.

The experimental design followed in this work is detailed in Figure 14. Due to the reduced lifespan of MPS VII mice, analysis after 6 months of age could not be performed.

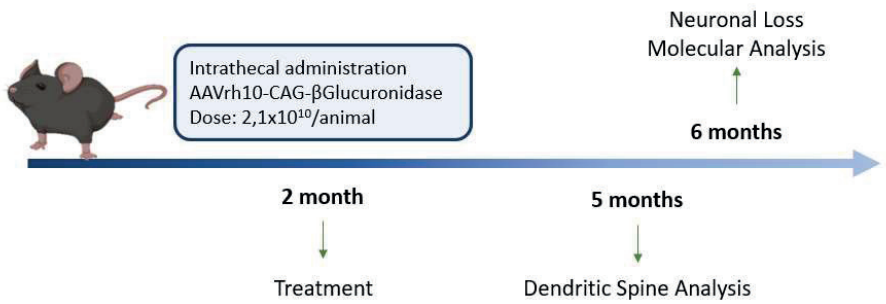

Figure 14: Experimental procedure

4.2. Anesthesia and euthanasia

Animals were anesthetized by different means depending on the procedure we wanted to perform. Before intrathecal administration, mice were anesthetized using an isofluorane (ISOFLO®, Ecuphar, Barcelona, Spain) vaporizer. The maintenance level of isofluorane oscillated from 2% for WT mice and 2,5% for GUSB animals but 4% of isoflurane was enough for all animals to induce general anesthesia. For perfusion, an intraperitoneal injection of mixed ketamine (Imalgene, Merial Laboratorios, Tarragona, Spain) and xylazine (Rompun, Bayer) was used. Both components were diluted in phosphate-buffered saline (PBS) to obtain a concentration of 100 mg/kg of ketamine and 10 mg/kg of xylazine. Higher doses of ketamine/xylazine had to be administered to GUSB animals in comparison to WT mice to obtain the same level of anesthesia.

Different methods of euthanasia were also performed depending on the specific use for the samples obtained. For fresh tissue samples euthanasia was done by decapitation after brief isoflurane inhalation to ensure a painless procedure. Samples were once obtained, frozen into liquid nitrogen and stored at -80 ºC. Perfusion with 4% paraformaldehyde (PFA, Sigma-Aldrich) was the method chosen to obtain fixed tissue samples. Firstly, the animals were anesthetized as previously stated and then the thoracic cavity was opened to expose the heart. Then a small cut was done in a lobule of the liver, and 10 mL of PBS followed by 20-50mL PFA were perfused through the left cardiac ventricle.

4.3. Intrathecal administration

To perform intrathecal administration, mice were first anesthetized using isoflurane. To ensure deep sedation, mouse reflexes were checking prior to the start of the procedure and just before administration. The back of the animals was shaved, and a 1,5cm long incision was done cutting the skin to expose the lumbar muscles. Using a binocular loupe, the epaxial muscles situated left of the column were separated to allow visualization and access to the intervertebral foramen between lumbar vertebrae L3 and L4. Ten µL

of AAV vector (diluted in PBS as necessary) were then slowly delivered into the intervertebral foramen, using a Hamilton syringe (Hamilton Company, Bonaduz, GR, Switzerland). The needle was directed to the brain of the mice and situated longitudinal to the spinal cord. Only 3-5 mm of the needle were introduced to prevent injury of the spinal cord, although an innocuous piercing of the outer meninges could be corroborated by a flick reflex in the tail of the animals.

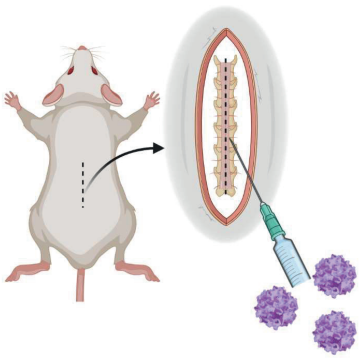

Figure 15 Schematic Representation of Intrathecal administration protocol.

To prevent efflux to peripheral tissues the needle was removed 20 seconds after the end of the delivery. Finally, the epaxial muscles were sutured using absorbable suture (Safil®, B.Braun, Rubí, Spain) and the skin was closed with Michel suture clips (Fine Science Tools GmbH, Heidelberg, Germany). Mice when then taken out of the isoflurane vaporizer and monitored until completely recovered from anesthesia.

4.4. Protein extraction

Brain samples were dissected into hippocampus (HC) and cortex (CX) samples. Directly after dissection or after being stored at -80 ºC, the samples were then processed by different methods depending on the subsequent analysis. Then protein concentration of all samples was determined by Pierce™ BCA Assay (ThermoFisher). All samples were stored at -20 ºC prior to analysis.

4.4.1. Total extracts

To obtain total protein extracts 300 to 500 µl of RIPA lysis buffer with Tergitol NP-40 (Sigma-Aldrich) and Protease Inhibitor Cocktail (Merck) was added depending on sample type and weight of the specific tissue. Samples were then sonicated 2 to 3 times for 10 seconds and homogenized. To separate cellular debris the homogenates were then centrifuged for 10 minutes at 4000g and the supernatant was collected.

RIPA lysis buffer: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA (Affymetrix, Santa Clara, CA, USA), 1% Tergitol type NP-40, 0.25% sodium deoxycholate (Sigma-Aldrich), 50 mM sodium fluoride (Sigma-Aldrich), 1 mM sodium ortovanadate (Sigma-Aldrich), 10 mM β-glycerophosphate disodium salt hydrate (Sigma-Aldrich), 5 mM sodium pirophosphate decahydrate (Sigma-Aldrich) and a protease inhibitor cocktail.

4.4.2. Synaptic fraction

For the purification of synaptic protein, the Syn-PER™ Synaptic Protein Extraction Reagent (Thermo Scientific™) was used following manufacturer's instructions. Briefly, samples were homogenized in Syn-PER reagent Protease with Inhibitor Cocktail (Merck).

Homogenates were centrifuged at 1200g for 10 minutes at 4ºC, and supernatant was transferred to another tube. To obtain the synaptosome pellet the supernatant was centrifuged for 20 minutes at 15000g. The subsequent supernatant containing the cytosolic fraction was transferred to another tube and the synaptosome pellet was resuspended in Syn-PER Reagent.

4.4.3. SPM and PSD fraction

Obtention of the pre-and post-synaptic fractions from brain samples was performed in collaboration with Alfredo Jesús Miñano, from the Institute of Neurosciences (INc). This technique allows to obtain very low protein

concentrations which is why only cortex samples but not hippocampus were used.

Cortex samples were homogenized in 600µL HEPES-Sucrose Buffer in a homogenizer for 25 seconds. The homogenates were then centrifuged at 1000xg for 10 minutes at 4ºC to separate nuclei and large debris in the pellet, which will be resuspended in 200 µL of HEPES-buffered sucrose.

The supernatant was centrifuged at 10000xg for 15 minutes at 4ºC to obtain the synaptosome fraction (pellet). A washed synaptosome fraction was obtained by washing the pellet in 1mL HEPES-Sucrose Buffer and centrifuging it in the same conditions again. The resulting pellet was lysed with the homogenizer for 10-15 seconds in 300µL of HEPES 4mM pH 7.4 and centrifuged at 25000xg for 20 minutes at 4ºC to pellet the synaptosome membrane fraction.

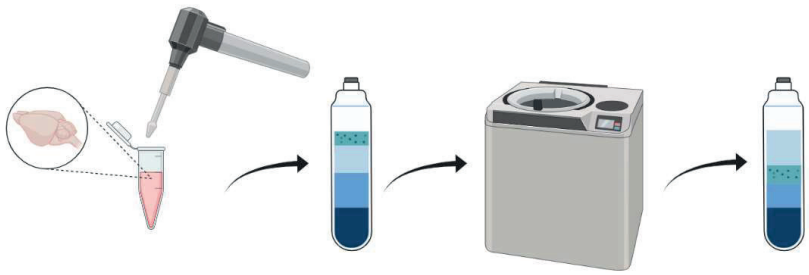

Figure 16 Schematic representation of the pre- and post-synaptic fraction separation protocol.

The resulting pellet was resuspended in 1mL of HEPES-Sucrose Buffer and loaded on a discontinuous sucrose gradient with 1,25mL of each 1.2M, 1M and 0,8M sucrose. After that, the gradient was centrifuged for 2h at 150000xg at 4ºC using a mini ultracentrifuge with a swinging bucket rotor. The PSD/SPM fraction could be found in the middle fractions as shown in figure 17.

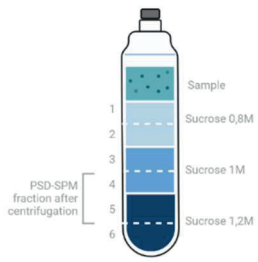

Figure 17: Sucrose gradient for SPM and PSD fractionation.

Therefore, 1mL of intermediate fraction was taken, diluted in 2,5mL of HEPES 4mM pH 7.4 and centrifuged at 150000xg for 30 minutes at 4ºC. The pellet was then resuspended in 200µL HEPES-EDTA solution and lysed for 15 minutes at 4ºC with occasional vortex. Finally, the lysed fraction was centrifuged at 32000xd for 20 minutes at 4ºC. The supernatant corresponded to the SPM fraction, and the pellet, the PSD fraction was resuspended in 60µL HEPES-EDTA solution. All samples were then stored at -20ºC.

HEPES 4mM Buffer: 13,96 mL H_2O, 600µL HPES 100mM pH 7.4, 150 µL Protease Inhibitor, 150 µL Phosphatase Inhibitor, 15µL NaF, 7,5µL NAPPi, 1,5µL Glycerophosphate

HEPES-Sucrose Buffer: 9,13mL H_2O, 600µL HPES 100mM pH 7.4, 4,8mL Sucrose 1M, 150µL Protease Inhibitor, 150µL Phosphatase Inhibitor, 150 µL sodium orthovanadate, 15µL NaF, 7,5µL NAPPi, 1,5µL Glycerophosphate

HEPES-EDTA Solution: 426µL H_2O,500µL HEPES 100mM pH 7.4, 4 µL EDTA 0,5M, 50µL Triton X-100 10%

Sucrose Gradient

Sucrose 0,8M: 4mL Sucrose 1M, 900µL H_2O, 50µL Protease Inhibitor, 50µL Phosphatase Inhibitor

Sucrose 1M: 5mL Sucrose 1M, 900µL H_2O, 50µL Protease Inhibitor, 50µL Phosphatase Inhibitor

Sucrose 1,2M: 3mL Sucrose 2M, 1,9mL H_2O, 50µL Protease Inhibitor, 50µL Phosphatase Inhibitor

4.4.4. Cellular fractioning

To separate the nuclear and cytosolic fractions, we used a simplified cellular fractionation protocol.

First, cells were harvested and resuspended in ice-cold PBS followed by centrifugation at 1000 xg for 5 minutes at 4 ºC. The supernatant was discarded, the pellet was resuspended in hypotonic buffer containing 0,1% NP-40 and the cell suspension was passed through a 27-gauge needle until all cells seemed lysed. The samples were then centrifuged at 1000 xg at 4ºC for 5 minutes to separate de nuclei (pellet) and the cytoplasm (supernatant).

The nuclei were then resuspended in RIPA buffer and the cytosolic fraction was centrifuged at 14000 xg at 4ºC for 3 minutes to pellet debris. The resulting supernatant and the nuclei were stored at -20ºC.

Hypotonic buffer: 20 mM Tris-HCl (pH 7.4), 10 mM KCl, 2 mM MgCl2, 1 mM EDTA and a protease inhibitor cocktail

Isotonic buffer: 20 mM Tris-HCl (pH 7.4), 150 mM KCl, 2 mM MgCl2, 1 mM EDTA and a protease inhibitor cocktail

4.5. Western Blot

Protein samples were first diluted to the same concentration and loading buffer was added prior to denaturation at 94 ºC for 5 minutes. Equal quantity of protein per sample (10 to 20 µg) was then loaded in a 10-12% SDS-polyacrylamide gel and separated by electrophoresis. Transference to an Amersham™ Hybond™ P 0.45 PVDF (polyvinylidene fluoride) blotting membrane (GE Healthcare, Chicago, IL, US) was performed using semidry electrotransfer system (Trans-Blot® Turbo™, Bio-Rad). Ponceau staining was used to confirm successful transference into the membrane and loading of equal amount of protein for each sample.

Membranes were then blocked for 1 hour at room temperature with tris-buffered saline + tween-20 (TBS-T) + 5% BSA to prevent unspecific binding of the antibodies and incubated overnight with primary antibody at 4-8 ºC with agitation. Then membranes were rinsed with TBST-T and incubated for 1 hour at room temperature with the secondary antibody, conjugated to horseradish peroxidase (HRP). Finally, after rinsing with TBST to remove unbounded antibody, membranes were washed with TBS to eliminate the detergent.

Detection of immunoreactive proteins was done using Westar ETA C Ultra 2.0 ECL substrate (Cyanagen) and the ChemiDoc MP Imaging System (Bio-Rad). Protein bands were quantified using the Image Lab software (Bio-Rad) and normalized by a housekeeping protein.

Antibody	Host	Dilution	Source
C1qA (ab155052)	Rabbit	1/500	Abcam
C3 (sc-28294)	Mouse	1/1000	Santa Cruz Biotechnology
GFAP (G3893)	Mouse	1/2000	Sigma (Merck)
Iba1 (ab5076)	Goat	1/1000	Abcam
Glutamate Receptor 1 (ab31232)	Rabbit	1/1000	Abcam
Gad67 (ab213508)	Rabbit	1/1000	Abcam
SNAP25 (GTX113839)	Rabbit	1/1000	GeneTex
pAKT (9271S)	Rabbit	1/1000	Cell Signaling
AKT (9272)	Rabbit	1/1000	Cell Signaling
pERK (9101)	Rabbit	1/1000	Cell Signaling
ERK (9102)	Rabbit	1/1000	Cell Signaling
Calbindin (ab11426)	Rabbit	1/1000	Abcam

Beclin1 (3738)	Rabbit	1/1000	Cell Signaling
α-Synuclein (610787)	Mouse	1/1000	BD
P62/SQSTM1 (P0067)	Rabbit	1/1000	Sigma (Merck)
Keap1 (10503-2-AP)	Rabbit	1/1000	Antibodybcn
GSTM1 (12412-1-AP)	Rabbit	1/1000	Proteintech
NfKB (ab16502)	Rabbit	1/1000	Abcam
Nrf2 (ab62352)	Rabbit	1/1000	Abcam
Actin (A-2066)	Rabbit	1/5000	Sigma-Aldrich
Tubulin ß3 (Tuj) (T2200)	Rabbit	1/1000	Sigma-Aldrich
GAPDH (14C10) (2118)	Rabbit	1/1000	Cell Signaling
NCAM (AB5032)	Rabbit	1/1000	Millipore
Homer1 (GTX103278)	Rabbit	1/1000	GeneTex
Dopamine D2 Receptor (AB5084P)	Rabbit	1/1000	Millipore
HRP-coupled anti-rabbit (P0399)	Swine	1/10000	Dako (Agilent)
HRP–coupled anti-mouse (31430)	Goat	1/10000	ThermoFisher
HRP-coupled anti-goat (P0160)	Rabbit	1/10000	Dako (Agilent)

Stacking gel: 3.9% Acryl/Bis 29:1 40% w/v solution (VWR, Radnor, PA, Canada), 0.5 M Tris-HCl pH 6.8, 0.4% SDS. Gel polymerization: 25 µL ammonium persulfate (VWR) and 5 µL of TEMED (Sigma-Aldrich).

Resolving gel: 10-12% Acryl/Bis 29:1, 1.5 M Tris-HCl pH 8.8, 0.4% SDS. Gel polymerization: 60 µL ammonium persulfate and 12 µL of TEMED.

6x Loading buffer: 0.35 M Tris-HCl pH 6.8, 3.3% glicerol (Sigma-Aldrich), 10% SDS (Affymetrix), 0.015% bromophenol blue (Sigma-Aldrich), 0.6M DL-dithiothreitol (Sigma-Aldrich).

Electrophoresis buffer: 25 mM Tris, 192 mM Glycine (Sigma-Aldrich), 1% SDS.

Transfer buffer: 25 mM Tris, 192 mM Glycine, 20% methanol.

TBS-T: 50 mM Tris, 136 mM NaCl, 40 mM KCl, 0.1% Tween-20 (Sigma-Aldrich), pH 7.4.

4.6. Histology

4.6.1. Immunohistochemistry

Anesthetized animals were perfused with 4% PFA-PBS buffer. Brain samples were post-fixated 24 to 48h in 4%PFA, cryoprotected with 30% sucrose and embedded in Tissue-Tek OCT compound (Sakura, Alphen aan den Rijn, Netherlands). Samples were then processed using a cryostat to obtain 12-µm-thick sections and mounted in Thermo Scientific™ SuperFrost Plus© Microscope Slides. For free-floating immunohistochemistry, 40-µm-thick sections were obtained and kept in PBS buffer in a 24 well plate and stored at -20 ºC.

Slides were warmed for 10 minutes at 37 ºC and hydrated with Tris 0,05M (Trizma® base, Sigma-Aldrich). Permeabilization was done with methanol (Panreac) followed by a 5-minute wash with Tris and a 10-minute incubation with Tris 0,05M + 0,3% Triton X-100 (sigma-Aldrich). Samples were then blocked for 1 hour at room temperature using TRIS 0.05M (Trizma® base, Sigma-Aldrich) + 0.3% Triton X-100 (Sigma-Aldrich) + 3% BSA (Sigma-Aldrich) + 5% FBS and incubated overnight at 4-8 ºC with primary antibody. Then sections were rinsed with Tris 0,05M + 0,05% Tween 20 (Sigma-Aldrich) 3 times for 10 minutes and incubated with secondary antibody for 1 hour at room temperature. After rinsing, Hoechst (Sigma-Aldrich) was used to counterstain cellular nuclei. Free-floating sections were then transferred to microscope slides, mounted using Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich) and let dry before storing them at -20 ºC.

Laser confocal microscope ZEISS LSM 700 was used to detect fluorescent signal and obtain snaps or Z-stacks of the areas of interest. Images were then analyzed using either ImageJ-Fiji or IMARIS software followed by a manual correction when necessary.

Antibody	Host	Dilution	Source
NeuN (ABN91)	Chicken	1/200	Sigma (Merck)
GFAP (G3893)	Mouse	1/500	Sigma (Merck)
Iba1 (ab5076)	Goat	1/200	Abcam
Calbindin (ab11426)	Rabbit	1/200	Abcam
Lamp1 (553792)	Rat	1/200	BD
Alexa Fluor 568 Goat anti-Chicken (A-11041)	Goat	1/400	Thermofisher
CY-3 conjugated AffiniPure donkey anti rabbit (711-165-152)	Rabbit	1/5000	Jackson ImmunoResearch
Alexa Fluor 568 Donkey anti-goat (A11057)	Donkey	1/200	Molecular Probes
Alexa Fluor 568 Goat Anti-Rat (A11077)	Goat	1/400	Molecular Probes

4.6.2. Immunocytochemistry

Cells were fixed using ice cold 2% PFA for 15 minutes and then rinsed with PBS. A 10-minute incubation with PBS- 0,3% Triton X-100Tween was done for permeabilization followed by blocking at room temperature for 1 hour with PBS + 0.3% Triton X-100 + 3% BSA + 5% FBS for blocking. Samples were then incubated overnight with primary antibody at 4-8 ºC, washed with PBS and incubated in the dark for 1h at room temperature with the secondary antibody, conjugated to different fluorophores. After rinsing Hoechst was used for counterstaining and slides were mounted with FluoromountTM.

Antibody	Host	Dilution	Source
Lamp1 (553792)	Rat	1/200	BD
P62/SQSTM1 (P0067)	Rabbit	1/200	Sigma (Merck)
α-Synuclein (610787)	Mouse	1/200	BD
Alexa Fluor 568 Goat Anti-Rat (A11077)	Goat	1/400	Molecular Probes
Alexa Fluor 488 Donkey Anti-Rabbit (A11077)	Donkey	1/400	Molecular Probes
Alexa Fluor 488 Chicken anti-Mouse (A21200)	Chicken	1/400	Thermofisher

4.6.3. Gene Gun

WT and MPS VII mice at 5 months of age were perfused with 4% PFA-PBS Buffer. Brains were quickly removed and rinsed with PBS. Later, both hemispheres were separated and 200-µm thick sections were obtained using a vibratome. Helios® Gene Gun System (Biorad) was used to deliver fluorescent dye directly to the hippocampus and entorhinal cortex. The experiments were performed at the IDIBAPS in collaboration with Silvia Ginés and Veronica Brito. Then the sections were rinsed with PBS, transferred to Thermo Scientific™ SuperFrost Plus© slides and mounted with Floromount™.

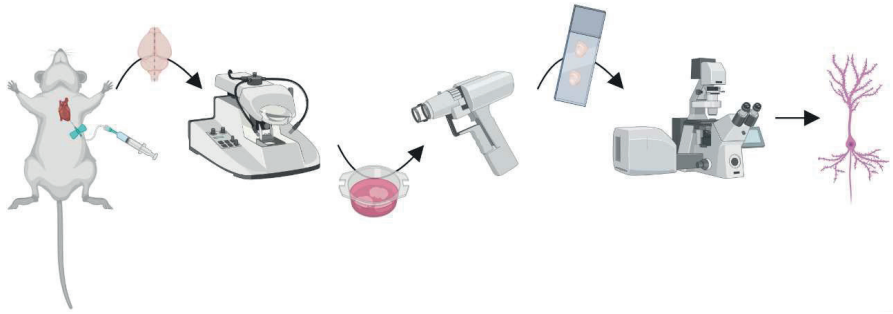

Figure 18 Schematic representation of the Gene Gun Diolostic staining protocol.

Fluorescent images were obtained using a Leica SP5 laser scanning confocal microscope with a ×63 objective. Dendritic segments from hippocampal CA1 and CA3 pyramidal neurons and from cortical pyramidal neurons from entorhinal cortex were selected for analysis. Spine density was manually analyzed using the ImageJ Plugin Cell Counter.

4.6.4. Golgi-Cox staining

For Golgi-Cox staining the Rapid Golgistain Kit (FD Neurotechnologies) was used following the manufacturer's instructions. Briefly, 5-month-old WT and MPS VII mice were killed by beheading, brains were removed and both hemispheres were separated. The samples were individually rinsed with PBS and incubated in the dark in a mix of solution A/B which had been prepared at least 24h in advance. The A/B solution was replaced the next day and incubated for 2 weeks, gently swirling the container every 3-4 days. Afterwards, brain hemispheres were submerged in solution C for 3 days at room temperature and processed using a vibratome to obtain 200-µm thick sections.

Later, sections were transferred to gelatin-coated slides, and rinsed with distilled water. Staining was performed by incubating the slides for 10 minutes with solution D/E, followed by another wash with distilled water. Tissue was then dehydrated by sequential 4-minute rinses of 50%, 75% and

95% ethanol and 4 incubations with 100% ethanol. Clearing was done by incubating the slides 3 times in xylene for 4 minutes. Finally, sections mounted with DPX and stored at room temperature and protected from light.

Bright field images were obtained using a Leica SP5 laser scanning confocal microscope with a ×63 objective. The areas analyzed and the methodology used were the same as in the Gene-Gun.

4.7. β-Glucuronidase Activity assay

Tail clips are cut in smaller sections, submerged in 150µL of lysis buffer and homogenized briefly by either manual disaggregation using a grinder or 5-second sonication (amplitude 40). Then samples are centrifuged at 4ºC for 10 minutes at 14000rpms to separate the remaining tissue. Protein concentration was then quantified using the BCA method to ensure equal amounts of protein were used for all samples in the activity assay. Dilution of the samples was done in lysis buffer.

In a 96 well opaque plate 10µL of each sample were incubated for 1 hour at 37ºC with Glucuronide (Sigma-Aldrich), the substrate of the β-Glucuronidase. After that, 200µL of activity stopping buffer were added and the absorbance was measured using the TECAN Spark spectrophotometer plate reader**.**

Lysis buffer: 20 mM Tris-HCl pH 7.4, 7,5 mM NaCl. pH should be adjusted to 7,5 using 1M NaOH

Activity stopping buffer: 200mM Na_2CO_3. Should be filtered before use.

4.8. Primary cultures

Sterilization of all surfaces and materials used should be performed to avoid contamination of the cultures. All solutions must be filtered before use. Seeding 24-well plates are incubated with poly-D-lysine for at least 2 hours before seeding of the cells.

To obtain E15 mice, a female pregnant mouse is euthanized by cervical dislocation and the uterus containing the embryos is carefully removed from the abdominal cavity and deposited in a PBS filled plate. The gestational sac is removed, each fetus is decapitated, and the heads are transferred to another plate with clean PBS. Using a stereoscope, the brain is removed from the skull and the cortex dissected from the rest of the brain. Careful removal of the meninges, striatum and thalamus is important to ensure integrity of the cortical neuron culture.

Cortex is then transferred using a Pasteur pipette to a 50mL falcon tube containing solution A. The tube is then briefly centrifuged (30") at 1500rpm and the supernatant is removed by aspiration. Solution B is quickly added, and the samples are incubated for 10' at 37 ºC with occasional mixing to improve tissue dissociation. After that, solution D is added before brief centrifugation at 1500rpm. Supernatant is then discarded and 2-3mL of solution C are added. A Pasteur pipette is then used to further dissociate the cells, which are then passed through a sterile cloth, homogenized again with the same pipette and transferred to a falcon tube containing solution E. Samples are then centrifuged 5 minutes at 1000rpm and the supernatant aspirated. After that, the pellet is resuspended in seeding medium and cells are counted using a 10 µL aliquot, Trypan Blue and the Neubauer chamber.

Cells are diluted to a concentration between 50000 and 300000 cells/mL and transferred to the seeding cells after a rinse with PBS 1X. Plates should be stored at 5% of CO_2 and 37 ºC. Two to four hours after seeding, the medium is changed to neurobasal. For maintenance of the cultures half of the medium should be removed every 3 to 4 days and fresh medium added. Cultures can be used after 7 to 10 divisions.

Krebs-Ringer Buffer 10X (KRB): 70.7g/L NaCl, 3.6g/L KCl, 1.66g/L KH_2PO_4, 21.4g $NaHCO_3$, 25.7g/L Glucose

Magnesium Stock solution: 3.8% $MgSO_4 \cdot 7H_2O$

Calcium Stock solution: 1.2% $CaCl_2 \cdot 2H_2O$

Solution A: 50 ml KRB 1X, 0.15g Bovine Serum Albumin (BSA), 0.4mL Magnesium Stock solution

Solution B: 10mL Solution A, 2.5g Trypsin

Solution C: 10mL Solution A, 0.8mg DNase, 5.2mg Trypsin inhibitor, 0.1mL Magnesium Stock solution

Solution D: 8.4mL Solution A, 1.6mL Solution C

Solution E: 5mL Solution A, 40µL Magnesium Stock solution, 6µL Calcium Stock solution

Seeding medium: DMEM (Life technologies), 1% Penicillin/Streptomycin (P/S) (Life technologies), 10% Fetal Bovine Serum (FBS)

Trypan Blue: 4.25g/mL NaCl 0.72M, 2 mg/mL Trypan Blue 0.2%. A 1:4 ratio should be used for mixing.

Maintenance Media: Neurobasal (Life technologies), 2% B27 (Life technologies), 1% Glutamax (Life technologies), 0.5% P/S

4.9. Cell culture

All the experiments in this project have been performed using the SH-SY5Y cell line, a neuroblastoma cell line presenting both floating and adherent cells. Since floating cells are mostly discarded during media changes, the adherent cells have been the population analyzed in this research. Moreover, adherent cells cand exhibit two morphological phenotypes generating a cellular population presenting both neuroblast-like and epithelial-like cells. Since increasing passage number leads to the loss of the neuronal-like features of SH-SY5Y cells (Kovalevich et al., 2021), cultures were continuously discarded, and low-passage cells used for further experiments.

4.9.1. Maintenance

SH-SY5Y cells were cultured in DMEM/F12 Medium (Invitrogen) with a 15% FBS, 1% of P/S and 1% of Non-essential amino acids (Sigma, M-7145).

To unfreeze cells, vials were rapidly thawed, and 7 mL of media was added. Then cells were centrifuged 5 minutes at 1000rpm, the supernatant aspirated to eliminate all the DMSO and resuspended in 1mL of growth media. Cells were then seeded in a 10cm plate containing 8mL of media.

For trypsinization, cells were rinsed two times with sterile PBS then incubated at 37 ºC for 2-3 minutes with 2,5mL of Trypsin-EDTA 1X (HyClone). Trypsinization was stopped by adding 7,5 mL of growth media, and cells were centrifuged 5 minutes at 1000rpm. Supernatant was then aspirated, and the cells were resuspended at the desired dilution.

4.9.2. Nucleofection

To obtain efficient transfection of large sized plasmids like those used in CRISPR/Cas9 genome editing in difficult to transfect cell lines like SH-SY5Y, electroporation or nucleofection are the only realistic options(Sondergaard et al., 2020).

We used the pSpCas9 (BB)-2A-Puro V2.0 (PX459; Addgene plasmid ID: 62988) plasmid (Ran et al., 2013) to provide all the required elements for gene editing and selection of transfected cells.

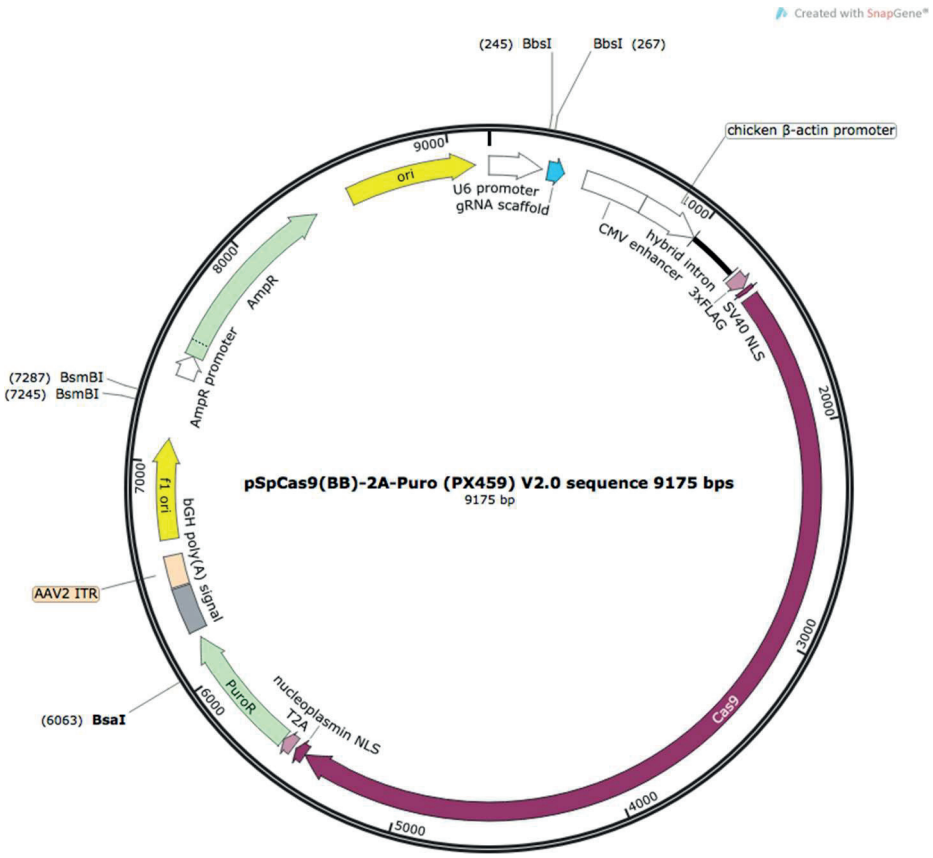

Figure 19: Map Image for pSpCas9(BB)-2A-Puro (PX459) V2.0. https://www.addgene.org/62988/

Cell cultures at approximately 75% confluence were trypsinized and dissociated. Cellular density was then measured using a Neubauer chamber to isolate fractions containing 106 cells. After centrifugation, cells were resuspended in 100µL of an electroporation solution in sterile 4mm nucleofection cuvettes (Lonza) where they were mixed with the CRISPR/Cas9 plasmid (PX459-derived construct). Cuvettes were introduced in an Amaxa™Nucleofector™II(Lonza) and an electric pulse was applied to the cells after selecting a suitable program. Then 500µL of DMEM/F12 medium was added to the cuvettes and the whole volume was transferred to 6-well plates.

To analyze the effects of nucleofection on cell viability, untransfected cells and cells nucleofected without plasmid DNA were used as negative controls.

Optimization of the nucleofection protocol was done using pCAG-GFP-WPRE-B11 (Unitat de Producció de Vectors - UAB) (7801 bp) and both Ingenio® Electroporation Solution (IES) (Mirus Bio) and Krebs-Ringer Solution were tested.

Krebs-Ringer Solution: 12.1 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 25.5 mM NaHCO3, 14.3 mM glucose

4.9.3. Puromycin Selection

The PX459-derived construct used in nucleofection contained a puromycin resistance the facilitate the detection of transfected cells. Since this plasmid does not integrate in the genome of host cells the resistance to puromycin is transient and selection must be performed before transfected cells start mitosis and the plasmid is diluted and lost.

Selection of transfected SH-SY5Y cells was done 24 hours after nucleofection using DMEM/F-12 with 2-3µg/mL of puromycin (InvivoGen). When mortality of the nucleofection negative controls reached 9-100% selective medium was replaced with maintenance medium.

4.9.4. Single-cell isolation and expansion of clonal populations

After selection, single cell isolation was performed to generate clonal lines. Transfected cells were trypsinized, dissociated and cellular density was measured using the Neubauer chamber. Cells were then diluted to a concentration of 0.5-1 cells/100µL, and 150µL of the diluted cell suspension was transferred into 96-well plates using a multichannel pipette. During the first 24h after seeding it is crucial to observe each seeded well under microscope to discard wells presenting more than one cell. However,

detection of wells presenting more than one cell is not always possible during the first 24 hours and therefore, isolated clones must continue to be checked daily to see if a secondary cell colony is formed. Amplification of the colonies to 24-well plates was done several weeks after seeding once they had reached an appropriate diameter. Colony dissociation allows for multidirectional cell growth and therefore, by using trypsinization we promoted clonal expansion. Amplification was done again in 6-well plates and finally in 100 mm plates.

4.9.5. Differentiation

Firstly, 6 or 12-well plates were incubated for 2h at room temperature inside a flux cabin with a 1:50 collagen solution (diluted in sterile distilled water). After trypsinization, cells were seeded at a 20000 cells/mL concentration in DMEM/F12 medium.

Twenty-four hours later the medium was changed to Neurobasal (Gibco) +B27 (Gibco)+ 10µM All trans Retinoic Acid. After 72h media was replaced, and then again at 48h but this time without B27 to further promote differentiation.

4.10. Image analysis and signal quantification

For the study of neuronal density, IMARIS software was used. For hippocampus analysis, the width of the neuronal layer was measured manually at 20 different points of the region, starting from the end of the dentate gyrus. To count neurons in cortex images, a spot mask was created using a predicted neuronal size previously determined by measuring neurons of diverse orientations in the images and calculating the media value. This mask was applied at the red channel (NeuN staining) followed by a manual correction of the calculated number, due to limitations of the software.

Overlapping of images could lead to overcounting of neurons located in the immediate borders of each image, these neurons were removed from the masks result.

To measure signal intensity Image J Fiji software was used. When appropriate, the area of interest was selected, and the image was confirmed to be 16-bit. An equal threshold was then applied to all images to dismiss background signal and the following parameters were measured: Area, Integrated Density, Area Fraction, Mean Gray Value and Limit to Threshold. The results were expressed as IntDen/Area which represents the mean intensity of the stained area corrected by the total area selected.

4.11. Statistical analysis

Statistical analysis presented in this thesis were performed using GraphPad Prism 9 software. Data are expressed as mean ± SEM and statistical signification depicted in the graphs corresponds to * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

To analyze one dependent variable between two groups we used two-tailed Student's *t*-test. One-way ANOVA was used to analyze one dependent variable among three groups, using Tukey *post hoc* test for pairwise comparisons between groups.

5. Results

5.1. Generation and validation of a KO GUSB SH-SY5Y cell line

We generated a novel GUSB KO neuronal cell line using the CRISPR/Cas9 technology as an MPS VII *in vitro* model. This system introduces precise mutations in the genome using small RNAs to guide the activity of the endonuclease Cas9. To boost the targeting capability and editing efficiency of the CRISPR/Cas9 system several strategies have been successfully employed including the use of polycistronic tRNA-gRNA expression cassettes (Xie et al., 2015). Using this methodology, multiple gRNAs are synthesized in a single transcript, in which they are flanked by tRNAs. This transcript is then cleaved by endogenous RNases releasing the guide RNAs. The following construct was cloned in the PX459 plasmid as a guide for the gene editing of GUSB gene (Figure 20).

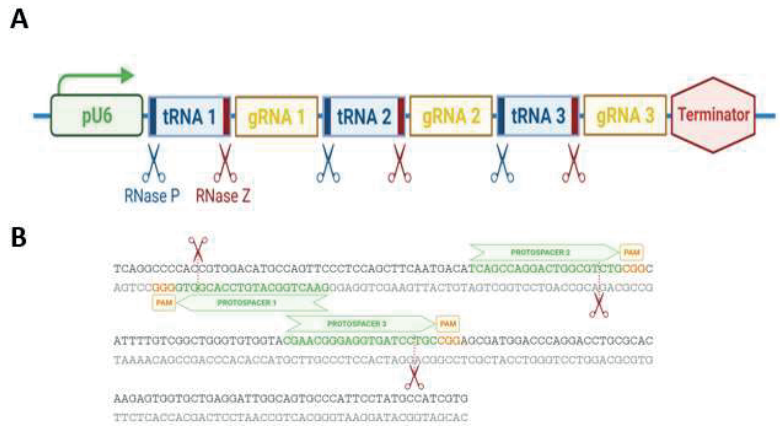

Figure 20: Schematic representation of the gRNA cassette used to generate the GUSB KO cell line. A) Polycistronic tRNA-gRNA expression cassette. Three gRNAs are synthesized in a single transcript, in which they are flanked by tRNAs. This transcript is cleaved by endogenous RNases P (expected cut sites in blue) and Z (red), thus releasing gRNAs. Note that genetic elements in the figure are not depicted in scale. B) Selected protospacers over exon 2 of GUSB. Protospacers are shown in green, PAMs in orange, and SpCas9 cut sites in red.

Optimization of the nucleofection in SH-SY5Y cell line was done using GFP to allow a quick analysis of transfection efficiency and was translated to the delivery of the plasmid containing the CRISPR/Cas9 system (PX459-PTG).

At 24 and 48 hours after transfection the protocol providing higher transfection efficiency and less cell mortality was the G004 protocol in combination with the Krebs-Ringer solution. Therefore, the same approach was employed with the PX459-PTG vector.

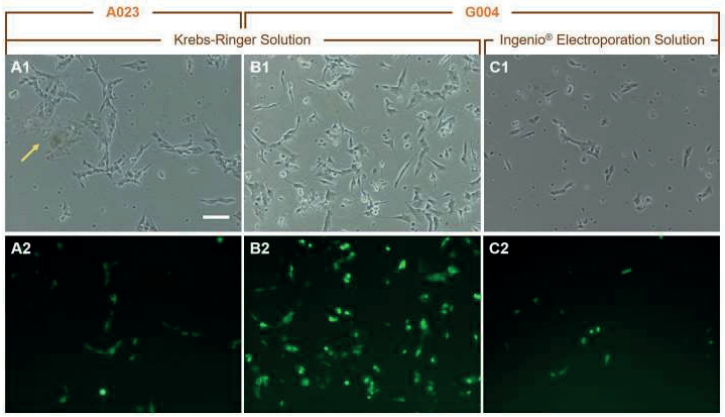

Figure 21: Optimizing nucleofection ofSH-SY5Ycells using pCAG-GFP-WPRE-B11. A1) Phase-contrast and A2) fluorescent image 24h after nucleofectionwiththeA023 program using KRS. Note that this program results in higher mortality rates, evident as the formation of multiple floating clumps of dead cells (yellow arrow). B1) Phase-contrast and B2) fluorescent image 24 h after nucleofection with the G004 program using KRS. C1) Phase-contrast and C2) fluorescent image 48 h after nucleofection withtheG004programusingIES.

Puromycin selection resulted in similar survival rates in non-transfected and transfected cells. But after removal of puromycin, only transfected cells were able to recover and continue growing whereas untransfected cells kept dying.

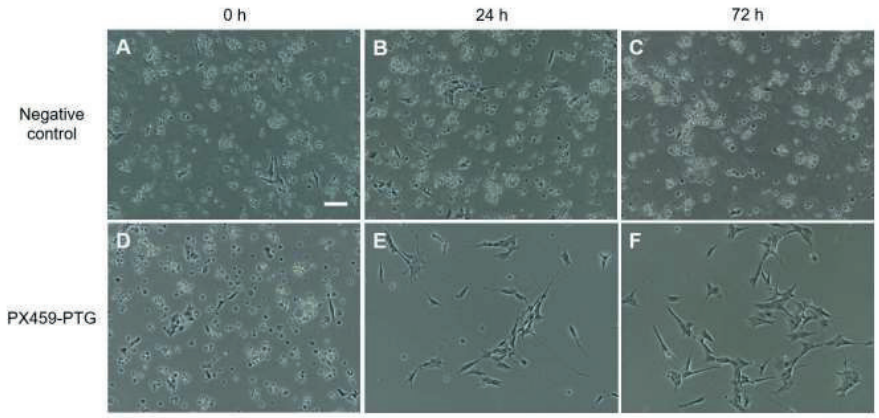

Figure 22: Effect of puromycin removal on Sh-SY5Y transfected and untrasnfected cells.

The expansion of isolated clonal populations was performed using conditioned medium from 50-70% confluent SH-SY5Y (Conde Guerri et al., 1989; Lo Furno et al., 2018).
To ensure that this cell line does not express β-Glucuronidase we first analyzed the β-Glucuronidase activity, comparing it to an SH-SY5Y cell line control.
As seen in Figure 1, the MPS VII cell line shows almost a complete loss of β-Glucuronidase function, retaining only a 1,25% of activity, and therefore can be used as a model for our studies.

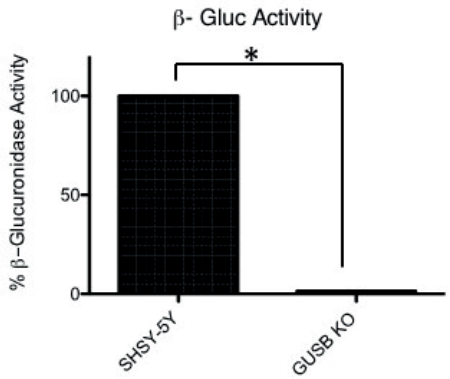

Figure 23: Percentage of β-glucuronidase activity measured by fluorometry in the SH-SY5Ycell line and the mutated cell line.

5.2. Quantification of neuronal loss

It is well stablished that hippocampus and entorhinal cortex play an important role in memory and cognition both in mice and in humans (Coutureau & Di Scala, 2009; Gerlei et al., 2021; Lisman et al., 2018; Rubin et al., 2014).

Although the mechanisms underlaying cognitive impairment in mucopolysaccharidosis type VII are not yet well understood, it is known that neuronal death takes place in the brain of mouse models for other mucopolysaccharidosis (Hamano et al., 2008; Pshezhetsky, 2015).

Taking that into consideration, we studied neuronal loss in some of the brain areas known to be more involved in cognition: the CA1 and CA3 of the hippocampus, the prefrontal cortex (CPF) and the entorhinal cortex (ECx) through the life of the animals at 1, 3 and 6 months of age.

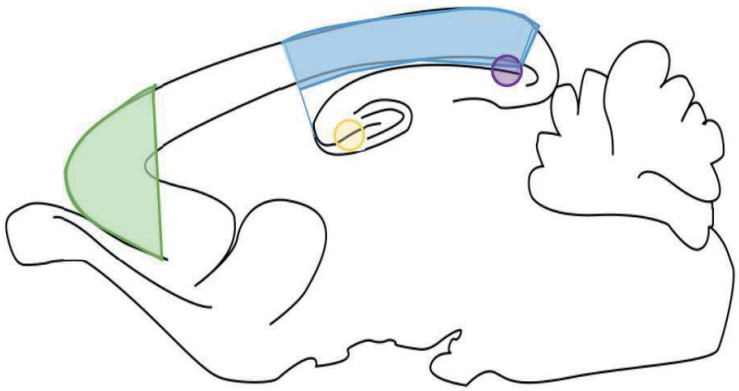

Figure 24: Brain scheme with analyzed regions delimited. PC (green) was delimited by the start of the olfactory bulb, and pictures were taken from that starting point until the end of the sample. For the EC (blue), the hippocampus was taken as reference, and a field was added at both sides. Two images were acquired to obtain information from all the width of the cortex. From the HC, two regions were analyzed: CA3 (yellow) and CA1(purple).

Neuronal density in both cortex areas was analyzed by immunodetection of the neuronal marker NeuN. For the analysis of the hippocampus, we analyzed the width of the neuronal layer to avoid errors in the quantification of the density due to overlapping of neurons in both studied areas.

We found different affectation depending on the age of the animal and the brain area analyzed. The prefrontal cortex and the CA1 region showed no differences between the MPS VII and the wild type (WT) mice at any age analyzed.

The width of the CA3 neuronal layer of 6-month-old GUSB animals was slightly thinner than in wild type animals. And the entorhinal cortex of the MPS VII animals exhibited a small decrease in neuronal density starting at 3 months of age and was more noticeable at 6 months.

Together, these results suggest that although some neuronal death might be taking place in the cortex and in the CA3 of the hippocampus of GUSB mice results were not statistically significant.

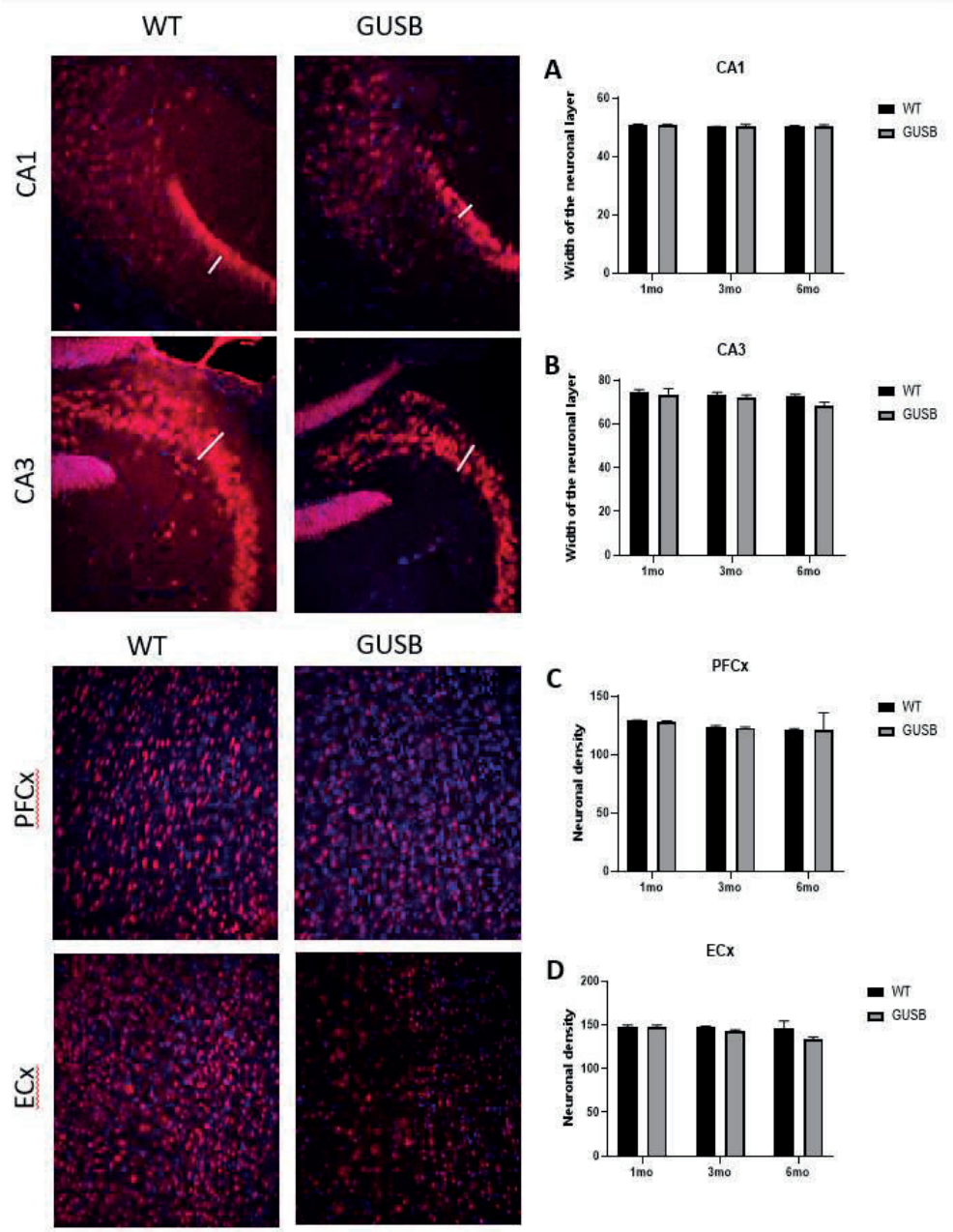

*Figure 25: Representative images of a WT (right) and a GUSB (left) mouse and graphical representation of neuronal density (MEAN±SEM) at the 3 ages studied of the A) CA1 B) CA3 in the hippocampus C) Prefrontal Cortex and D) Entorhinal Cortex region (WT/GUSB 1/3mo (n=3) , WT 6mo (n=**4**) , GUSB 6mo (**n=4**)).*

5.3. Dendritic spines analysis

In disorders with cognitive impairment showing no overt neuronal death, synaptic alterations have been proposed as the main driver of the pathology. Several mouse models of different mucopolysaccharidoses, including an iPSC model of MPS VII present abnormal localization and/or expression of synaptic proteins (Bayo-Puxan et al., 2018; Wilkinson et al., 2012). Moreover, synaptic impairment has also been described in diseases with neuronal loss, suggesting that synaptic loss or malfunction could be a process preceding neuronal death (Cardozo et al., 2019).

To study synaptic involvement in our mouse model, we first addressed the question whether animals at the late stages of their life presented abnormalities in the number of dendritic spines in either cortex or hippocampus. Multiple techniques have been developed to analyze neuronal and synaptic morphology with axonal and dendritic arborization. In this work we used Golgi-Cox and Gene-Gun staining because the reduced percentage of stained neurons and, in the case of Gene-Gun, the ability to only stain neurons in our regions of interest facilitated the obtention of good quality images with almost no overlapping of dendritic processes making the quantification easier and more reliable.

Using the Gene-Gun technique to obtain high-resolution images of dendrites in 5-month-old MPS VII mice and their littermate wild type controls we observed a significant decrease in spine density in the hippocampus and in the cortex, suggesting a possible factor causing the neurological symptoms of MPS VII.

This decrease, statistically significant in all regions, was found to be more exacerbated in the cortex, which correlated with the increased number of cell death described above.

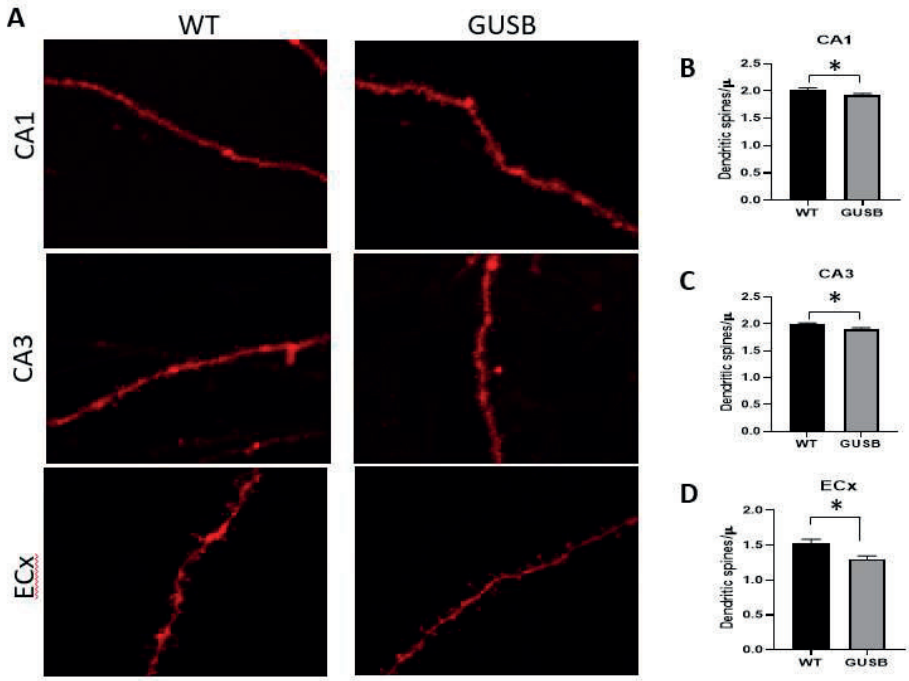

*Figure 26: A) Representative Images of neuron dendrites from a WT (right) and a GUSB (left) mouse and histogram showing the quantitative analysis of dendritic spine density (MEAN±SEM) of B) CA1 C) CA3 D) Entorhinal cortex (*p-value < 0.05 WT (n=4) , GUSB (n=5)).*

The decrease in synaptic density in the CA1, CA3 and ECx of GUSB animals was further confirmed by Golgi-Cox staining techniques. More importantly, in treated GUSB animals we found an increase in dendritic spine density when compared with untreated GUSB mice. These differences disappeared when compared with wild type animals. Thus, these results indicate that gene therapy can revert the synaptic loss found in MPS VII mice.

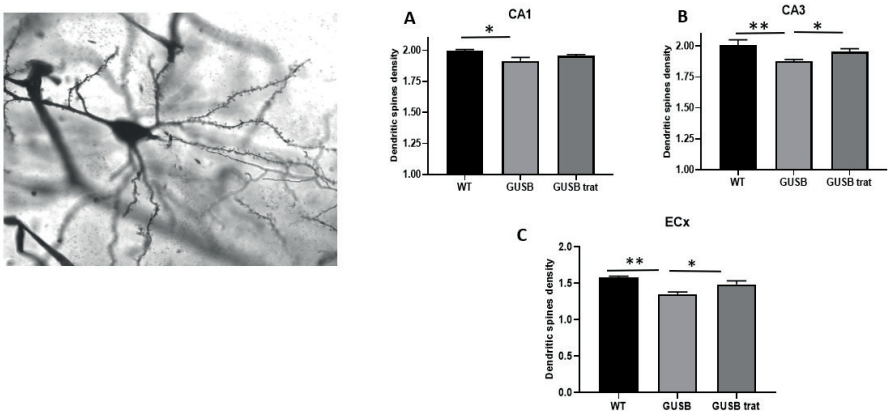

*Figure 27: Representative Image of a Golgi Cox-stained mouse cortex neuron and histograms showing the quantitative analysis of dendritic spine density (MEAN±SEM) of A) CA1 B) CA3 C) Entorhinal cortex (*p-value < 0.05 **p-value < 0.01 WT(n=4), GUSB (n=4), GUSB trat (**n=4**)).*

5.4. Primary cultures and cell line differentiation

Neuronal differentiation of iPSCs cells into neurons has been done with varying rates of success in different mucopolysaccharidoses, including MPS VII (Bayo-Puxan et al., 2018; Beneto et al., 2020; Lehmann et al., 2021). Taking this into consideration, we decided to analyze whether the GUSB KO cell line could be successfully differentiated.

Although it seemed that WT cells started the process faster, in the end there were no apparent differences between cell lines suggesting that both can reach a mature phenotype. However, a study of mature neuronal markers needs to be performed before conclusions can be reached.

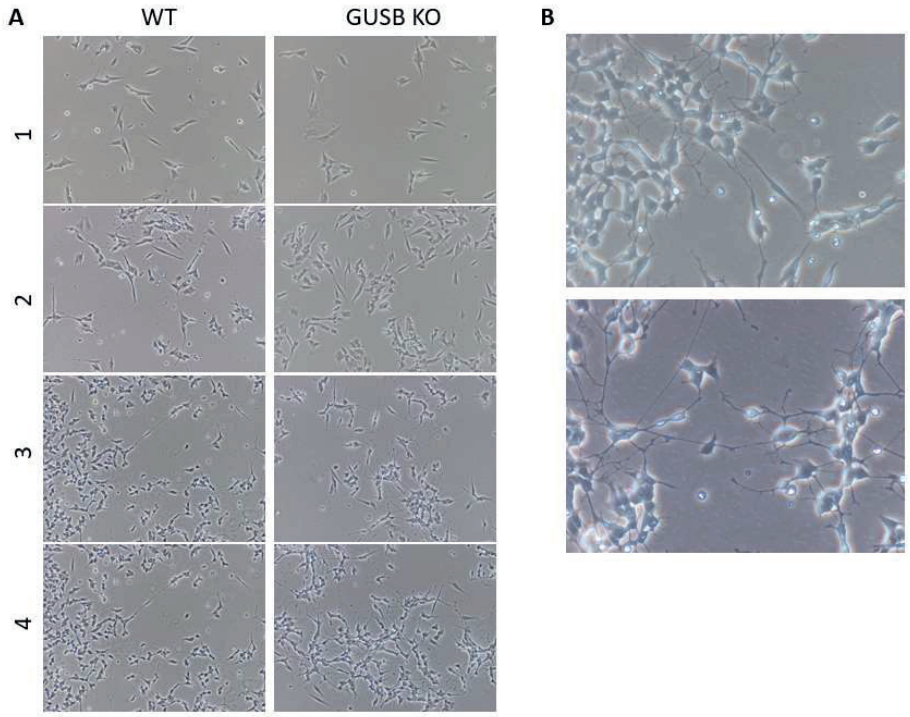

Figure 28: A) Representative images of SH-SY5Yand GUSB KO SH-SY5Ycell line cultures at different stages of differentiation. B) Higher magnification images of differentiated SH-SY5Y(Top) and GUSB KO SH-SY5Ycell cultures (Bottom).

We then analyzed primary cortical neurons and used β-Tubulin immunohistochemistry to mark all dendritic processes. As seen with the cell lines, the differentiation seemed to start faster in the WT cultures as evidenced by the longer dendritic processes at day 3. Then both cell cultures seemed to progress equally up to day 12. At that time, it is apparent that WT primary cultures develop into neurons with increased arborization when compared with their GUSB counterparts. This could correlate with our previous results that showed decreased dendritic spine density in GUSB mice, although more detailed studies need to be done.

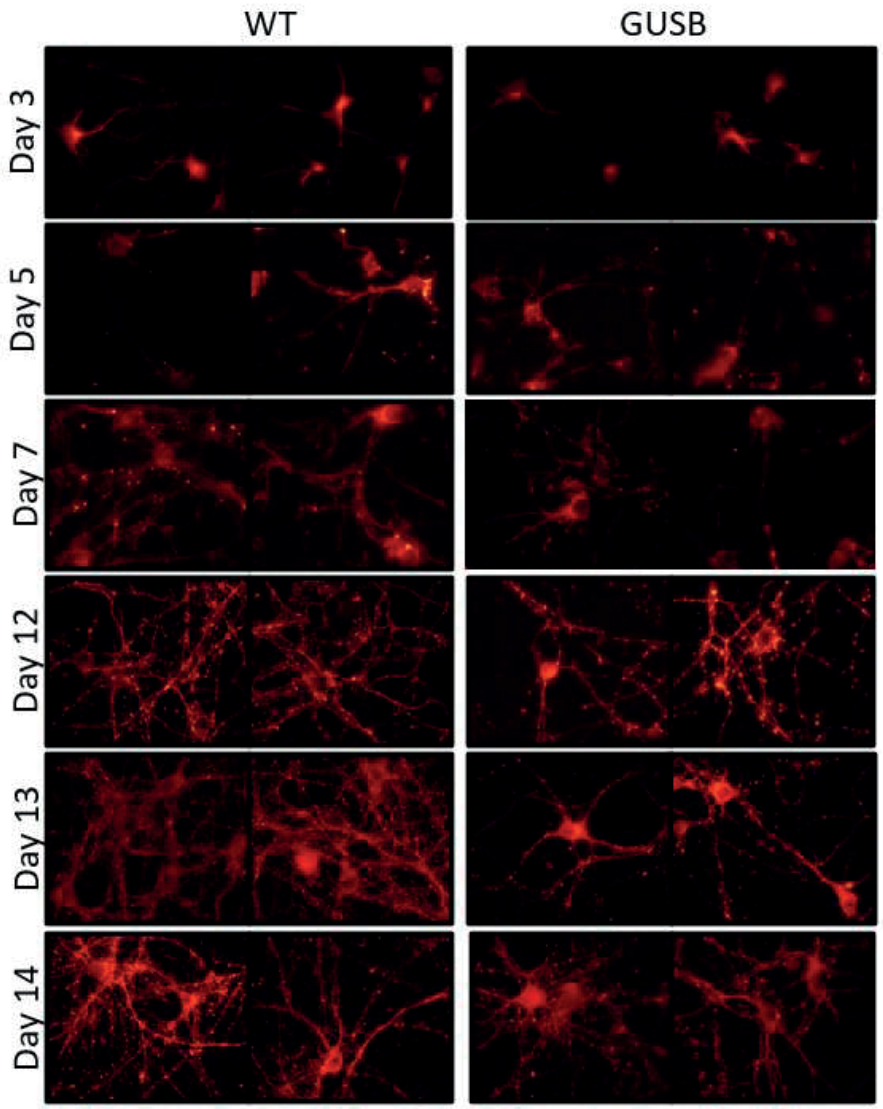

Figure 29: Representative images of β-Tubulin immunohistochemistry from cortical neuron primary cultures obtained from WT and GUSB mice at different stages of differentiation.

5.5. Study of neuronal function

To further unveil pathways involved in the MPS VII neuropathology, we studied several proteins fundamental for a correct neuronal function.

It has been described that glutamate receptors are involved in neurotoxicity and neurodegeneration (Lau & Tymianski, 2010). To study glutamate transmission we analysed the Glutamate Receptor 1 which mediates fast excitatory synaptic transmission. We used western blot to analyse protein levels in total extracts of mice cortex and hippocampus. There were no differences between WT and GUSB mice at neither 1 nor 3 months of age in the hippocampus. However, we could see a significant decrease in Glutamate Receptor 1 (GluR1) protein levels in the cortex of GUSB animals starting at 1 month of age. This decrease was maintained through all ages analysed being close to a 40% reduction at 6 months.

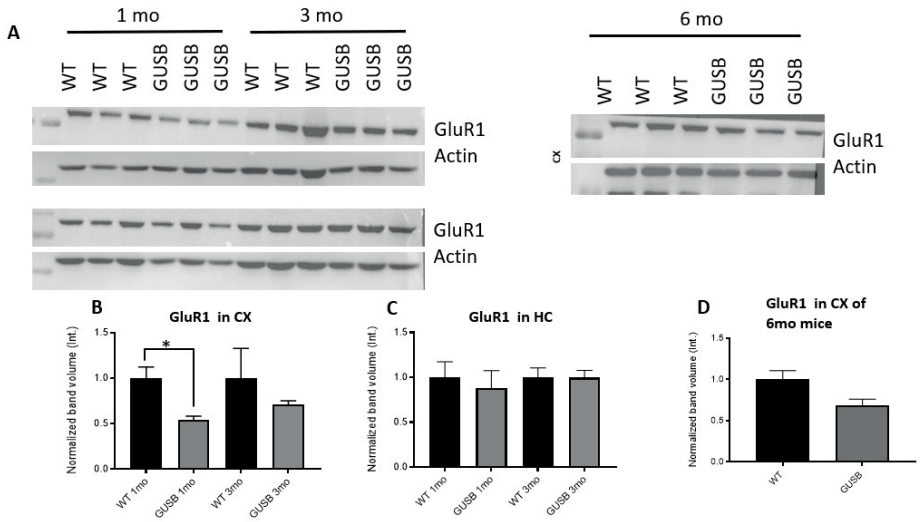

*Figure 30: : : A) Western blot analysis of Glutamate Receptor 1 protein levels in protein extracts of 1, 3 and 6 month old WT, GUSB mice and histogram showing the quantitative analysis in B) CX of 1 and 3 mo mice, C) HC of 1 and 3mo animals and D) CX of 6mo mice (MEAN ±SEM) (*p-value < 0.05 WT (**n=3**) , GUSB (**n=3**),).*

We also studied the levels of Glutamate receptor 1 after gene therapy in GUSB mice, and at first, we did not see a recovery. However, after cellular fractionation to isolate the pre-and postsynaptic fractions in cortex samples, we found that Glutamate receptor 1 levels were significantly restored after gene therapy since no statistically significant differences were seen between WT and GUSB treated mice.

Unfortunately, this enrichment was not possible in hippocampal samples due to the reduced size of this tissue.

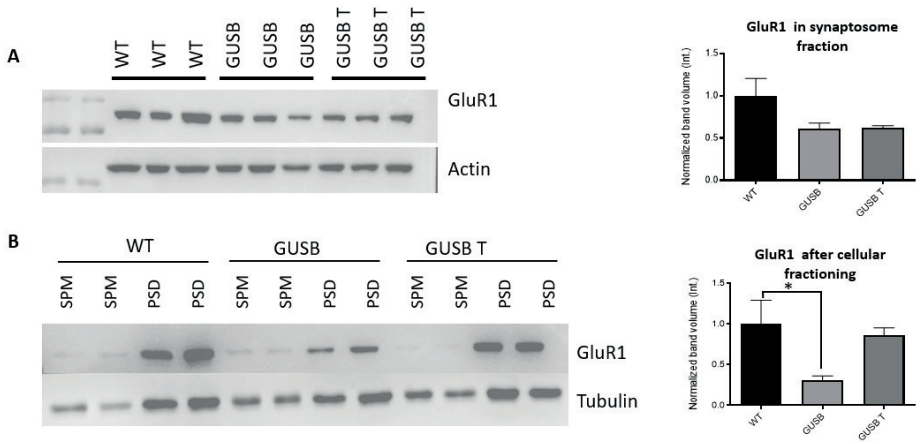

*Figure 31: Western blot analysis of Glutamate Receptor 1 protein levels in A) synaptosome extracts (WT (n=3) , GUSB (n=3), GUSB T (n=3)) and B) presynaptic (SPM) and postsynaptic (PSD) extracts (WT (n=2) , GUSB (n=2), GUSB T (n=2)) of 6 month old WT, GUSB and GUSB treated mice and histogram showing the quantitative analysis (MEAN±SEM) (*p-value < 0.05).*

Since cell cultures facilitate the study of neuronal markers due to the lack of other cell types, we used the GUSB KO mutant SH-SY5Y cell line and analysed several proteins related to synaptic function.

Using the cell line, we could further confirm the reduction in Glutamate receptor 1 levels found in the GUSB animals. Additionally, we could also detect an almost 40% reduction in the levels of GAD67 a glutamate decarboxylase essential for the synthesis of gamma-aminobutyric acid (GABA).

Moreover, we found a decrease of SNAP25 levels in the GUSB KO neurons. SNAP25 is a component of the SNARE complex and not only plays an important role in neurotransmission but also in neuronal spine morphogenesis and density (Tomasoni et al., 2013; Vadisiute et al., 2022).

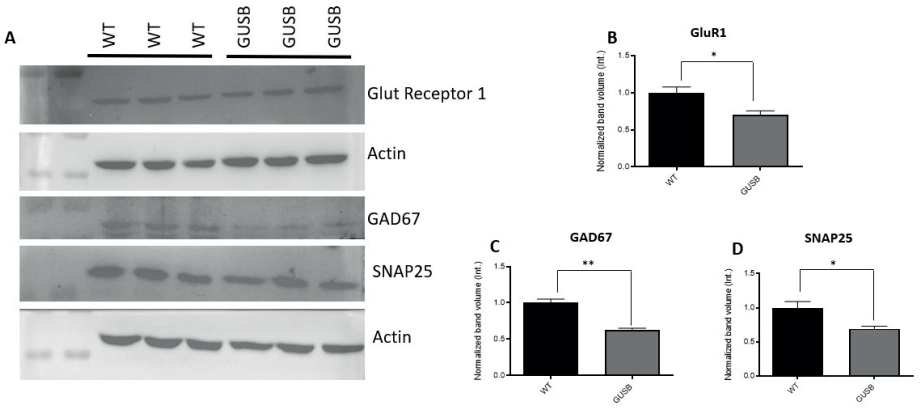

*Figure 32: A) Western blot analysis of neuronal function related proteins in SH-SY5Y and GUSB KO SH-SY5Y cell line protein extracts and histogram showing the quantitative analysis of B) Glut. R1, C) GAD67 and D) SNAP25 (MEAN±SEM) (*p-value < 0.05 **p-value < 0.01 WT (n=3) , GUSB (n=3), GUSB T (**n=3**)).*

Altogether, this suggests that neuronal function might be impaired as a result of the alteration of several mechanisms essential for its activity including synthesis, release and uptake of neurotransmitters.

We have also analysed other proteins related to neuronal function, but we did not observe significant alterations in the MPS VII model indicating the susceptibility of some specific proteins to be affected in this disorder. Proteins whose levels were not altered in MPS VII mice include the neural cell adhesion molecule (NCAM), a protein involved in dendritic growth and synaptic plasticity (Brennaman & Maness, 2010) and the Dopamine D2 receptor. Dopamine is one of the main neurotransmitters and is involved in a multitude of functions including motor coordination and memory. Moreover, abnormalities in the dopaminergic system have been described in several neurodegenerative disorders (Rangel-Barajas et al., 2015).

When analysing Homer1, a scaffolding protein with numerous postsynaptic functions, we saw a non-significant decrease in protein levels in the cortex of 6-month-old GUSB mice. Interestingly, Homer1 is known to modulate Ca^{2+} trafficking by interacting with several glutamatergic receptors(Reshetnikov & Bondar, 2021) including GluR1 which we have described as reduced in the cortex of GUSB mice.

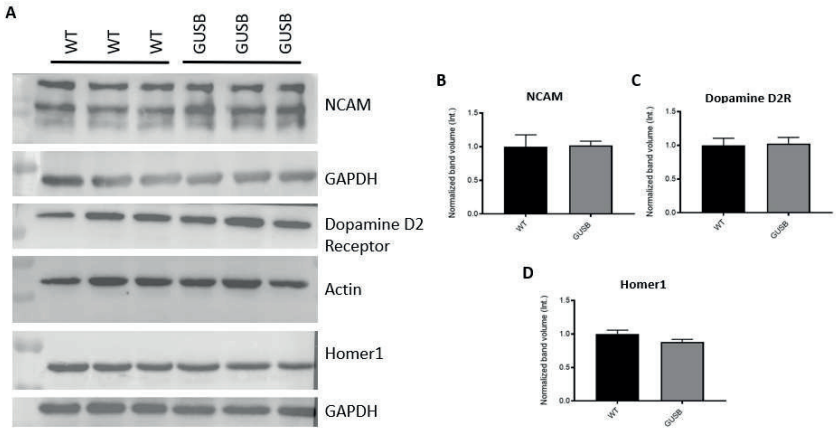

*Figure 33 : Western blot analysis of neuronal function related proteins in cortex samples of 6-month-old GUSB and WT animals and histogram showing the quantitative analysis of B)NCAM, C)Dopamine D2 Receptor and D)Homer1 (MEAN±SEM) (WT (**n=3**) , GUSB (**n=3**), GUSB T (**n=3**)).*

Calcium signaling has been suggested to play a role in the development of lysosomal storage disorders (Lloyd-Evans & Platt, 2011), and since Ca^{2+}traffick is increasingly relevant for a correct function of neurons we analyzed calcium homeostasis in MPS VII using Calbindin a calcium binding protein as marker. The GUSB KO neuronal cell line showed almost a 75% reduction in calbindin levels while in the cortex of 6-month-old MPS VII mice the reduction was only about a 25% showcasing possible different affectations depending on cell type. Importantly, we were able to restore calbindin protein levels after treatment.

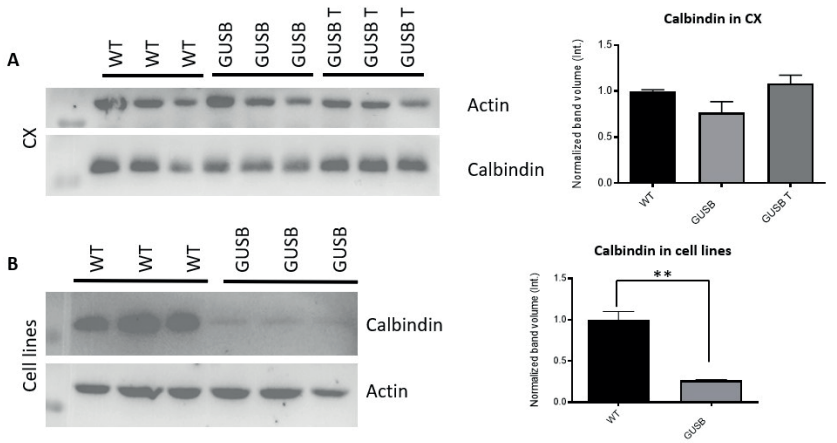

*Figure 34: Western blot analysis of Calbindin protein levels in A) cortex protein extracts of 6 month old WT, GUSB and DUSB treated mice and B) SH-SY5Yand GUSB KO SH-SY5Ycell cultures and histogram showing the quantitative analysis (MEAN±SEM) (**p-value < 0.01 WT (**n=3**) , GUSB (**n=3**), GUSB T (**n=3**)).*

We confirmed our findings using immunohistochemistry of 6-month-old animals. As shown in figure 35, GUSB animals show a reduction in calbindin signal not only in the cortex, but also in the cerebellum. This is highly relevant because it implies an affectation of Purkinje cells which have not been the focus of study in MPS VII.

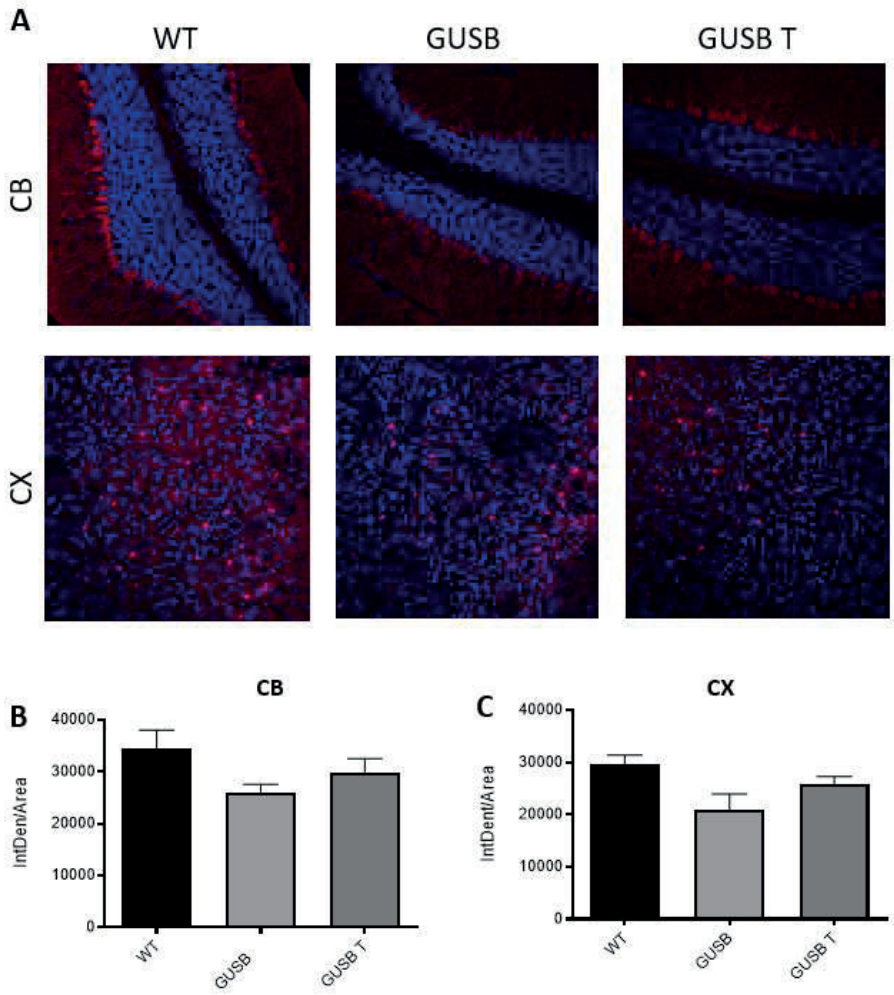

*Figure 35: A) Representative Images of Calbindin immunohistochemistry from 6 month old WT, GUSB and a GUSB treated mice and histogram showing the quantitative analysis of signal intensity (MEAN±SEM) of B) Cerebellum C) Cortex (*p-value < 0.05 WT (n=3) , GUSB (n=3)).*

Calbindin is a calcium binding protein involved in the regulation of the calcium homeostasis, and the reduced levels we found seem to indicate that calcium signaling is dysregulated and could also be playing an important role in the neuropathology of this disease.

Reduced calbindin expression has been linked to aggregation of α-Synuclein (Rcom-H'cheo-Gauthier et al., 2014) and therefore we evaluated α-Synuclein levels in our MPS VII models. We found noticeably increased signal of α-Synuclein in primary cultures of GUSB embryo cortical neurons and elevated levels of this protein in the cortex of 3-month-old MPS VII mice.

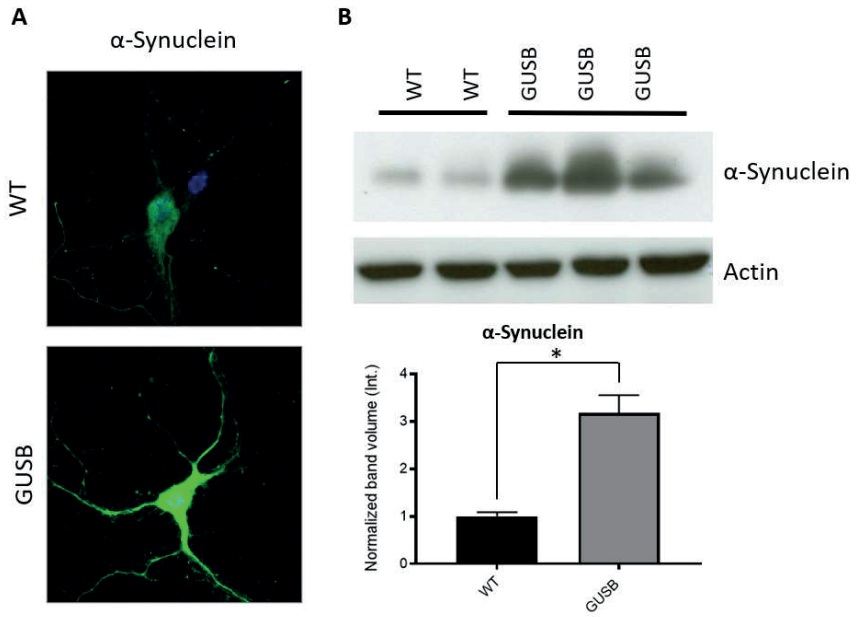

*Figure 36: A) Representative images of α-Synuclein immunocytochemistry from primary cultures using Hoechst to mark the nucleus. B) Western blot analysis of α-Synuclein in the cortex of 3-month-old WT and GUSB mice and histogram showing the quantitative analysis (*p-value < 0.05 WT (n=2) , GUSB (n=3)).*

5.6. Inflammation and complement system analysis through the disease development

The immune system and the inflammatory response have been commonly implicated in neurodegenerative diseases. Mild or moderate astrogliosis has been described in several mucopolysaccharidosis including MPS VII in which whole transcriptome analysis of the brain of the murine model detected increased expression of multiple inflammatory genes including GFAP(Parente et al., 2016; Wilkinson et al., 2012).

An overall increase in inflammation has been detected in mucopolysaccharidosis, but the progression of the phenotype has not been studied with much detail. Therefore, we analyzed several markers at 1, 3 and 6 months of age to establish the inflammatory status of mucopolysaccharidosis type VII through the disease progression.

First, we analyzed the glial fibrillary acidic protein (GFAP) by western blot and found an increase in protein levels at all ages analyzed. Surprisingly, the levels of GFAP in cortex samples were higher in the 1-month-old mice than at 3 or 6 months. Hippocampus showed only a slight but significant increase at 1 month but the difference in GFAP protein levels between WT and GUSB mice increased much more at 3 and 6 months.

It is interesting to notice that, once again the cortex seems to be more affected than the hippocampus.

When we analyzed the treated MPS VII mice we found that GFAP protein levels were restored in both areas analyzed meaning that gene therapy can reduce the astrocytosis of MPS VII to healthy conditions, as our group previously described (Pages et al., 2019).

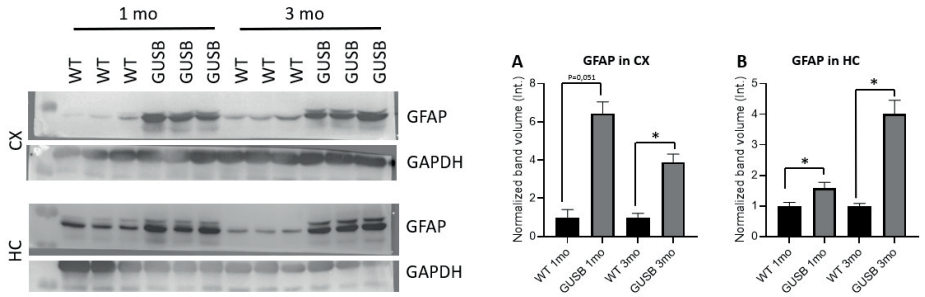

*Figure 37: Western blot analysis of GFAP protein levels in protein extracts of 1- and 3-month-old WT and GUSB mice and histogram showing the quantitative analysis (MEAN±SEM) of A) CX B) HC (*p-value < 0.05 WT (n=3), GUSB (n=3)).*

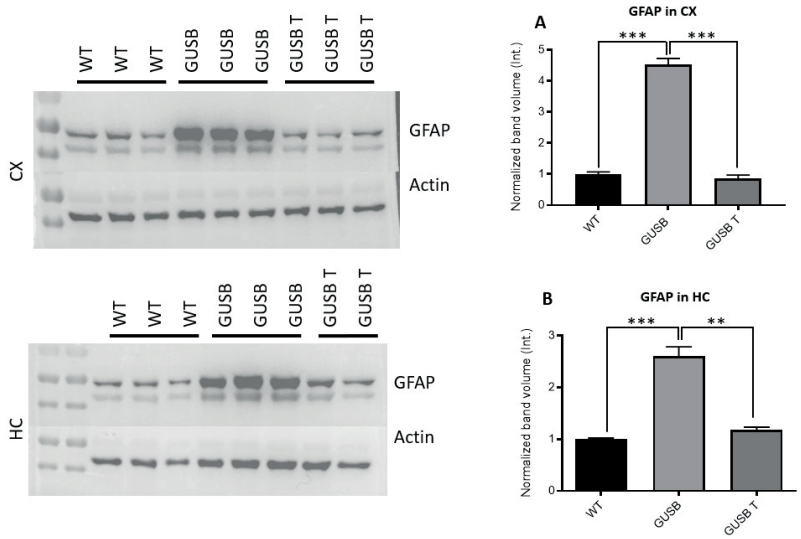

*Figure 38: Western blot analysis of GFAP protein levels in protein extracts of 6-month-old WT, GUSB and GUSB treated mice and histogram showing the quantitative analysis (MEAN±SEM) of A) CX B) HC (**p-value < 0.01 ***p-value < 0.001 WT (n=3), GUSB (n=3)).*

Concomitantly, GFAP immunohistochemistry of the entorhinal cortex seemed to corroborate our findings, showing higher signal in GUSB animals at all ages analyzed and being 3 months of age the time point with the smaller difference between genotypes.

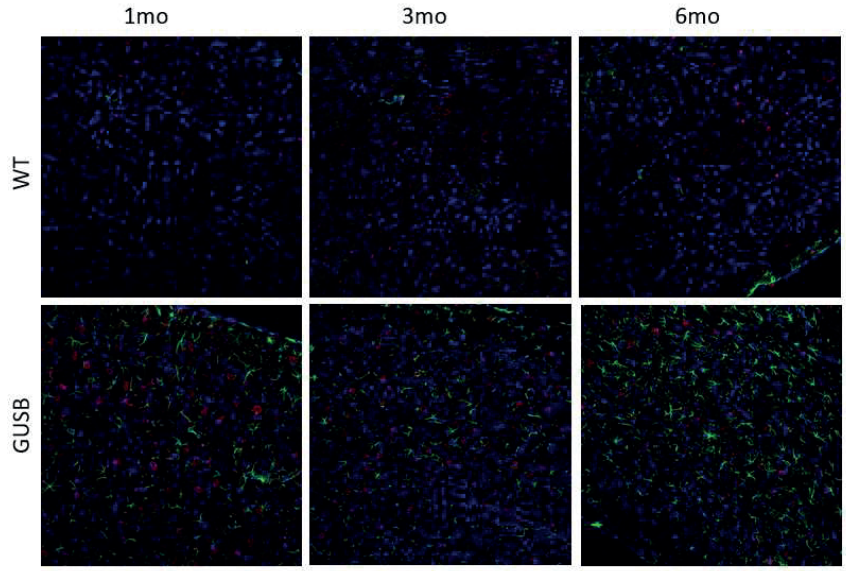

Figure 39: Representative images of GFAP(green) and Iba1(red) immunohistochemistry of 1-, 3- and 6-month WT and GUSB mice entorhinal cortex.

Since not only astrocytosis but also activation of the microglia have been described to play a critical role in driving neuroinflammation in lysosomal storage disorders (Bellettato & Scarpa, 2010) we studied Iba1, a microglial marker. Using immunohistochemistry, we could see a noticeably increase in Iba1 signal in the cortex of the MPS VII mice starting at 1 month of age. As for GFAP signal, in the CA1 and CA3 of the hippocampus, the signal was only slightly higher at 1 month of age but the difference between WT and GUSB mice increased at 3 months. At that time, Iba1 signal appears to be similar in all three regions of the MPS VII mice brain and significantly higher than in the WT counterparts.

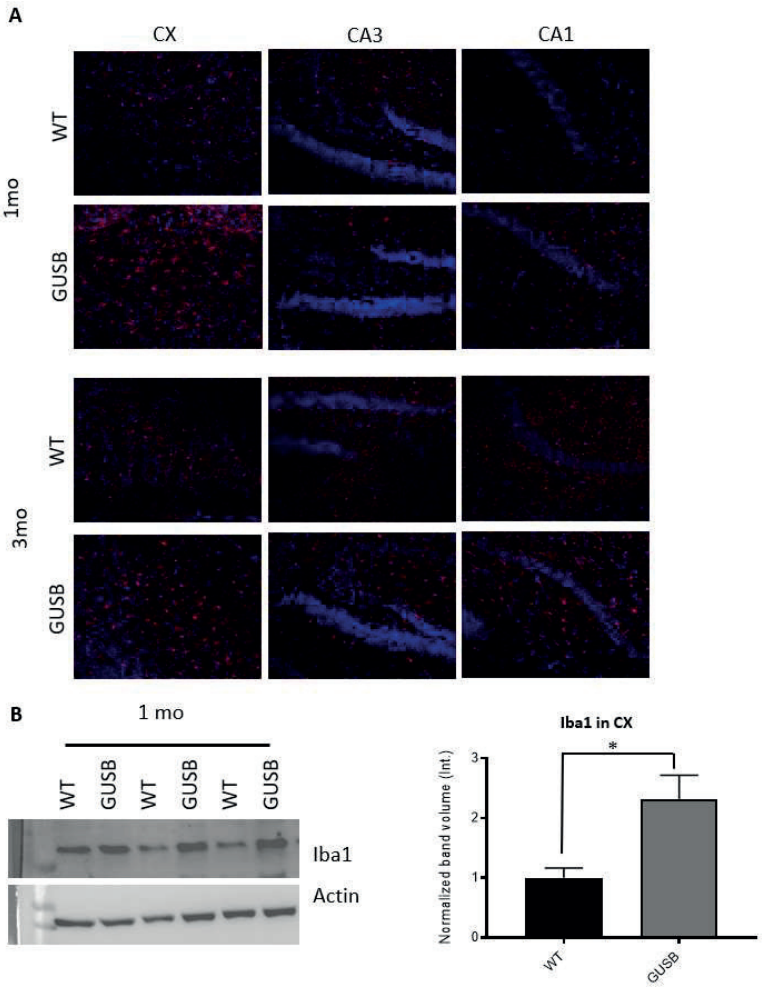

*Figure 40: A) Representative images of Iba1 immunohistochemistry in WT and GUSB mice at 1 and 3 months of age. B) Western blot analysis of Iba1 protein levels of 1 month old WT and GUSB mice and histogram showing the quantitative analysis (MEAN±SEM) (*p-value < 0.05 WT (**n=3**) , GUSB (**n=3**)).*

When analyzed at 6 months of age we could see higher signal of Iba 1 in all areas analyzed. Here, cortex did not seem to be more severely affected than neither hippocampal region. The treated GUSB animals showed reduced levels of Iba1 but not a complete recovery, and interestingly the hippocampus showed more improvement than the cortex.

This could suggest that although Iba1 is increased almost equally at all regions studied, the increased general affectation of the cortex makes the recovery of the WT phenotype after treatment harder to achieve.

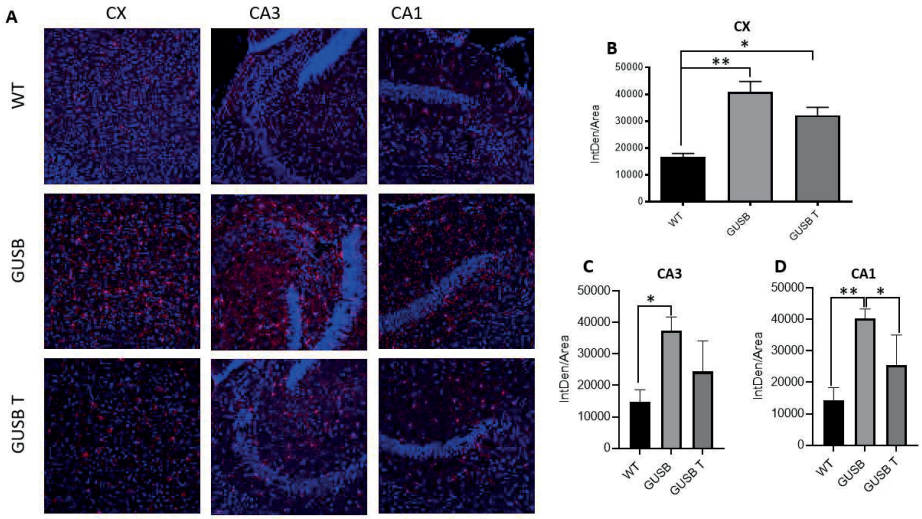

*Figure 41: A) Representative Images of Iba1 immunohistochemistry from 6 month old WT, GUSB and a GUSB treated mice and histogram showing the quantitative analysis of signal intensity (MEAN±SEM) of B) Cortex C) CA3 and D) CA1 (*p-value < 0.05 **p-value < 0.01 WT (n=3) , GUSB (n=3) , GUSB T (**n=3**)).*

On the other hand, dendritic spines are dynamic structures believed to be important for the adequate development and refinement of neuronal circuits and ultimately for proper cognitive function. As an example, LTP has been associated with spine enlargement while LTD with their shrinkage (Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004). Aberrant dendritic spine plasticity is found in several neurodevelopmental disorders like Fragile X, Rett and Angelman syndromes and epilepsy and autism (Bagni & Zukin, 2019; Phillips & Pozzo-Miller, 2015). Microglia is involved in modifying immature neuronal circuits by engulfing and eliminating synaptic structures like axons and dendritic spines, a process known as synaptic pruning.

Disruption of microglia pruning leads to defects in synaptic development (Schafer et al., 2012) and to neurodevelopmental disorders like autism and schizophrenia (Koyama & Ikegaya, 2015; Sellgren et al., 2019).

The classical complement cascade is known to be involved in synapse pruning (Kim et al., 2017). While the complement pathway encompasses a high number of proteins, C1q expression is critical due to its function as the initiator of the classical complement pathway. Moreover, aberrant levels of this protein have been linked to cognitive decline in aging (Reid, 2018). Another essential factor of the complement cascade is the C3 protein which is the point of convergence of the different pathways and can directly act to promote phagocytosis.

Thus, we studied these two components of the complement pathway to determine whether it could be playing a role in the MPS VII neuropathology.

We analyzed the protein levels of C1q in the synaptosome fraction of GUSB and wild type animals. In both areas, cortex (CX) and hippocampus (HC), we found an increase in C1q protein levels starting at 1 month of age that became more pronounced 3 months.

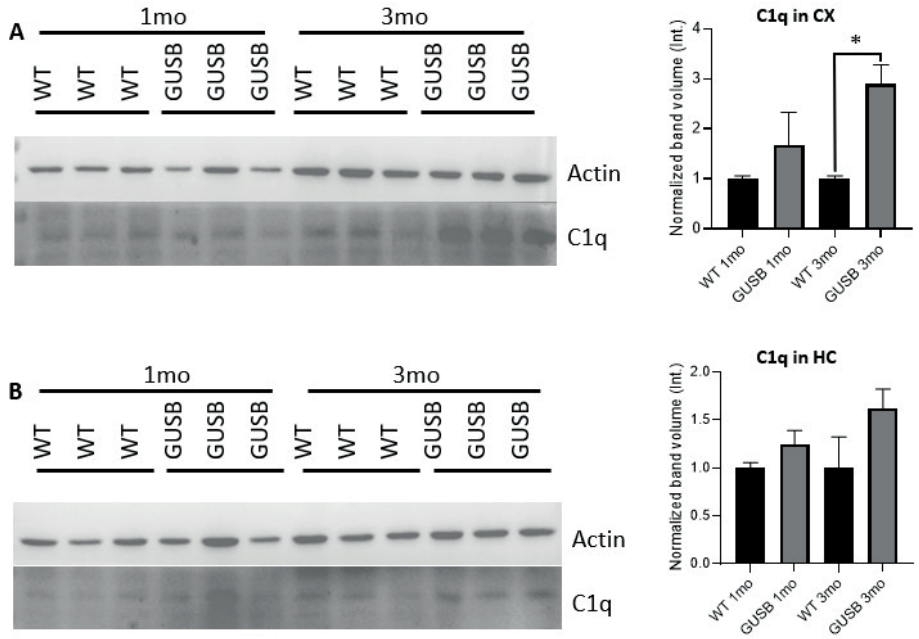

*Figure 42: Western blot analysis of C1q protein levels in total protein extracts of 1- and 3-month-old WT and GUSB mice and histogram showing the quantitative analysis (MEAN±SEM) of A) HC B) CX (*p-value < 0.05 WT (n=3), GUSB (n=3)).*

C1q protein levels in hippocampal total protein extracts of 6-month-old animals confirmed increased levels of C1q in GUSB mice. Furthermore, at 6 months of age the levels of C1q were higher in the synaptosome fraction of the neurons, supporting the hypothesis that it could be related to the decreased number of dendritic spines.

More importantly, in GUSB animals treated by gene therapy we were able to decrease C1q protein level in both total extracts and synaptosome fraction samples of GUSB treated animals.

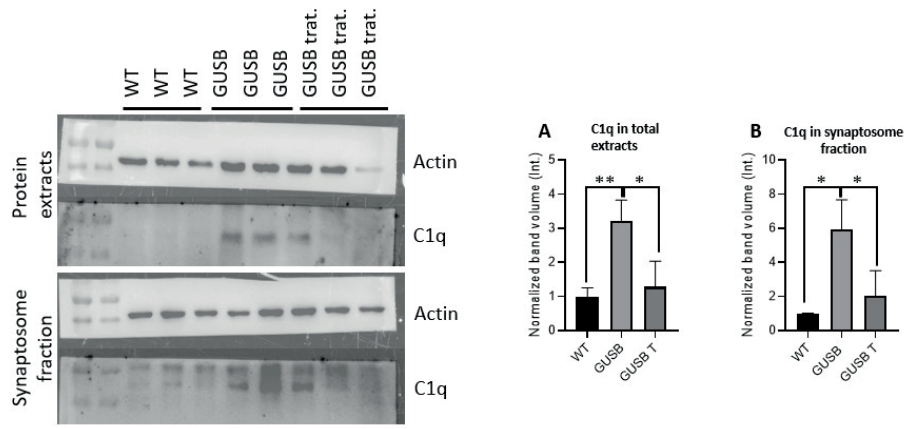

*Figure 43: Western blot analysis of C1q protein levels of 6-month old WT, GUSB and GUSB treated mice and histogram showing the quantitative analysis (MEAN±SEM) of A) protein extracts B) synaptosome extracts (*p-value < 0.05 **p-value < 0.01 WT (n=3) , GUSB (n=3)).*

In addition, we observed increased levels of C3 protein in the hippocampus of 6-month-old GUSB animals, suggesting that the complement pathway is upregulated in these animals and could be playing an important role in the pathology.

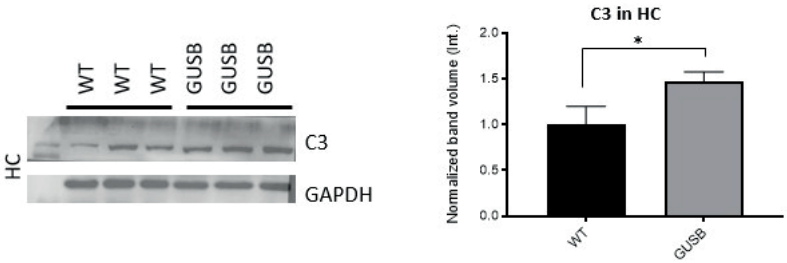

*Figure 44: Western blot analysis of C3 protein levels of 6-month-old WT and GUSB mice and histogram showing the quantitative analysis (MEAN±SEM) (*p-value < 0.05 WT (n=3) , GUSB (n=3)).*

5.7. Analysis of oxidative stress in GUSB KO SH-SY5Yneuronal cells

Mitochondrial dysfunction (Plotegher & Duchen, 2017; Saffari et al., 2017) and perturbed calcium homeostasis (Kiselyov & Muallem, 2008) have been reported in neurons and astrocytes of several lysosomal storage disorders. Both processes can lead to the leaking of reactive oxygen species (ROS) and to a critical energy crisis activating cell death signaling pathways which would result in the death of the affected cell. For that reason, we analyzed this mechanism in MPS VII using the neuronal cell line uncovering several dysregulated processes.

To study oxidative stress, we followed a dual approach. On the one hand, we analyzed the enzymes in charge of the clearance of ROS by studying the levels of GSTM1, a Glutathione S-transferase responsible for the metabolization of oxygen reactive species and other xenobiotics. On the other hand, we analyzed the main signaling pathways involved in oxidative stress response: Keap1-Nrf2 and NFKB.

Using total protein extract of the GUSB KO SH-SY55Y cell cultures we found a 50% reduction in Keap1 and GSTM1 proteins by western blot (Fig. 45 A-C).

Besides, we detected increased NfKB in the MPS VII cell model, suggesting an overactivation of this pathway (Fig. 45 A&D).

Since Keap1 acts as inhibitor of Nrf2 preventing its translocation to the nucleus, we then analyzed Nrf2 expression in nuclear and cytosolic fractions. Both, in WT and GUSB cell lines, we found most Nrf2 protein located in the nucleus rather than in the cytosol. In the cytosolic compartment we could not see any differences, but there was an increase in Nrf2 protein levels in the nucleus of GUSB KO cells, consistent with the decrease in Keap1 (Fig. 45 E&F). This indicates an overactivation of Nrf2, presumably in response to a higher oxidative stress environment.

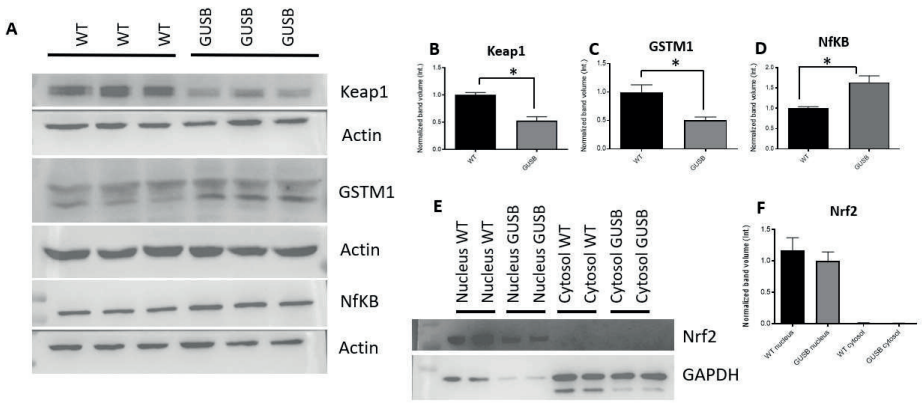

*Figure 45: A) Western blot analysis of oxidative stress related proteins in SH-SY5Yand GUSB KO SH-SY5Ycell line protein extracts and histogram showing the quantitative analysis of B) KEAP1, C) GSTM1 and D) NFKB. E) Western blot analysis of NRf2 in nuclear and cytosolic fractions of SH-SY5Yand GUSB KO SH-SY5Ycell line protein extracts and **F)** histogram showing the quantitative analysis (MEAN±SEM) (*p-value < 0.05 WT (**n=3**) , GUSB (**n=3**)).*

5.8. Cellular signaling analysis

Cellular signaling is an extremely complex process that has been linked to neurodegeneration and intensively studied in recent years (Brady & Morfini, 2010; Zugaza, 2021). Previous studies have suggested that both ERK and AKT are important for embryonic brain development yet control different aspects of neurogenesis: ERK promotes neuronal differentiation and AKT induces neuronal progenitor proliferation and survival (Pucilowska et al., 2012; Samuels et al., 2008; Zhang et al., 2013) and they are altered in human neuronal progenitors of Rett syndrome (Mellios et al., 2018). Erk is also altered in Fragile X mental retardation (Xing et al., 2016).

Akt signaling is known to promote inflammation and apoptosis in neurodegenerative processes (Malagelada et al., 2008). Taking this into consideration, we analyzed its expression and activation in our MPS VII models.

Using cortex samples from 6-month-old mice, we found a 50% reduction in the levels of both AKT and pAKT, suggesting a decreased expression of this protein. The ratio between pAKT and AKT which is indicative of the activation of the pathway was almost no different between WT and GUSB animals.

Gene therapy treatment was not able to restore Akt levels but resulted in an increased pAKT. These results suggest that, although our therapeutic approach could not increase Akt expression it contributed to a higher activation of the pathway, resulting in pAKT levels similar to those found in WT samples.

In protein extracts from GUSB KO and WT SH-SY5Ycellular cultures we observed the same pattern. The MPS VII model presented a reduction, in this case greater than 50%, of both pAKT and AKT.

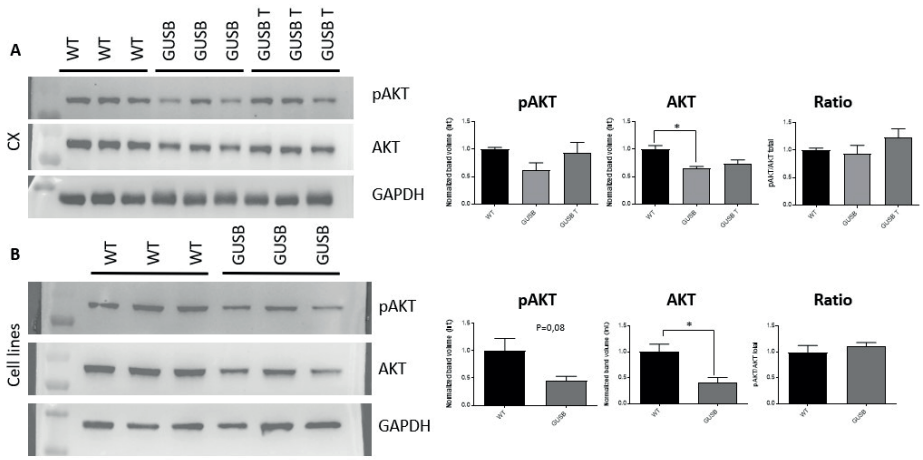

*Figure 46: Western blot analysis of AKT and pAKT protein levels in protein extracts of A) cortex samples of 6-month-old WT, GUSB and GUSB treated mice and B) independent cultures of SH-SY5Yand GUSB KO SH-SY5Ycells and histogram showing the quantitative analysis and the pAKT/AKT ratio (MEAN±SEM) (*p-value < 0.05 WT (**n=3**) , GUSB (**n=3**), GUSB T (**n=3**),).*

Another crucial pathway in cellular signalling is the Erk pathway, which participates in central nervous system homeostasis through a myriad of functions. This pathway is part of an intricate network involving different mechanisms such as inflammatory and oxidative stress response and neuronal plasticity among others.

Similarly to Akt, cortex samples of GUSB animals presented a reduction in both Erk and pErk protein levels. In this case, however, the reduction of pErk was more important than that of total Erk, which was less than 25%. Therefore, GUSB mice also presented reduced pErk/Erk ratio suggesting decreased activation of the pathway. Treated animals showed increased levels of both pErk and Erk, as well as higher pErk/Erk ratio. These results indicate that expression of corrected GUSB resulted in an overexpression and overactivation of the Erk signalling pathway.

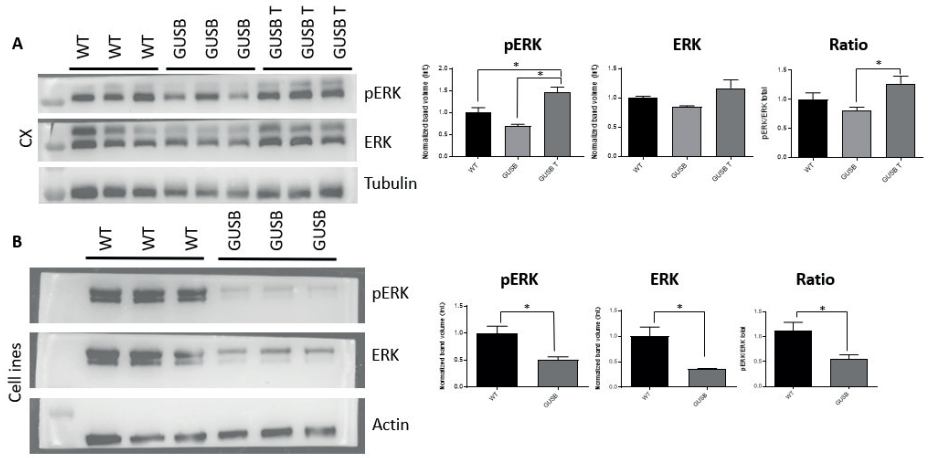

*Figure 47: Western blot analysis of ERK and pERK protein levels in protein extracts of A) cortex samples of 6 month old WT, GUSB and GUSB treated mice and **B)** independent cultures of SH-SY5Yand GUSB KO SH-SY5Ycells and histogram showing the quantitative analysis and the pERK/ERK ratio (MEAN±SEM) (*p-value < 0.05 WT (**n=3**) , GUSB (**n=3**), GUSB T (**n=3**),).*

5.9. Metabolism and autophagy evaluation

Impaired autophagy is a well-known hallmark of lysosomal storage disorders. Although it has been well characterized in several mucopolysaccharidosis this is not the case for MPS VII in which the more detailed study was published last year using a *Drosophila model* (Rigon et al., 2020). Therefore, we aimed to characterize the autophagy alterations using the different models available in our hands.

Using immunohistochemistry we studied Lamp1 accumulation as a lysosomal marker at different time points of the disease development and we found a progressive increase in Lamp1 signal in GUSB mice. Besides, MPS VII animals presented noticeably higher Lamp1 signal in cortex than in either region of the hippocampus, suggesting greater affectation.

Western blot analysis confirmed these findings in the cortex, with Lamp1 levels being close to 6 times higher in GUSB animals when compared to WT mice at 3 months of age.

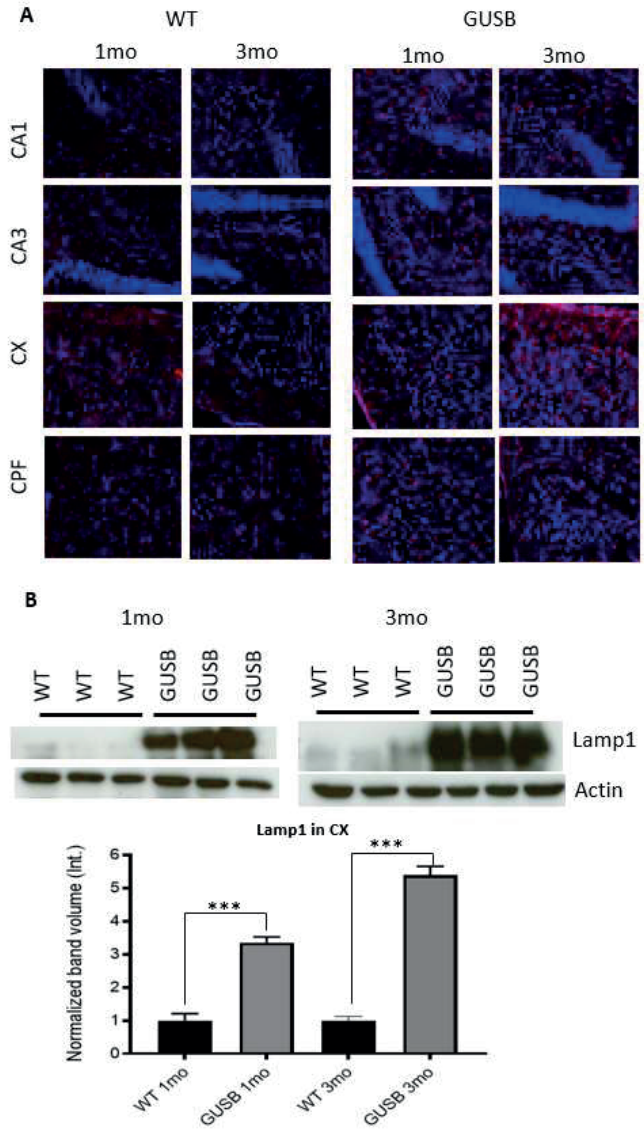

*Figure 48: A) Representative images of Lamp1 immunohistochemistry from several brain regions of 1- and 3-month-old WT and GUSB mice. B) Western blot analysis of Lamp1 in cortex samples and histogram showing the quantitative analysis (MEAN±SEM) (*p-value < 0.05 WT (n=3) , GUSB (n=3)).*

We further corroborated those finding in the primary cultures, which also exhibited increased Lamp 1 signal. Then we used the cell line model to further analyze autophagy and found an overall increase in autophagy related protein levels. When analyzing protein levels by western blot we detected significantly higher levels of p62 in both the GUSB KO cell line and the cortex of MPS VII mice.

Beclin 1, a protein involved in several steps of the autophagic process was also found to be increased by almost 50% in the MPS VII cell line. Similar results were obtained in cortex samples of 1-month-old animals in which a smaller but significant increase in Beclin levels was found suggesting enhanced initiation of autophagy and/or an impaired progression of the autophagy which would then lead to a reduced degradation of the autophagic material. Furthermore, the fact that these alterations are already present as such ages indicates that abnormal autophagy is an early event in the progression of the disease.

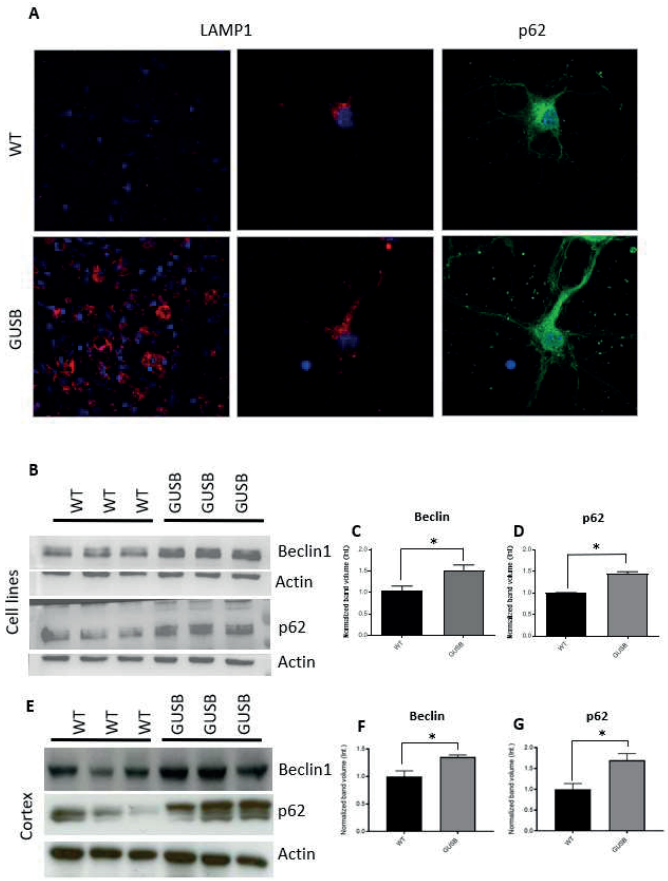

*Figure 49: A) Representative images of Lamp1 and p62 immunocytochemistry from primary cultures. Pictures on the top line were taken with less zoom to provide general observation. Zoom was increased in the rest of the images to obtain detailed localization in the cells. B) Western blot analysis of autophagy related proteins in SH-SY5Yand GUSB KO SH-SY5Ycell line protein extracts and histogram showing the quantitative analysis of C) Beclin1 D)p62 E)Western blot analysis of autophagy related proteins in cortex of WT and GUSB animals and histogram showing the quantitative analysis of F) Beclin1 G)p62 (MEAN±SEM) (*p-value < 0.05 WT (n=3), GUSB (n=3)).*

Increased expression of other autophagic proteins in the cortex and the hippocampus of 1 and 3month old GUSB mice further confirm that altered autophagic flux is present in the early stages of the disease. LC3 plays a critical role in maturation of the lysosomes, and therefore its progressive accumulation in the neurons, which has also been described in a iPSC model of the disease (Bayo-Puxan et al., 2018) could be due to a defect in the autophagosome-lysosome function. Another protein suggested to play a role in the fusion process is Lamp2a, the levels of which are also increased in GUSB mice although it is only significant in cortex samples.

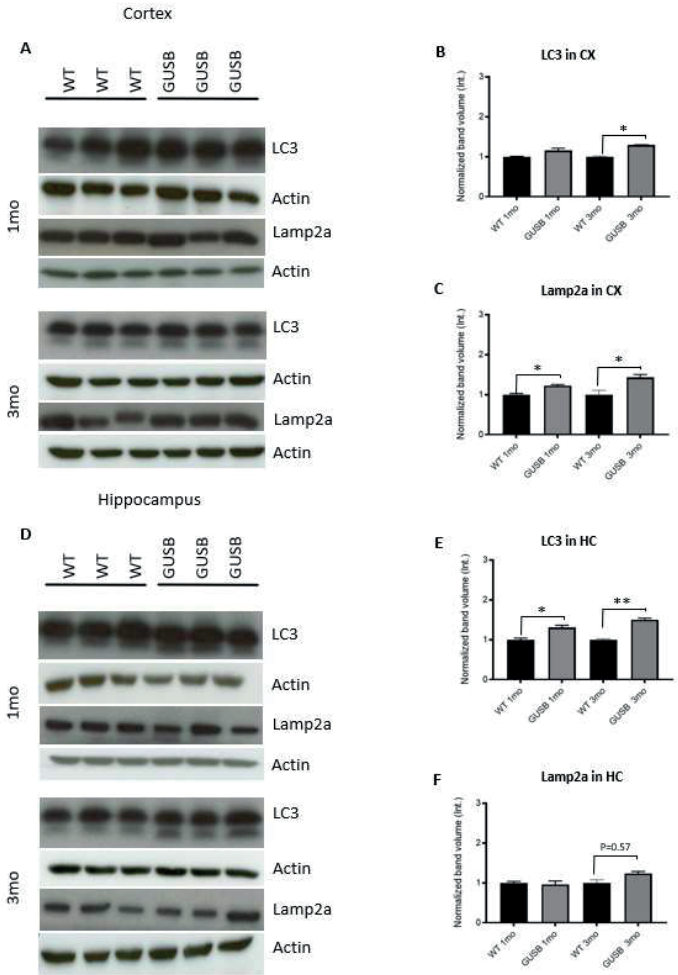

*Figure 50:Western blot analysis of autophagy related proteins of 6-month-old WT and GUSB animals in A) Cortex and B) Hippocampus and histogram showing the quantitative analysis of B), E) LC3 and C), D) Lamp2a (MEAN±SEM) (*p-value < 0.05. **p-value<0.01 WT (n=3), GUSB (n=3)).*

6. Discussion

Mucopolysaccharidosis type VII is an autosomal recessive inherited disease classified as an ultrarare disorder due to its low prevalence (less than 1 in 100,000,000 newborns (Orphanet Report Series: prevalence of rare diseases). Caused by a lack of β- Glucuronidase activity, this progressive and multisystemic disorder presents high heterogeneity of clinical symptoms Some of the most common manifestations of MPS VII include skeletal abnormalities, cardiovascular and pulmonary complications developmental delay and progressive cognitive impairment. Patients start presenting symptoms in early childhood and have short life expectancy usually featuring a poor quality of life. Currently, therapies directed to ameliorate the symptoms are the standard of care for Mucopolysaccharidosis type VII as there are no curative treatments available that can fully correct the pathology.

A recent study published by our group demonstrated that intrathecal administration of an AAV encoding the human β-Glucuronidase achieves supraphysiological levels of GUSB expression throughout the brain and the peripheral tissues, leading to a complete correction of the histopathological and biochemical hallmarks of the disease, doubling their life span. Importantly, the biochemical correction led to a significant improvement in the physical, cognitive and emotional characteristics of MPS VII (Pages et al., 2019).

However, since the cognitive correction was not complete in the MPS VII mouse model we aimed to study why this could be happening by analyzing the underlying mechanisms of the pathological process and the effect our gene therapy strategy has on them.

Animal models, while fully mimicking the human pathology of MPS VII present an intrinsic source of variability that may difficult the obtention of relevant but small alterations. To study a reliable but simple model, using cell cultures is a viable option.

The use of cell lines started as early as 1977, when hybrid cells between mouse cells and human fibroblasts with β-glucuronidase deficiency were used to study structural gene mutations and their ability to generate an active enzyme(Chern, 1977).

A few years later, cell lines with mucopolysaccharidosis were developed using electroporation in human fibroblast (Okamoto et al., 1992).

However, the use of cell models for MPS VII has not been relevant until much later with the advances in induced pluripotent stem cells (iPSC) technologies. Disease-specific iPS cells from mouse models have been generated for MPS VII along with other diseases. MPS VII-iPSC presented deficient β-glucuronidase activity and accumulation of GAGs. However, this iPS cells could not correctly form embryoid bodies, suggesting a possible impairment of embryonic development in MPS VII patients (Meng et al., 2010).

More recently, MPS VII iPSC-derived neurons have been used to study lysosomal and network alterations. This cellular model presented the main characteristics of the disorder and the addition of recombinant β-glucuronidase to these neurons was able to restore the phenotype similar to a healthy control cell culture. Other findings using this model cultured as 3D neurospheroids include reduced neuronal activity, upregulated GFAP gene expression and downregulation of GABAergic neuron markers (Bayo-Puxan et al., 2018).

Not only stablished cell lines can be used as cellular models for MPS VII, but primary cultures also represent an interesting alternative. Primary cultures of brain cells established from newborn MPS VII mice have been stablished and used to evaluate β-glucuronidase activity, GAG accumulation and the effect of a retroviral vector-mediated transfer of a corrected copy of the GUSB gene. They found that corrected β-glucuronidase expression could be maintained for more than 2 months in nondividing glial cell cultures(Taylor & Wolfe, 1997).

Murine MPS VII skin fibroblast have also been used to develop a fluorescence-activated cell sorter-based assay to analyze β-glucuronidase activity in viable cells after retroviral gene transfection. This allows cells presenting higher levels of enzymatic activity to be isolated and further cultured before transplantation of the gene-corrected cells(Lorincz et al., 1999).

Although the use of animal models is well stablished in Sly syndrome, cellular models have only been used sparingly and no stable cell model has been obtained before.

One of the highlights of this thesis is the combination of the animal model, primary cultures and a stablished cell line to study the neurodegeneration in MPS VII. For this, we generated and validated the first cellular model for MPS VII using CRISPR/Cas9 technology that allows us to study the effects of β-Glucuronidase deficiency exclusively in the neuronal population.

In our analysis of neuronal death in the MPS VII mouse model we found a decrease in the density of the neurons and the width of the neuronal layer in the entorhinal cortex and the CA3 of the hippocampus respectively, although statistics are not significant. In the prefrontal cortex and the CA1 of the hippocampus we did not find any indication of neuronal death.

On the one hand, since MPS VII is a progressive disease, the stage of the disorder in which neuronal loss plays a critical role may just be starting and its development could be happening at different speed between brain regions. It would be interesting therefore to study these animals at later ages. However, one of the complications when working with this animal model is that GUSB mice have a considerably reduced half-life (4.5 months) compared to WT mice, probably due to respiratory problems. During this thesis we used 5- or 6-month-old animals because is the maximum time most of the GUSB animals survive even when provided with easier access to food and water, and it may not be late enough in disease development for neuronal death to be a prominent feature in their CNS.

On the other hand, neuronal loss may not be playing a significant role in MPS VII neuropathology. Several studies analyzing neurodegeneration in 4- to 6-month-old MPS mice showed no increase in apoptosis (Settembre et al., 2008; Wirths & Zampar, 2020), although other cell death mechanisms were not analyzed to confidently discard neuronal death. This is consistent with the initial decrease in neuronal density we found at 6 months. Besides, the same study showed that although non-neuronal cells in the brain presented the same levels of GAG storage, the same could not be said for neurons. This was specially marked in the hippocampus where CA1 presented few vacuoles and CA2, CA3 and CA4 presented high amounts of GAGs accumulated and cytoplasmatic vacuoles (Levy et al., 1996). This increased affectation of the CA3 correlates to our findings, with a greater neuronal density decrease in the CA3 of GUSB animals compared to WT than in the CA1 region.

Although both hippocampal regions participate in encoding and retrieval of extinguished fear, only CA1 seems to be essential for context dependent

retrieval (Ji & Maren, 2008). Furthermore, reduced activity in open field test and severe problems to interact with the environment have been described in the mouse model of MPS VII (Ariza et al., 2014). This suggests that another reason that could be involved in the differences between hippocampus regions could be their distinct participation in some brain functions which could lead to neuroprotective processes taking place differently.

Other studies have shown that neurodegeneration in MPS VII mice brain is characterized by ubiquitin and neurofilament inclusions following a specific pattern. Furthermore, areas of increased reactivity were found in specific structures (Heuer et al., 2002). All this supports the assumption that the neurodegenerative process is not equal through the brain but specific for each brain region. Besides their functional relevance, the prominent alterations exhibited in hippocampus and entorhinal cortex were a reason for the selection of these brain areas as study target through this thesis (Ariza et al., 2014).

For other disease with a prominent neurological affectation, the involvement of neuronal death in the pathology is widely variable. Although in Down syndrome the loss of neurons has been described linked to increased expression of apoptosis-related genes (Sawa, 1999), in Rett syndrome patients only abnormal neuronal morphology but not neuronal death have been detected (Kyle et al., 2018). In this progressive neurodevelopmental disorder, the mechanism proposed to cause the intellectual disability is neuronal dysfunction associated to alterations in dendritic spines which have described in the patients and animal models of this disease(Xu et al., 2014). In Fragile X syndrome, the most common inherited form of cognitive impairment, the neuropathology has been increasingly attributed to altered neuronal circuit excitability and neuronal dysfunction rather than cellular death(Contractor et al., 2015).

The same could be happening in our MPS VII mouse model which would mean that the main cause of cognitive deficits in the model is not neuronal death but neuronal disfunction. Neurodegeneration is a highly complex process with many mechanisms preceding cell death. Neuronal death does not happen immediately as a consequence of macromolecule accumulation. It is the result of molecular and morphological changes in the cell which impair their functionality and finally leads to death.

Neuronal dead is not strictly needed for neuropathological symptoms to develop. Reduced neuronal functionality could easily cause miscommunication between neurons leading to cognitive impairment without cell death. In the case of MPS VII mice, a described factor that could be affecting neuron communication is demyelination which has been described in certain areas of the MPS VII mouse model brain(Kumar et al., 2014).

Other mechanisms that could be impairing neuronal function and exerting a detrimental effect on cell function could be the presence of meganeurites and axonal spheroids that have been described in neurons of other lysosomal disorders (Bellettato & Scarpa, 2010), and the buildup of lipid droplets caused by the disfunction in lysosomal activity (L. Liu et al., 2015).

Multiple studies have found structural abnormalities in the synapses of several lysosomal storage disorders further suggesting that synaptic disfunction could be playing an important role in the neurological pathology of this type of diseases. Our analysis showed a small but significant decrease in dendritic spine density in the pyramidal neurons of both the hippocampus and the cortex of 5-month-old MPS VII mice. The affectation was not equal in all regions with cortex presenting a more severe phenotype. This correlates with our neuronal death findings and suggest that cortex is more affected than hippocampus in MPS VII presenting faster progression of the disease. Even though dendritic spine loss did not affect all brain regions equally in our mouse model, intrathecal administration of an AAV9 vector resulted in a reversion of the phenotype in both cortex and hippocampus.

Abnormal spine morphology has been detected in several animal models of lysosomal storage disorders including mucopolysaccharidosis type II and Tay-Sachs' disease (Dwyer et al., 2017; Sambri et al., 2017). Moreover, the same studies described alterations in frequency and amplitude of miniature EPSCs and IPSCs, reduced synaptic vesicles and abnormal localization of pre- and post-synaptic proteins. A mouse model for Niemann-Pick type C disease is also characterized by alterations in synaptic structure and function with impairment of long-term synaptic potentiation (D'Arcangelo et al., 2016) but presents important neuronal loss providing a possible explanation of how the neuropathology might be developed in the cases of lysosomal storage disorders with more severe CNS affectation (Peake et al., 2011; Wang et al., 2010). In the case of Gaucher's disease, a Drosophila model was used to

analyze neurodegeneration and major synaptic loss was described (Kinghorn et al., 2017).

Further analysis of synaptic function could prove fundamental to understand MPS VII pathology. One option would be to use electrophysiological analysis, which has been successfully used to study synaptic transmission at molecular, cellular and network levels. Recently, the development of new optical techniques such as 2-photon microscopy, optogenetics, genetically-encoded calcium indicators (GECIs) and voltage probes has offered an alternative way to investigate synaptic function(Glasgow et al., 2019).

Although all these studies point to synaptic dysfunction as a reason behind the neurological symptoms of mucopolysaccharidosis type VII and lysosomal storage disorders in general, how each specific enzymatic deficit leads to these synaptic abnormalities has not been detailed.

Since synaptic function is complex and tightly regulated, it seems highly unlikely that lysosomal deficits directly trigger dysfunction but rather that disruption of cell homeostasis at several levels leads to synapse alteration. Among others, abnormalities in pre and post synaptic membranes, reduced vesicular transport (Xu et al., 2010), deficient vesicle (Virmani et al., 2005) and SNARE complex formation (Fraldi et al., 2022) could be processes affecting synaptic function. Furthermore, lysosomal deficiency could be impairing the exocytosis and recycling of synaptic vesicles generating even more metabolic burden on synapses. Therefore, synaptic pathology in Sly syndrome and lysosomal storage disorders could be hypothesized as the result of an interplay of multiple factors.

The overlapping of neurons when using Golgi-cox staining prevented us from analyzing the arborization complexity in MPS VII mice brain. However, using primary cultures we detected a noticeable reduced arborization of neurons from GUSB mice suggesting that other than decreased spines, lower number of dendritic branches may also be involved in the neuropathology of Mucopolysaccharidosis type VII.

Furthermore, neuronal models derived from iPSCs have been developed for multiple mucopolysaccharidosis and differentiation into neurons has had varying degrees of success. While neuronal and astrocytic differentiation could be achieved from MPS IIIC iPSCs (Beneto et al., 2020), this was not the case for MPS IIIA in which neural differentiation was impaired (Lehmann et

al., 2021). Our cell line model could be differentiated without overall morphological differences compared to the WT which would suggest that differentiation is not altered in MPS VII. Nonetheless, further testing should be performed to ensure that complete differentiation into functional neurons has been achieved. These results are in agreement with the correct neural differentiation described in MPS VII iPSCs (Bayo-Puxan et al., 2018).

To further understand how synaptic function is affected in MPS VII we studied the expression of Glutamate receptor 1. Analysis of the expression of the different glutamate receptor subtypes in the MPS VII mouse model has provided contradictory results.

One article studied levels of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor subunits (GluR1 and GluR2) and found a reduction in the hippocampus starting at 4 weeks of age. Only in the case of GluR2 this deficit increased with age. Furthermore, the same study described reduced levels of the NR1 receptor subunit of NMDA (Liu et al., 2007). However, a study analyzing the hippocampus proteome of 6-month-old GUSB mice discovered increased expression of the metabotropic glutamate receptor 5 but did not find any significant reductions in the levels of other glutamate receptors (Parente et al., 2016). Moreover, we decided to analyze the expression both at the cortex and the hippocampus in case the affectation was variable between regions.

In the hippocampus of 1-month old GUSB mice we found a slight reduction in GluR1 receptor levels, but this no significant reduction was not present when mice were analyzed at 3 months. This contradicts the findings of Liu et al. but surprisingly; we detected a reduction in GluR1 levels in the cortex of mucopolysaccharidosis type VII animals. An almost 50% reduction was detected at 1 month but the difference between phenotypes was smaller at 3 months. However, increased variability at the 3-month wild type samples might be obscuring an accurate estimation. When studied at 6 months, an almost 40% reduction in levels of GluR1 was detected which would be similar to the decreases found by Liu et al. in the hippocampus. We further confirmed these findings by analyzing GluR1 expression in the cellular model of MPS VII in which we found the same levels of reduction.

Interestingly, when we used total protein extracts to analyze the effect of our therapeutic approach in the levels of glutamate receptor 1, we did not observe any improvement. However, when analyzing the pre-and post-

synaptic regions separately, we found elevated GluR1 levels in treated animals in the post-synaptic density, the region where these proteins is mostly located. This difference could be due to the enrichment of specific synaptic proteins obtained through the fractionation protocol promoting the use of this technique in the future to provide more accurate results.

Glutamate receptors have been involved in the neuropathology of multiple neurodegenerative disorders such as Alzheimer's disease, Parkinson's disorder and even Huntington disease, and modulation of these receptors has been proposed as a therapeutic strategy. However, further study is needed to elucidate the specific mechanisms through which alterations in glutamate receptors participate in cognitive impairment (Crupi et al., 2019).

Moreover, glutamate receptors are also involved not only in neurodegenerative diseases but other neurological disorders such as Fragile X syndrome, Rett syndrome and autism. Rett syndrome is characterized by frequent epilepsy attacks due to neuronal hyperexcitability. In this disease, higher levels of several glutamate receptors have been described in several regions of the patient's brain(Nisar et al., 2022), but decreased levels of the metabotropic glutamate receptors 2 and 3 have also been described in patients and the mouse model (Vermudez et al., 2022). This difference observed between receptor subtypes showcases the need for a detailed analysis of the different types of receptors in MPS VII to evaluate the specific alterations for each one.

Similar findings have been described in autism, in which both hyperfunction of hypofunction of glutamate receptors have been associated with an autism spectrum disorder phenotype (Nisar et al., 2022). In Fragile X syndrome it has been proposed that altered glutamate receptor-dependent plasticity contributes to the development of the pathology and pharmacological interventions modulating the expression of the glutamate receptor could reverse many phenotypes in the animal model (Ronesi et al., 2012).

Interestingly, a study in the mouse model found decreased levels of the metabotropic glutamate receptor 5 in the postsynaptic density in association with altered balance of the different isoforms of Homer (Giuffrida et al., 2005). Although the decrease in Homer1 levels we detected in the cortex of GUSB animals is not significant, further study of the different isoforms should be performed to analyze the possible interaction between Homer1 and the glutamate receptor in MPS VII.

One of the mechanisms critical for neurotransmitter release which has been involved in the neuropathology of lysosomal storage disorders is the formation and correct activity of the SNARE complex.

Using the cellular model, we studied the SNARE complex and detected an almost 50% reduction in SNAP25 levels in the GUSB KO cell line. Decreased availability of SNAP25 at synaptic terminals could lead to a decline in neurotransmitter release leading to altered communication between neurons suggesting another possible mechanism responsible for the cognitive deficits in mucopolysaccharidosis type VII.

SNAP25 is an essential protein in neurons and total loss of this protein is known to produce cell death in both CNS and dorsal root ganglia (DRG) neurons (Santos et al., 2017). Interestingly, even though SNAP25 expression has been found reduced in multiple brain regions of Alzheimer's disease patients (Furuya et al., 2012), increased protein levels in the cerebrospinal fluid (CSF) have been related to synaptic damage that precedes neuronal loss and detection assays are being optimized to use as biomarker for Alzheimer's disease (Agliardi et al., 2019; Brinkmalm et al., 2014; Mila-Aloma et al., 2021).

The way in which SNAP25 is involved in neuronal function and neurodegeneration is still being studied (Delgado-Martinez et al., 2007). Nonetheless, changes in SNAP 25 expression have been described in numerous and heterogeneous disorders involving cognition such as autism spectrum disorders (Braida et al., 2015), schizophrenia (Barakauskas et al., 2016; Yang et al., 2017) and Down syndrome (Weitzdoerfer et al., 2001), which confirms its importance for a correct neuronal function. Furthermore, it's role in synaptic plasticity and morphogenesis (Antonucci et al., 2013; Fossati et al., 2015) could make reduction of SNAP25 one of the mechanisms involved in the loss of dendritic spines in our mouse model of MPS VII.

On another hand, we found reduced levels of GAD67, a glutamate decarboxylase essential in the synthesis process of gamma-aminobutyric acid (GABA), suggesting an impaired production of neurotransmitters may also be involved in the neuropathology of Sly syndrome. The expression of GAD67 has been proposed to play an important role in the development of social withdrawal in several psychiatric pathologies like autism and schizophrenia (Fatemi et al., 2002; Volk & Lewis, 2005). Therefore, it could be one of the mechanisms impairing behaviour and more specifically breeding capability of

GUSB animals. Haplodeficiency of GAD67 has been found to impair social behaviour mostly related to the processing of olfactory stimuli in mice but does not have any impact in locomotor activity nor anxiety-like behaviour (Sandhu et al., 2014). However, the same haploinsufficiency reduced amyloid pathology and rescued olfactory memory deficits in a mouse model of Alzheimer's disease (Y. Wang et al., 2017).

GAD 67 has also been hypothesised to play a role in activity-dependent synaptic plasticity and regulation of inhibitory synaptic transmission through alteration of the GABA content in synaptic vesicles (Lau & Murthy, 2012). Moreover, reduced levels of GAD in the brain lead to excitotoxicity by decreasing GABA and increasing glutamate levels in the synaptic vesicles (Nisar et al., 2022).

As expected, these results combined suggest some synapse abnormalities in mucopolysaccharidosis type VII affecting different players and leading to a dysregulation through all neurotransmission process: synthesis of the neurotransmitters, release into the synaptic cleft, and uptake at the post-synaptic membrane.

Calcium homeostasis has been proposed to be altered in lysosomal storage disorders and play an important role in the progression of the disease but has not been studied in detail yet in MPS VII. Niemann-Pick type C (NPC) has been associated to defective lysosomal Ca^{2+} uptake and release which leads to the disruption in endocytosis and lipid accumulation that characterize the disorder (Lloyd-Evans & Platt, 2011). In fibroblasts from mucopolysaccharidosis type IV disrupted Ca^{2+} signaling has also been described and linked to disruptions in membrane trafficking which is essential for a correct autophagy (LaPlante et al., 2004). Moreover, in a murine model of MPS I alterations in both Ca^{2+} and H^{+} homeostasis were suggested to result in increased apoptosis involving permeabilization of the lysosomal membrane (Pereira et al., 2010).

In this thesis we have analyzed the levels of Calbindin, a cytosolic calcium binding protein involved in regulation of calcium homeostasis due to its Ca^{2+} buffering capacity in neurons. Our results showed decreased Calbindin levels in the cell model for MPS VII and in several regions of the GUSB mouse brain. Physiological levels of this protein were restored upon gene therapy treatment suggesting a correction of calcium homeostasis as well. During normal aging Calbindin its known to decrease through the brain although

with variable affectation and it has been suggested to turn neurons more vulnerable to pathological processes (Bu et al., 2003). Reduced calbindin has been described in some but not all patients of MPS II and MPS III (Hamano et al., 2008).

Furthermore, interaction between calcium and α-Synuclein has been proposed to play a role in the development and progression of other neurodegenerative disorders such as Parkinson's disease. Using a unilateral mouse lesion model of this specific disorder α-Synuclein aggregates were present almost exclusively in Calbindin-negative neurons. Moreover, in several studies Calbindin-positive neurons were found to be less likely to undergo apoptosis resulting in a relative sparing of this neurons during Parkinson's disease neurodegenerative process (Rcom-H'cheo-Gauthier et al., 2014).

Since we also found increased α-Synuclein signal in the cortex of our mouse model and in the primary cell cultures of MPS VII it would be interesting to perform a progressive analysis of both α-Synuclein and calbindin expression at different time points to analyze if the related vulnerability described in Parkinson's also happens in Mucopolysaccharidosis type VII.

Accumulation of α-Synuclein is also a feature of other LSD including Gaucher's disease (Mazzulli et al., 2011) and MPS IIIA. Interestingly, one study demonstrated that lack of α-Synuclein does not affect the early course of Mucopolysaccharidosis Type IIIA suggesting that by itself, this protein is not the starting cause of the neurodegeneration process(Soe et al., 2019).

Our findings in relation to GFAP expression are consistent with previous research that has described activation of inflammatory pathways as an early and progressive event in the disease, with up-regulation of cytokines, chemokines and GFAP increasing with age (Richard et al., 2008). Furthermore, it has been proposed that both astrocytosis and microglia activation are events preceding neuronal death in other neuropathic lysosomal storage disorders (Bellettato & Scarpa, 2010; Rama Rao & Kielian, 2016).

However, the region-specific differences we uncovered here had not been described yet. The heightened increase in GFAP in the cortex of GUSB animals correlates perfectly with our findings in both neuronal and synapse loss being more prominent in this brain region.

Astrocyte activation has been described as the first process to take place leading to activation of the microglia which can then trigger neuronal death exacerbating the neurodegenerative process (Bellettato & Scarpa, 2010). The fact that 1- and 3-month-old MPS VII mice exhibited only slightly increased Iba1 levels, but prominent Iba1 signal could be detected at 6 months at both cortex and hippocampus further confirms this timeline for mucopolysaccharidosis development in the CNS. Interestingly, the changes between brain areas observed for GFAP were not found when analyzing Iba1.

One pathway that could possibly relate inflammation and the synaptic deficits seen in MPS VII murine model is the complement cascade. Therefore, we were interested in analyzing the classical activation of the pathway using the levels of C1q as a marker and we found interesting results. C1q expression is higher in GUSB animals and the difference between phenotypes increases with age suggesting a progressive overactivation of the pathway.

At 6 month of age which is the latest stage analyzed in this thesis we found that the increase in C1q expression was more pronounced in the synaptosome fraction rather than in whole protein extracts. This suggest that C1q could be activating the complement pathway locally in the synapse inducing an aberrant synaptic pruning. Further evidence of complement activation is the increase in C3 levels found in the hippocampus of MPS VII mice.

The involvement of the complement pathway in the pathogenesis of other neurological disorders like Alzheimer's disease, in which increased complement factors can be found in the brain and CSF, has been proved by human genetic association studies(Hansen et al., 2018). C1q deletion in an Alzheimer's disease mouse model reduced astrocyte-synapse interaction and synapse engulfment rescuing synapse density. In neurodegenerative diseases like AD, PD, HD or ALS, C1q is induced by microglia to activate astrocytes to A1 phenotype, which in turn overregulate C3 and become neurotoxic (Liddelow et al., 2017). Furthermore, inhibition of C3 in several mouse models of the disease resulted in reduced plaque-associated synapse loss, amelioration of neuronal loss and improved neurophysiological and behavioral measurements (Wu et al., 2019). In frontotemporal dementia (FTD) the loss of progranulin, a pleiotropic factor involved in inflammation and lysosome function leads to the activation of the complement pathway

and increased synaptic pruning, offering another possible mechanism linking lysosome function to neurodegeneration. Moreover, gene delivery of this protein in models of Alzheimer's and Parkinson's -like diseases inhibited the progression of the phenotype (Chitramuthu et al., 2017; Lui et al., 2016). It would be interesting to analyze the expression of this secreted glycoprotein in our model to see if it could play a part in the neuropathology of MPS VII.

Using gene therapy, we were able to substantially reduce the levels of C1q in the hippocampus of MPS VII treated animals which could be one of the mechanisms behind the restoration of dendritic spine density found in the treated GUSB animals. This is especially relevant because it means that early treatment reaching the CNS of mucopolysaccharidosis type VII patients could lead to a rescue of the neurological phenotype.

Mitochondrial dysfunction and oxidative stress have been linked to multiple lysosomal storage disorders (Stepien et al., 2022) and our results show that dysregulation of the oxidative stress response is also a feature of mucopolysaccharidosis type VII.

Glutathione S-transferase-micro1 (GSTM1) is part of a glutathione S-transferases superfamily in charge of the metabolization of a wide range of xenobiotics and reactive oxygen species. Therefore, the decreased levels of GSTM1 exhibited by the GUSB KO cell line could be partially responsible for an impaired clearance of ROS. Abnormal glutathione homeostasis and glutathione-dependent enzyme activity have been increasingly implicated in neurodegenerative disorders, both in its induction and progression (Johnson et al., 2012).

Furthermore, genetic polymorphisms of glutathione S-transferases have been associated with increased risk of schizophrenia (Kim et al., 2015; Pinheiro et al., 2017; Shao et al., 2020) and Alzheimer's disease (de Mendonca et al., 2016; Jafarian et al., 2018). In Parkinson's disease, the link between GSTM1 polymorphisms and increased risk of developing the disease is not as evident and may be dependent on cytochromes P450 (CYPs) polymorphisms (Fan et al., 2022; Rebai et al., 2021; Wang et al., 2016).

Furthermore, it would be interesting to analyze the expression of GSTM2 in our cellular model because it has been described that the absence of GSTM1 can be compensated by overexpressing GSTM2 maintaining physiological glutathione S-transferase activity levels (Bhattacharjee et al., 2013).

In the cellular model for MPS VII the Nrf2-Keap1 system is overactivated as demonstrated by both the 50% reduction in Keap1 and the increased Nrf2 levels. Keap1 is an Nrf2 inhibitor and therefore higher levels of this protein lead to inhibition of Nrf2 as a transcriptional factor. However, nuclear translocation of Nrf2 is a critical process for its activity as transcriptional factor, and therefore location of Nrf2 in the cell can be used as a marker of transcriptional activity.

For that reason, in our analysis we separated the cytosolic and nuclear fraction to be able to see the location in which Nrf2 was accumulating. As stated in the results, the levels of Nrf2 in the cytosol were low and indistinguishable between genotypes, but GUSB KO cell cultures presented reduced levels of Nrf2 in the nucleus. These results suggest that although response to oxidative stress might be increased due to Keap1 reduction, the downstream events resulting in the expression of antioxidant genes may not be taking place as Nrf2 is not increasingly located in the nucleus. These results are in agreement with those found in a meta-analysis of Parkinson's and Alzheimer's disease in which Nrf2 expression was upregulated, but its target genes were all downregulated (Q. Wang et al., 2017).

Other than that, Nrf2 activation has been proposed to have a neuroprotective effect by increasing antioxidant capacity and clearance of misfolded proteins and decreasing neuroinflammation (Dinkova-Kostova et al., 2018). As such activation of Nrf2 has been suggested as a strategy to treat and ameliorate the symptomatology of neurodegenerative diseases (Aflaki et al., 2016; Yamazaki et al., 2015). Furthermore, studies have described molecular crosstalk between Nrf2-Keap1 system and autophagy, another mechanism known to be impaired in MPS VII (Stepkowski & Kruszewski, 2011).

Oxidative stress and autophagy are also related through NF-κB, a pleiotropic regulator of cellular biology activity acting as a transcriptional factor by binding to specific promoter regions in the genome. In a macrophage model of Gaucher disease elevated levels of p62 have been described leading to activation of NF-κB in the nucleus which results in increased expression of inflammatory cytokines. (Aflaki et al., 2016). This transcription factor is known to play a central role in the regulation of both inflammation and cellular death signaling, increasing its relevance in neurodegenerative disorders (Singh & Singh, 2020). However, the specific role of NF-κB is

exceedingly complicated to describe as it has been proposed to have both neuroprotective and pro-apoptotic effects with both activation and inhibition strategies being proposed as therapeutic approaches (Kaltschmidt et al., 2022; Singh & Singh, 2020).

In our case, we detected increased NF-κB levels in the MPS VII cell model, suggesting an overactivation of the pathway which could be either an early neuroprotective response or an enhancer of the neurodegeneration increasing neuroinflammation. Another interesting insight in the increased levels of NF-κB found in the GUSB KO cell cultures comes from the previously described decrease of Nrf2 in the nucleus of the cells. The existence of an interplay between these two pathways is well known and using Nrf2 knock out cells NF-κB activity increases resulting in higher cytokine production (Lingappan, 2018; Pan et al., 2012). Therefore, it could be hypothesized that the elevated levels of NF-κB we found in our cellular model could be at least partially caused by the reduced activity of Nrf2.

One of the recurring questions regarding mucopolysaccharidosis is how GAG accumulation affects intracellular trafficking and metabolism. Most lysosomal storage disorders are linked to autophagy defects; however, basal autophagy has been suggested to protect from early onset of neurodegeneration(Hara et al., 2006). One of the mechanisms proposed to lead to cell death in MPS IIIA is the defective fusion of autophagosomes and lysosomes which would result in accumulation of poly-ubiquitinated proteins and dysfunctional mitochondria (Settembre et al., 2008).

Our characterization of the autophagic phenotype in the early stages of mucopolysaccharidosis type VII corraborates some of the described events in other mucopolysaccharidoses. Progressive increase in Lamp1 signal was detected in cortex and hippocampus of GUSB animals at 1 and 3 months of age proposing autophagy disfunction as an early event in the development of the disease.

Once again, the affectation we detected was greater in the cortex than in the hippocampus which is why we used cortex samples to further analyze autophagy related proteins.

Lamp1 has been found increased in neurons and glial cells of animal models for MPS I, MPS IIIB and MPS IIID which indicate that aberrant autophagy might be a shared mechanism amongst all mucopolysaccharidoses which

would make any finding more relevant for its translatability (Baldo et al., 2015; Ohmi et al., 2003; Takahashi et al., 2021).

In a *Drosophila* model of MPS II, a glia specific knockdown of iduronate 2-sulfatase (IDS) was not enough to affect Lamp1 levels suggesting that a complete abolishment of enzymatic activity is needed for the development of autophagic disfunction (Rigon et al., 2020). These results suggest that gene therapy would most likely be able to correct this specific defect. Nonetheless, study of autophagy in treated and non-treated animals at later ages should be performed for confirmation.

Although it is commonly assumed that lysosomal storage disorders are based on a deficient endolysosomal trafficking pathway which leads to the accumulation of lysosomal-originated vesicles inside the cells, other studies propose defects in the secretory pathway as alternative mechanisms leading to the development of the disorders (Ballabio & Gieselmann, 2009).

For instance, in a mouse model of MPS IIIB the storage vesicles accumulated in cortical neurons were not related to endocytosis nor autophagy but to defective Golgi trafficking. This uncovers another mechanism that would be interesting to study in our model to analyze whether Golgi structure and function is altered in Sly Syndrome (Vitry et al., 2010). Unpublished data from previous researchers of our group suggested a possible involvement of the Golgi apparatus but not endoplasmic reticulum in the pathology of MPS VII.

Beclin 1 is another critical protein in autophagy due to its involvement in every major step in the process from autophagosome formation to autophagososme/endosome maturation (Kang et al., 2011). The increase in Beclin1 levels we found in both the cellular and the murine model of MPS VII are indicative of either increased initiation of autophagy or impaired progression which would result in accumulation of immature autophagosomes.

Although increased Beclin1 is a feature of Niemann-Pick disease, changes in Beclin1 expression have not been described in mucopolysaccharidoses (Settembre et al., 2008).

In traumatic brain injury Beclin1 expression is rapidly increased near the injury site which may suggest that in early stages elevated Beclin1 are caused by overactivation of autophagy as a protection mechanism (Erlich et al.,

2006). However, a study which mimicked Alzheimer's disease by generating Aβ toxicity in a neuronal cell line found that although autophagy is induced in response to Aβ treatment it does not provide any significant protection against apoptosis. Moreover, knockdown of Beclin1 showed protective effects against Aβ treatment with less cells undergoing apoptosis (Saha et al., 2021). Therefore, we could hypothesize that Beclin1 is increased in early stages of MPS VII as a protective mechanism against GAG accumulation but overactivation of autophagy ends up contributing to the worsening of the pathology.

Another marker of autophagic flux is p62, which we also found increased in all models of MPS VII analyzed in this thesis. It has been described that α-Synuclein can lead to increased p62 levels and microglial autophagic impairment which contributes to neuroinflammation (Tu et al., 2021), providing another link between the different alterations we have described in MPS VII.

Furthermore, immunoreactivity of p62 together with α-Synuclein in spheroidal structures was described as indicative of impaired axonal trafficking, although its effect in neuronal function remains unclear (Beard et al., 2017). As our primary cultures present increased p62 also in the dendritic processes, it would be interesting to analyze if axonal dystrophy is also a feature of MPS VII.

Another study using the murine model for MPS II found that inhibition of autophagy reduced neuronal degeneration but did not suppress damage to microglia cells, suggesting that regulation of autophagy could be used as co-treatment for mucopolysaccharidoses (Maeda et al., 2019).

Interestingly, p62 accumulation may be one of the mechanisms triggering the activation of NF-κB, further rounding the results we have obtained in the cellular model of MPS VII. Furthermore, p62 also interacts with the Nrf2/Keap1 pathway at different levels. On the one hand the direct binding of p62 to Keap1 promotes Nrf2 release and activation. On the other hand, it has been described that p62 is one of the Nrf2 target genes which would create a positive feedback loop between these two pathways (Stepkowski & Kruszewski, 2011).

One of the most conflicting findings of our work has been the reduced levels of total and phosphorylated Erk in both, the cortex of MPS VII mice and the

GUSB KO cell line. The ERK pathway has not been explored in mucopolysaccharidoses, and its status in other lysosomal storage disorders is highly variable.

In opposition to our findings, increased autophagy in a mouse model for G(M1)—gangliosidosis occurred with increased Akt-mTOR and Erk signaling (Takamura et al., 2008). Nonetheless, a study using cDNA expression microarrays in samples from Niemann-Pick C1 disease (NPC) detected downregulation of the ERK-MAPK kinase pathway, but its effect in the development of the diseases is not clear (Hetmanczyk-Sawicka et al., 2020).

Increased Erk phosphorylation, especially in microglia, has been proposed to enhance neuroinflammation leading to neurodegeneration in Alzheimer's and Parkinson's disease (Albert-Gasco et al., 2020; Chen et al., 2021; Kim & Choi, 2010). Moreover, suppression of Erk activity in rat hippocampus has been shown to alleviate Alzheimer's disease pathology suggesting new therapeutical approaches (Muraleva et al., 2021).

It is also known that the Erk pathway is a key regulator of autophagy and activation of Erk leads to increased levels of p62 (Kim et al., 2014). Additionally, increased Erk activity has been described in rats showing autistic-like behaviors resulting in impaired autophagic activity and dendritic spine loss in hippocampal neurons (Choi et al., 2020).

Since the models studied in this thesis present high levels of p62 but reduced Erk activation other mechanisms must be responsible for the increased autophagy in Mucopolysaccharidosis type VII. Some of these mechanisms are oxidative stress which has been found increased in MPS VII and could be affecting p62 expression and the JNK/c-Jun pathway, which would need to be studied to detect any alteration in our models(Go et al., 2017; Puissant et al., 2012).

Contradictory results have been found in ALS in which some studies using transgenic SOD1 mice describe a down-regulation of Erk and up-regulation of JNK leading to dysregulation in axonal transport (Perlson et al., 2009), and other find overactivation of the pathway to be involved in the development of the neurological symptoms and even propose inhibition of the Erk signaling pathway as a preventive strategy (Sahu et al., 2021).

Furthermore, Erk activation is required to obtain nuclear localization of Nrf2 (Zipper & Mulcahy, 2003) , therefore the reduction in nuclear Nrf2 we found in MPS VII mice could be caused by the reduced levels of Erk activation.

Intriguingly, Erk signaling has been found downregulated in humans and various chronic animal models of depression which has been linked to decreased sexual behavior (Feng et al., 2003; Wang & Mao, 2019). Therefore, although the effect of reduced Erk phosphorylation in the neurodegeneration exhibited by MPS VII mice remains unclear, it provides an explanation for the impaired matting behavior of this animals.

The involvement of Akt signaling pathway in neurodegenerative disorders seems to be much more straightforward and goes in accord with the results we have obtained. This pathway is known to be involved in apoptosis and inflammation, with activation of Akt promoting both processes.

Reduced Akt phosphorylation has been described in Parkinson's disease, and stimulation of Akt activity has been proposed to have a beneficial effect (Arab et al., 2021; Malagelada et al., 2008). In Alzheimer's disease Akt signaling pathway has been proposed to be suppressed in early stages to promote autophagy but overactivated at later points, and therefore both positive and negative modulators of Akt activation have been studied and found to be beneficial for Aβ clearance and cognitive function (Long et al., 2021; Razani et al., 2021; Y. Wang et al., 2020; Yang et al., 2020).

However, in Neuronal Ceroid Lipofuscinoses, combined upregulation of both the Akt and the Erk pathway has been described which emphasizes the difference in mechanisms between disorders sharing neuropathological symptoms and the need for specific research in each case (Vidal-Donet et al., 2013).

Interestingly, it has been described that reactive oxygen species can cause redox modification of Akt in the synapses, leading to compromised Akt signaling and impairment of activity-dependent protein translation, a necessary function for synaptic plasticity (Ahmad et al., 2017). Since we have also found increased oxidative stress in our MPS VII models, we could hypothesize that the excessive ROS are involved in the reduction of phosphorylated Akt we detected in the cortex of the GUSB animals, and this could be one of the factors contributing to the spine pathology.

Although the mechanisms underlying neurodegeneration involve a wide range of processes, in this thesis we provide a comprehensive analysis of a myriad of pathways dysregulated in this disorder and their role in the development of the neuropathological symptoms. More importantly, we have demonstrated that our therapeutical approach is able to restore physiological values in most of the factors and processes analyzed and therefore could have beneficial effects in human patients.

7. Conclusions

1. Nucleofection is a reliable strategy to deliver large plasmids to the hard to transfect SH-SY5Y cell line.
2. A synthetic PTG cassette bearing GUSB-specific gRNAs achieved 99% reduction in β-Glucuronidase activity in the SH-SY5Y GUSB KO justifying its use as a Mucopolysaccharidosis type VII model.
3. GUSB mice exhibit an age-dependent neuronal loss in the CA3 of the hippocampus and the entorhinal cortex, but it is not significant enough to explain the neuropathology of MPS VII.
4. MPS VII mouse model show a significant decrease in dendric spines in both CA1 and CA3 of the hippocampus at 5 months of age. This decrease is exacerbated in the pyramidal neurons of the entorhinal cortex.
5. Gene therapy using intrathecal administration of an AAVrh10 encoding a functional β-Glucuronidase is able to restore the dendritic spine density present in GUSB mice.
6. The MPS VII neuronal cell line could be morphologically differentiated, but primary cultures of GUSB mice cortex show reduced arborization.
7. Glutamate Receptor 1 levels are decreased in cortex but not in hippocampus of GUSB mice at 1, 3 and 6 months of age. This reduction was restored after gene therapy.
8. SH-SY5Y GUSB KO cell line shows reduced levels of glutamate decarboxylase (GAD67) and SNAP25, proteins related to neuronal function.
9. No differences were observed between WT and GUSB mice in the expression of NCAM and Dopamine D2 receptor in the cortex of 6-month-old animals, and the scaffolding protein Homer 1 showed a non-significant reduction in MPS VII mice.

10. Calbindin levels are reduced in cortex and cerebellum of the GUSB mice, as well as in the KO GUSB cell line. The expression was restored after gene therapy.
11. Increased accumulation of α-Synuclein has been detected in the cortex of GUSB mice and in primary cultures of MPS VII cortical neurons.
12. GUSB mice exhibit higher brain inflammation compared to WT as demonstrated by significantly increased of both GFAP and Iba1. This rise in inflammation is more pronounced in the cortex than in the hippocampus. AAVGUSB treatment achieved a complete restoration of GFAP levels and a partial recovery of the Iba1 levels.
13. The complement system protein C1q shows age dependent increased in GUSB animals when compared to WT littermates. Furthermore, C3 levels were also higher in the hippocampus of MPS VII mice at 6 months of age indicating increased activation of the pathway. Our therapeutic strategy resulted in a normalization of the C1q protein level in hippocampus.
14. Increased levels of NF-κB and overactivation of the Nrf2 pathway indicates abnormally increased oxidative stress in the KO GUSB SH-SY5Y cells. Moreover, the MPS VII neuronal cell line exhibits reduced levels of GSTM1 suggesting deficient clearance of reactive oxygen species.
15. Cellular signaling is dysregulated in MPS VII as showcased by the reduction in Akt and Erk signaling found in the cellular and animal models of the disease. Treatment of the animals resulted in increased Erk and Akt activation.
16. Mucopolysaccharidosis type VII presents enhanced initiation and/or an impaired progression of autophagy in the CNS as demonstrated by increased levels of several autophagy-related proteins.

8. Bibliography

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