

PhD Thesis / Tesi Doctoral

**INTRATHECAL ADMINISTRATION OF AAVrh10 CODING FOR
 β -GLUCURONIDASE CORRECTS BIOCHEMICAL AND HISTOLOGICAL
HALLMARKS OF MUCOPOLYSACCHARIDOSIS TYPE VII MICE
AND IMPROVES BEHAVIOR AND SURVIVAL**

Author / Autora:

Director / Directora:

Gemma Pagès i Pi

Assumpció Bosch Merino

Doctorat en Bioquímica, Biologia Molecular i Biomedicina
Departament de Bioquímica i Biologia Molecular
Universitat Autònoma de Barcelona

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ABBREVIATIONS

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AAV	adeno-associated virus
ALD	adrenoleukodystrophy
ANOVA	analysis of variance
β-gluc	β-glucuronidase
β-hex	β-hexosaminidase
BBB	blood brain barrier
BG	basal ganglia
bp	base pairs
Bs	brainstem
BSA	bovine serum albumina
Cb	cerebellum
CNS	central nervous system
cv	cervical
Cx	cortex
D-PBS	Dulbecco's phosphate-buffered saline
DMEM	Dulbecco's modified eagle medium
DRG	dorsal root ganglia
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERT	enzyme replacement therapy
FBS	fetal bovine serum
fg	femtograms
Fwd	forward
GAG	glycosaminoglycan
GFP	green fluorescence protein
GL	granular layer
Hc	hippocampus
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
Ht	hypothalamus
HTZ	heterozygote
IC	intracranial
ICM	intra-cisterna magna
ICV	intra-cerebroventricular
IF	immunofluorescence
IgG	immunoglobulin G

IT	intrathecal
ITR	inverted terminal repeat
IV	intravenous
kDa	kiloDalton
LAMP-1	lysosome-associated protein 1
LINCL	late infantile neuronal ceroid lipofuscinosis
Imb	lumbar
LMP	lysosomal membrane protein
low-th	lower thoracic
LPL	lipoprotein lipase
m	month
μ-CT	micro-computed tomography
M6P	mannose 6-phosphate
mg	milligram
MHC	major histocompatibility complex
μl	microliter
ML	molecular layer of the cerebellum
MLD	metachromatic leukodystrophy
MPR	mannose-6-phosphate receptor
MPS VII	mucopolysaccharidosis type VII
MW	molecular weight
NAb	neutralizing antibody
NHP	non-human primate
NS	nervous system
OB	olfactory bulb
OMIM	online mendelian inheritance in man
ORF	open reading frame
PCR	polymerase chain reaction
Pf	prefrontal
PFA	paraformaldehyde
PL	Purkinje layer
PNS	peripheral nervous system
PS	pilot study
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
rAAV	recombinant adeno-associated virus
rcf	relative centrifugal force
RLU	relative light units
RMA	repeated measures analysis of variance
rpm	revolutions per minute
RT	room temperature

Rv	reverse
s	second
SC	spinal cord
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERCA2	sarcoplasmic/endoplasmic reticulum calcium ATPase 2
SPF	specific pathogen free
T	temperature
TBS	tris buffered solution
TEMED	N, N, N', N', tetrametil-etilen-diamine
TGN	<i>trans</i> -Golgi network
Th	thalamus
th	thoracic
TMB	3,3',5,5' tetramethylbenzidine
up-th	upper thoracic
vg	viral genomes
VTA	ventral tegmental area
WB	western blot
WPRE	woodchuch hepatitis virus posttranscriptional regulatory element
WT	wild type

INTRODUCTION

INTRODUCTION

Gene therapy is a promising therapeutic approach for many diseases that do not have any available treatment. Among them, diseases caused by the lack of function of a specific gene are good candidates for gene therapy strategies. Thus, the aim of this work is to develop a gene therapy strategy for mucopolysaccharidosis type VII (MPS VII), an ultrarare monogenic disease affecting both central nervous system and peripheral organs, for which there is not any available treatment.

1. GENE THERAPY WITH AAV VECTORS

In the design of a gene therapy approach, the ultimate aim is to use a vector that transduces the target tissues required, while avoiding any deleterious effects due to the host immune system. In this sense, adeno-associated viral vectors (AAV vectors) have several features that make them very attractive as gene therapy vectors: they are able to transduce dividing and quiescent cells –though mainly transducing the later–, they provide long term expression of the transgene, they are not able to autonomously replicate without a helper virus, and wild type AAV infections are not pathogenic.

Since they were first used for gene delivery, AAV vectors have been presented as a promising tool for central nervous system (CNS) transduction due to their capacity of transducing quiescent cells (reviewed by Xiao et al. (1997)). In the last 15 years, great efforts have been invested in the characterization of the tropism of different serotypes of AAV vectors in diverse animal models, with the aim to use them as gene therapy vectors. Lately, and after the rise of immunogenicity concerns related to AAV vector administration, there is also a growing research field covering this topic.

In this part of the work we will first present the AAV vectors and their actual status in the clinics. Then we will discuss the current research field in AAV vector tropism and administration routes, and finally we will present the related immunogenicity issues.

1.1 ADENO-ASSOCIATED VIRAL VECTORS

AAV is classified in the parvovirus family, which comprises some of the smallest DNA animal viruses. The parvovirus capsid diameter is approximately 25 nm and it is composed entirely of protein and DNA. For a productive infection, AAVs require coinfection with helper viruses (e.g. adenovirus, herpes simplex virus, vaccinia virus, human papilloma virus), and the AAV infection by itself is not pathogenic in humans.

The AAV genome is a linear, single-stranded DNA of 4,680 nucleotides. The DNA contains two genes that are transcribed from three different promoters: the gene *Rep* encodes four replication proteins (Rep78, Rep68, Rep52, Rep40); the gene *Cap* encodes the three proteins that will conform the capsid (VP1, VP2, VP3) and an assembly-activating protein (AAP), which is transcribed from an alternative ORF and is required for capsid assembly (Sonntag et al. (2010)). These genes are flanked on either side by 145 bp inverted terminal repeats (ITR) that are required for DNA packaging (**Figure 1**). When AAV encounters a host cell it enters by receptor-mediated endocytosis, and it is transported to the nucleus. There, the genome is released and converted to double stranded DNA by Rep proteins and cellular DNA synthesis machinery. In the absence of a helper virus, wild type AAV dsDNA can remain in circular episomal form or can be integrated into a specific region of human chromosome 19, after ITR mediated concatamerization. AAV transcription and DNA replication can be reactivated by helper virus infection or cellular stress, thus completing the AAV replication cycle (Grieger and Samulski (2012), Ojala et al. (2014)).

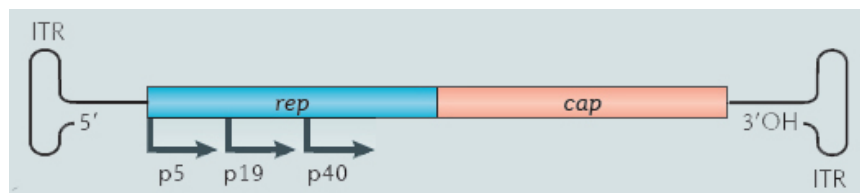


Figure 1: Wild type AAV genome (Adapted from Kotterman and Schaffer (2014))

Recombinant AAV vectors (rAAV) are one of the most important and safe viral gene delivery vectors and they are simple to design. Since the ITR are the only cis-acting elements necessary for genome replication, integration and packaging, the rAAV are

produced replacing the *Rep* and *Cap* genes with an expression cassette containing a promoter, the therapeutic gene and a polyadenylation sequence. In the production process of the rAAV, the *Rep* and *Cap* genes are provided in trans from a different plasmid lacking ITRs, which prevents their packaging and avoids the formation of wild type AAV particles. The helper virus genes required for AAV replication and production are also provided *in trans* (Figure 2).

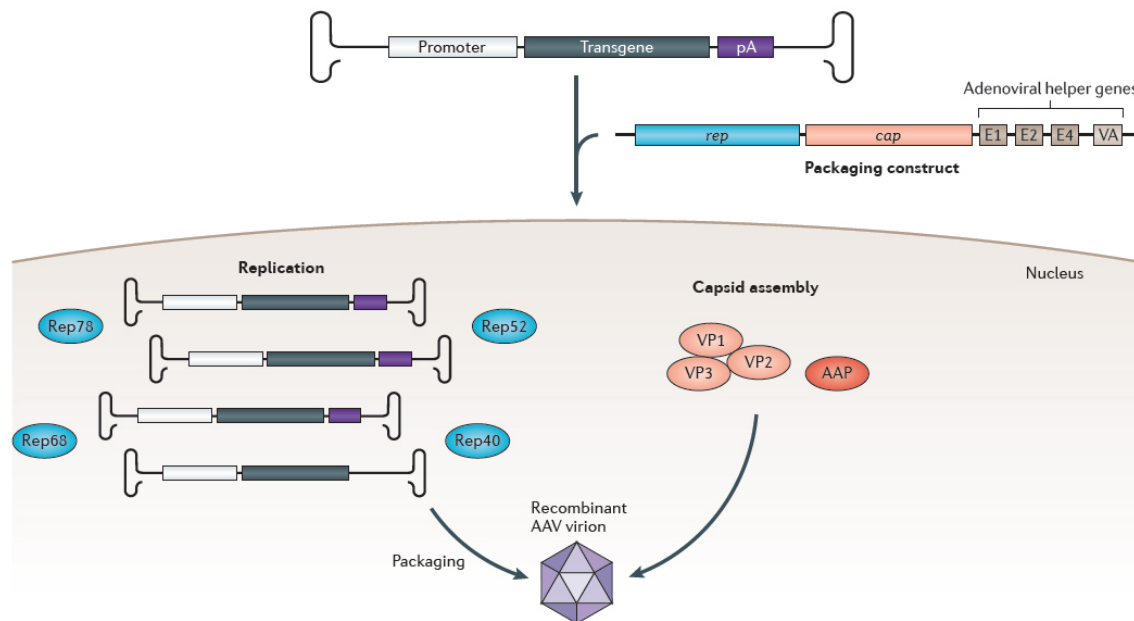


Figure 2: Recombinant AAV vector production scheme (From Kotterman and Schaffer (2014))

Although rAAV vectors present many advantageous features, they have a limited packaging capacity due to the small size of the AAV genome. It has been described that the vector cassette including the ITRs should not exceed 4.7 - 5.0 kb, being 4.4 - 4.7 kb the maximum transgene cassette length for efficient rAAV production (Grieger and Samulski (2012)). In contrast to wild type AAV, rAAV integration is inefficient and vector genomes remain the transduced cells in episomal form (McCarty et al. (2004)).

An important number of AAV serotypes have been identified. The first AAV serotypes were described as contaminants in human adenoviral isolates (AAV1 to AAV6). Later on, Gao et al. (2002) isolated AAV7 and AAV8 from rhesus macaque samples and, subsequently, they performed an exhaustive work to detect and characterize AAV

latent infections in human and non-human primate tissue samples. They isolated over 90 new AAV serotypes and classified them in phylogenetic clades (Gao et al. (2004)). The capsid proteins of the different AAV serotypes isolated from primates present 51-87% identity between clades, with the variability mostly located in the surface exposed regions of the capsid (**Figure 3**) (Zinn and Vandenberghe (2014)).

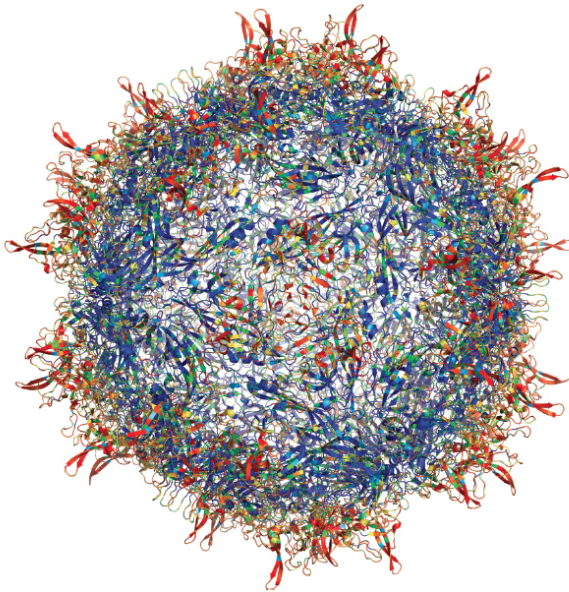


Figure 3: Variability of the AAV capsids plotted on the structure of the AAV2 capsid. The blue end of the spectrum represents more conserved positions while the red end represents more variable positions. (From Zinn and Vandenberghe (2014))

These surface exposed regions of the AAV capsids determine the interactions with the host cell surface, where several glycans, such as heparan sulfate or sialic acid, have been identified as primary receptors for many AAV serotypes (Huang et al. (2014)). This specific binding to cell surface glycans determines the differential transduction of each AAV among the diverse cell types and, therefore, the different tropism exhibited by the different serotypes.

Characterizing the specific tropism of each AAV serotype is of great interest before using them as vectors for gene therapy strategies. But not only the naturally occurring AAV serotypes are being studied: engineering of new AAV variants or mutant strains allows the generation of AAV vectors with different characteristics. These vectors can be generated either by rational design of AAV capsids after the knowledge of their structure and features, or by directed evolution of AAV capsids using capsid libraries and screening methodologies. These engineered AAV vectors could address the challenging issues of AAV gene delivery and efficacy: enhancing

tropism for target cells and detargeting the off-target tissues, providing the ability to cross epithelial barriers such as blood-brain barrier, modulating the interactions with the immune system, and increasing the packaging capacity (Kotterman and Schaffer (2014)).

1.2. A STEP FORWARD: AAV VECTORS INTO THE CLINICS

Until January 2015, 127 gene therapy clinical trials used AAV vectors to deliver the therapeutic genes. It accounts for 5.9% of total gene therapy clinical trials and represents an increase compared to 2012, when AAV-based clinical trials were 4.9% (Ginn et al. (2013), Gene Therapy Clinical Trials Worldwide, provided by The Journal of Gene Medicine, January 2015 update, www.abedia.com/wiley). Initially, AAV2 and AAV1 were the serotypes preferentially used in the clinical trials, although several trials with serotypes 5, 8, 9 and rh10 are currently ongoing. These trials include diseases like Duchenne muscular dystrophy, macular degeneration, Parkinson disease, spinal muscular atrophy, cystic fibrosis and some lysosomal storage diseases such as MPS type III, Pompe disease and late infantile neuronal ceroid lipofuscinosis (www.clinicaltrials.gov).

AAV gene therapy has demonstrated its safety and efficacy in clinical trials for monogenic diseases such as lipoprotein lipase (LPL) deficiency (Stroes et al. (2008) and subsequent publications), Leber's congenital amaurosis (Hauswirth et al. (2008) and subsequent publications), and hemophilia B (Nathwani et al. (2011b)). Moreover, it has also been successful for treating an idiopathic disease such as severe heart failure by the administration of an AAV vector coding for the SERCA2 gene (Jaski et al. (2009)).

In 2012, the European Commission granted marketing authorization for the first AAV therapeutic product, Glybera®. It is designed for LPL deficiency and is authorized only under exceptional circumstances. The product is administered intramuscularly and consists of an AAV1 vector containing an expression cassette with a high activity form of the human LPL protein (Ylä-Herttuala (2012), www.uniqure.com/products/glybera).

1.3. AAV VECTOR RESEARCH: ADMINISTRATION ROUTES AND VECTOR TROPISM

The characterization of the tropism of the AAV vectors is a main issue in gene therapy research. It is the purpose of a high number of publications that use and compare different AAV serotypes, administration routes, vector purification methods and vector doses in diverse animal models. In consequence, it is also the topic of many review articles (e.g. Asokan et al. (2012), Murlidharan et al. (2014), Zinn and Vandenberghe (2014)). Currently, serotypes AAV1 to AAV9, followed by AAVrh10, are the serotypes better characterized and most used in gene therapy preclinical assays. The tropism of an AAV vector mainly depends on the serotype, but it is also dependent on the administration route.

The delivery of the AAV vector into the organism can be done either systemically or directly into a specific organ, depending on the target disease. The AAV administered into a specific organ is expected to mainly transduce cells in this organ, where the therapeutic protein can either do its function or be secreted to the bloodstream. An example of the first case would be the approach used for Leber's congenital amaurosis clinical trials: they used the subretinal injection of an AAV2 coding for the lacking RPE65 gene, and the vector transduced the retinal pigment epithelial cells, restored the protein function and lead to therapeutic benefits (Acland et al. (2005)). On the other hand, Glybera®, the gene therapy product for LPL deficiency, is an example of secretion of the therapeutic protein to the bloodstream: the AAV1 vector is injected intramuscularly, it transduces muscle cells and the recombinant LPL protein is expressed and released into the bloodstream, where its enzymatic activity is required (Ross et al. (2006)).

The systemic administration of AAV vectors is an easier and less invasive approach than the administration to a specific organ. The broad biodistribution of the AAV vector after intravascular delivery favors the transduction of different organs and tissues throughout the whole body. However, the targeted organs mainly depend on the AAV serotype, and some serotypes present wider transduction patterns than others after intravascular injection. AAV8 is an example of liver-preferential tropism after intravascular delivery in humans: the clinical trial for hemophilia B was based

on intravenous delivery of AAV8 that mainly transduced hepatocytes, from where the clotting factor IX protein was secreted to the bloodstream and led to therapeutic benefits (Nathwani et al. (2011a)). In contrast, AAV9 displayed a broader transduction pattern in the initial preclinical assays in mice, transducing many peripheral organs, such as liver, heart and skeletal muscle (Inagaki et al. (2006)).

The transduction of multiple organs after IV AAV delivery can be of interest in the case of monogenic diseases that affect multiple organs, such as MPS VII. However, this disease affects peripheral organs, skeletal structures and also the CNS. The CNS can be specifically targeted by direct AAV administration into the brain parenchyma, but that would restrict the therapeutic gene expression into the brain. Therefore, a gene therapy approach that could deliver the therapeutic protein to the peripheral organs and also to the CNS would be of great interest.

One of the major issues of gene therapy and other therapeutic approaches that target the CNS is the presence of the blood-brain barrier (BBB). This BBB is an endothelial barrier present in capillaries that course through the brain, and it is in close contact with astrocytes. These endothelial cells present different characteristics than those in most peripheral tissues: they have low rate of endocytosis and they are coupled by tight junctions. These features allow their function as the BBB: to impede the entry from blood to brain of virtually all molecules. Only small and lipophilic molecules can cross the BBB and enter the brain without any active transport. Other essential nutrients and macromolecules, such as glucose, amino acids or transferrin, can enter the brain by specific membrane transporting proteins (Rubin and Staddon (1999)). In mice, but not in humans, the tight junctions are not completely formed at birth, so in neonatal mice the permeability of the BBB is greater than in adults (Stewart and Hayakawa (1987)).

Many therapeutic agents, such as recombinant proteins or viral vectors, find a barrier to enter the CNS from the bloodstream. In the case of gene therapy for MPS VII, the therapeutic protein could be produced in peripheral organs and secreted to the bloodstream, but it would not reach the CNS due to the BBB presence. Therefore, an AAV vector that could cross the BBB and transduce cells in both CNS and peripheral

organs would be of great interest. **Figure 4** represents the different administration routes used for AAV delivery to the CNS that will be discussed:

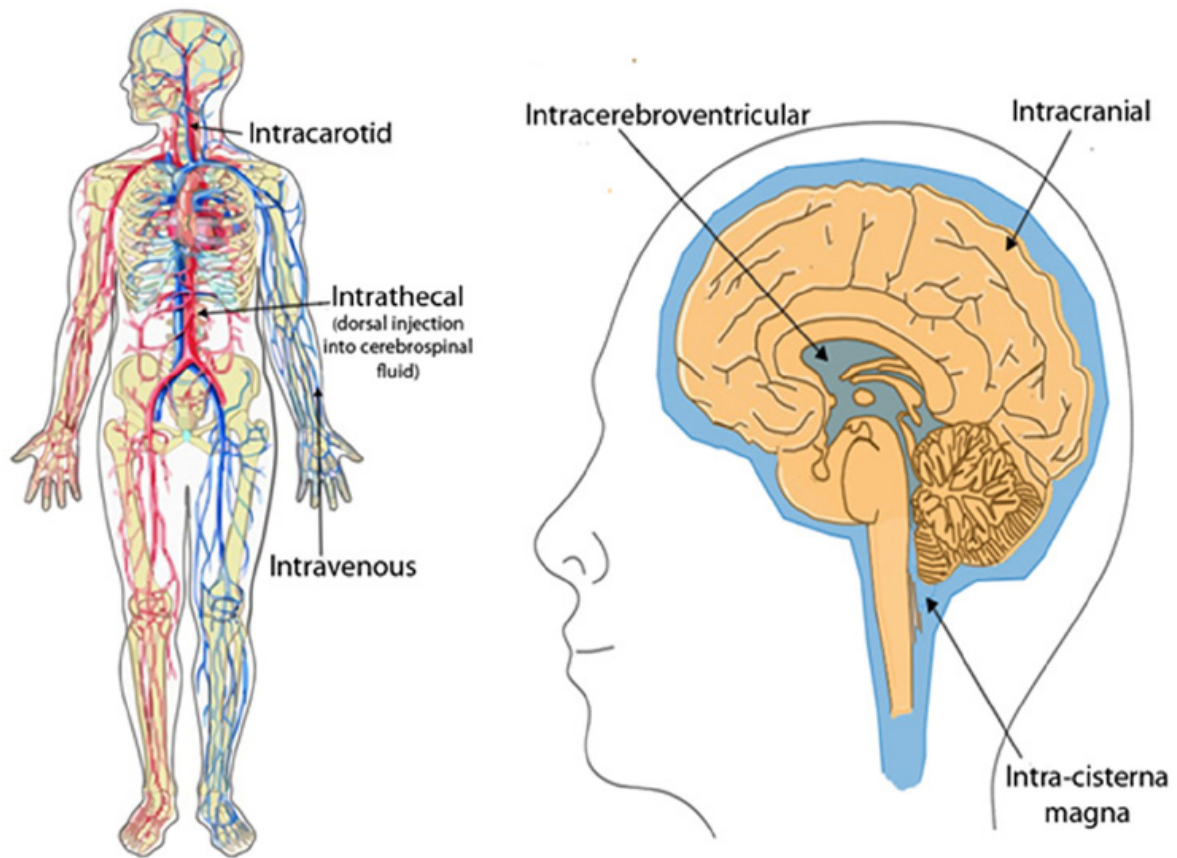


Figure 4: Administration routes to reach the CNS. Intracarotid and intravenous routes deliver the vector to the bloodstream. Intracranial injection delivers the vector to the brain parenchyma. Intracerebroventricular, intra-cisterna magna and intrathecal routes deliver the vector to the cerebrospinal fluid (CSF) (from Ojala et al. (2014)).

1.3.1. Intravascular AAV administration

Foust et al. (2009) described the capacity of AAV9 to transduce cells in the CNS when administered intravenously (IV), reaching the brain and the spinal cord. It was the first evidence that an AAV serotype could cross the blood-brain barrier (BBB) in adult mice, thus proposing IV administration as a less invasive route than intracranial injection (IC), in order to target the CNS by gene therapy. They reported that IV administration of AAV9 in adult mice achieved transduction of brain and spinal cord, where 90% of transduced cells were astrocytes, besides vascular cells and some localized neurons. In spinal cord, they reported the transduction of 65% of total

astrocytes. However, a more recent work by Gray et al. (2011) described the transduction of neurons and astrocytes in different proportions depending on the brain area (2:1 in hippocampus and striatum; 1:1 in cortex). When injected to non-human primates (NHP), AAV9 preferentially transduced astrocytes in the brain by intravenous (Gray et al. (2011), Bevan et al. (2011)) or intracarotid injection (Samaranch et al. (2012)), while transducing motor neurons and glial cells in the spinal cord, and sensory neurons in dorsal root ganglia (Bevan et al. (2011)).

An experimental approach that allows high and widespread transduction of the CNS is the perinatal intravenous administration of AAV vectors, which is the delivery to fetuses or newborns. It has been published in different works in mice (e.g. Foust et al. (2009), Hu et al. (2010), Rahim et al. (2011), Zhang et al. (2011)) and also in NHP (e.g. Bevan et al. (2011), Mattar et al. (2012)) and reviewed by Karda et al. (2014). These authors propose perinatal intravenous gene therapy for the treatment of neurodegenerative diseases that also present systemic affection. However, the need of prenatal diagnosis for the use of these therapies makes them less feasible nowadays, because most of the clinical cases are diagnosed at more advanced stages.

The IV administration of AAV9 coding for therapeutic genes to adult mice has been used in diverse mouse models of neurological diseases including MPS. In a mouse model of MPS IIIB, Fu et al. (2009) reported normal or even supraphysiologic levels of the therapeutic protein in brain and in different somatic organs, which were stable between 6 and 9 months after treatment. This led to an improvement in the histopathological traits of the disease both in CNS and in somatic organs that were translated to behavior amelioration and increased survival. Ruzo et al. (2012) performed a similar approach in MPS IIIA mice achieving 65% increase of lifespan. Eight months after treatment, they got 10% of the therapeutic enzymatic activity in male and 8% in female brains, which normalized the pathological signs in most of the brain areas. In peripheral organs they got normal or higher levels of enzymatic activity and correction of the cellular pathological traits. More recently, Murrey et al. (2014) performed an IV approach in normal non-human primates with AAV9-NAGLU – the lacking gene in MPS IIIB that codes for a secreted protein– and reported high and sustained levels of the therapeutic protein both in CNS and peripheral organs:

2.9-12-fold above endogenous levels in somatic organs and 1.3-3-fold in brain, 6 months after injection.

Besides mucopolysaccharidoses, intravenous administration of AAV9 has been performed in a mouse model of Huntington disease, in which the expression of an iRNA for mutant huntingtin prevented atrophy of key brain regions and mice weight loss (Dufour et al. (2014)). Moreover, in a mouse model of amyotrophic lateral sclerosis the intravenous injection of AAV9 coding for a therapeutic gene reached cortical and spinal neurons and prevented progressive motor dysfunction (Yamashita et al. (2013)).

Even with these good results, when proposing the intravenous administration of AAV vectors as a gene therapy strategy that could allow targeting the CNS, some issues must be taken into account. An important issue is the preexisting immunity against AAV capsids in the host serum. Since the vector is delivered directly to the bloodstream, the preexisting immunity could preclude AAV transduction (see section 1.4.1).

Besides, when AAV vectors are capable to cross the BBB after IV injection in adult mice, they are also efficient in transducing many peripheral organs. As it was stated before, this feature is of interest in some multiorgan diseases, but the broad peripheral transduction is not desirable in therapies to treat disorders with affectation restricted to the CNS. In these cases, such as amyotrophic lateral sclerosis, the off-target transgene expression could have unknown effects. At present, different strategies are being tested in order to avoid either AAV transduction or transgene expression in peripheral tissues after IV administration (reviewed by Bourdenx et al. (2014)). However, the direct administration into the CNS could be a better approach for these neurodegenerative diseases.

1.3.2. Direct AAV administration to the CNS

Direct administration to the CNS can be done either by intracranial injection, delivering the vector into the brain parenchyma, or by injection to the cerebrospinal

fluid (CSF) in different anatomic localizations: the cerebral ventricles, the cisterna magna or the lumbar vertebral area (intrathecal) (see Figure 4).

1.3.2.1 Intracranial administration

Early it was demonstrated that AAV2 was capable to transduce neurons after injection into the brain parenchyma, although only around the area of injection (Bartlett et al. (1998)). Soon after, Davidson et al. (2000) reported that 15 weeks after intrastriatal injection, AAV5 was capable to transduce 5000 times more cells than AAV2, which were widely dispersed throughout the injected hemisphere. Contrasting to AAV2, AAV5 transduced both neurons and astrocytes, while AAV4 only transduced ependymal cells. Later on, AAV8 appeared to achieve stronger expression than AAV1, 2 and 5 when injected in the hippocampus or the substantia nigra of rat brains, transducing neurons but not astrocytes (Klein et al. (2006)).

When AAV8 was showing up to be a good candidate for CNS gene therapy, the work of Gao et al. (2004) brought to light a number of novel AAV serotypes that could potentially compete with AAV8 in CNS transduction, such as AAV9 and AAVrh10. Intracranial injections of AAV9 or AAVrh10 in rodents demonstrated neuronal transduction without apparent glial targeting, being AAV9 the most efficient serotype when compared also to AAV1 or AAV8. In addition, it was demonstrated that all these serotypes undergo axonal transport to distal parts of the brain, achieving larger CNS transduction (Cearley and Wolfe (2006), Cearley and Wolfe (2007), Klein et al. (2007), Castle et al. (2014)). When injected in the brain parenchyma of large animals such as dogs and NHP, AAV9 and AAVrh10 are able to transduce large volumes of brain, where they mainly target neurons, although in dogs it was detected a significant astrocyte transduction by AAV9 (Masamizu et al. (2011), Sondhi et al. (2012), Swain et al. (2014), Rosenberg et al. (2014)). Two studies performing intracranial injections with some of the last identified serotypes (e.g. AAVhu32, AAVhu11, AAVpi2, AAVrh8, AAVcy5, AAVrh20, AAVrh39, AAVrh43) achieved transduction of larger volumes of brain than with AAV9 (Cearley et al. (2008)) or with AAV8 (Lawlor et al. (2009)). With some exceptions, the different AAV vectors

mainly transduced neuronal cell populations, and some serotypes were also able to transduce astrocytes and oligodendrocytes.

The intracranial injection of AAV vectors has led to successful results in many preclinical assays in different animal models of neurodegenerative diseases. Two recent examples could be the use of AAV5 and AAV9 in a mouse model of Alzheimer disease (Carty et al. (2013)) or the administration of AAVrh8 to a feline model of Sandhoff disease (McCurdy et al. (2014)). More importantly, some clinical trials have administered AAV vectors intracranially to patients for the treatment of diseases like MPS IIIA (Tardieu et al. (2014)) or late infantile neuronal ceroid lipofuscinosis (Crystal et al. (2004), www.clinicaltrials.gov: NCT00151216, NCT01161576 and NCT01414985), two neurodegenerative lysosomal storage diseases.

It is important to notice that, when compared to intravenous administration, intracranial AAV delivery allows the transduction of large areas of the brain using relatively low doses of AAV vector: e.g. Cearley and Wolfe (2007) used 1.2×10^{10} vg per mouse for intracranial injection, while Foust et al. (2009) used 4×10^{12} vg per mouse for intravenous injection. Therefore, the dose-scaling of the IV administration to achieve CNS transduction in larger animals, and in humans, would require great quantities of vector to be produced and very high doses to be administered, which extremely increases technical costs and raises biosafety concerns about this therapy. However, for widespread distribution in the brain, the intracranial injection may be performed by several injections that require perforating the skull, disrupting the BBB and inserting the needle through the brain parenchyma, making the overall strategy extremely invasive.

1.3.2.2. Administration to the CSF

The administration of the AAV vector to the CSF is an alternative approach for CNS transduction that could address the issues of the high dosage required for intravenous delivery and the invasiveness of intracranial approaches. This strategy could also prevent the initial contact of the AAV vector with the preexisting immunity in the host serum and allow a single injection procedure.

Cells in the choroid plexus, lining the cerebral ventricles, produce the CSF, which fills the ventricles and the subarachnoid space. The subarachnoid space is the space between arachnoid mater and pia mater, the two more internal meninges that enclose the brain, spinal cord, dorsal root ganglia and spinal roots. The classical theory describes that CSF circulates throughout all the subarachnoid space, around the brain and along the spinal cord, until it is resorbed by specific structures present in the arachnoid mater (arachnoid villi and granulations) and drained to the venous circulation via the superior sagittal sinus (**Figure 5**). However, other findings demonstrate that CSF is also drained from the subarachnoid space to the lymphatic system via the olfactory and respiratory submucosa in the cribriform plate (Irani (2009)).

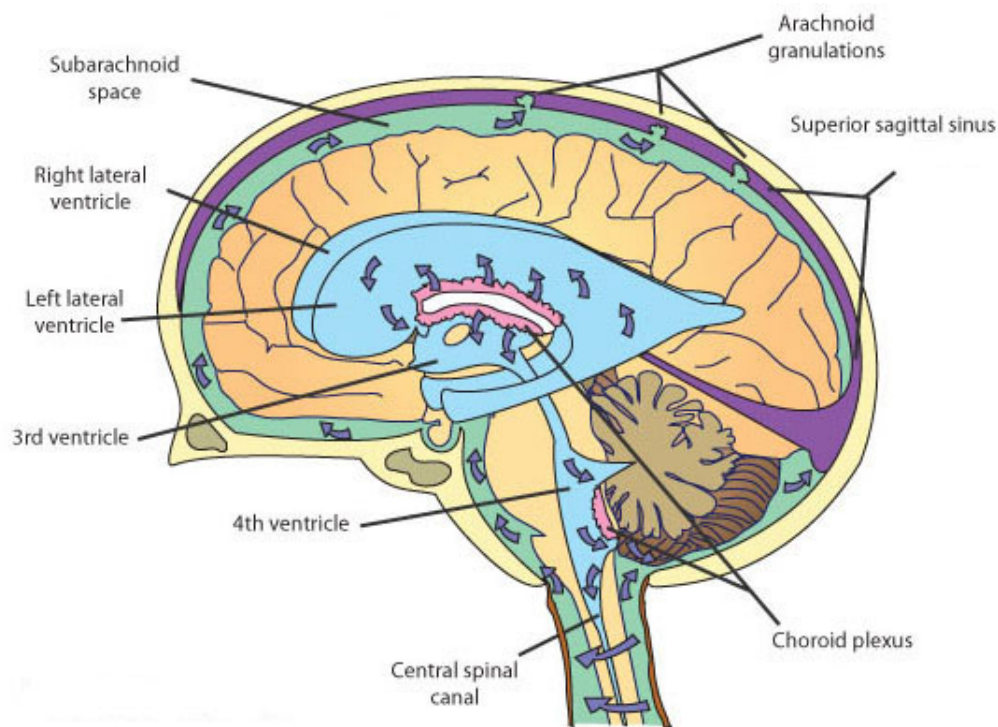


Figure 5: CSF circulation. CSF formation in the choroid plexus, flow in the ventricles and throughout the subarachnoid space, and resorption to the venous circulation by arachnoid granulations. (Adapted from www.drummy.com)

The CSF has many physiological functions: mechanical protection of neural structures, relative immunological protection, metabolic support to the CNS, and removal of metabolic waste products, among others (Irani (2009)). The arrival of CSF metabolites to the brain parenchyma, as well as the clearance of waste products from

the brain parenchyma, was thought to occur by diffusion through the interstitial fluid. However, recent findings have described the glymphatic system, a brain-wide pathway for fluid transport (Iliff et al. (2012), Iliff et al. (2013)). Briefly, CSF flows into the brain parenchyma by the paravascular sheaths, a pathway immediately surrounding vascular smooth muscle cells and bounded by perivascular astrocytes, which is also called the Virchow-Robin space. CSF flows into the brain interstitial space by the para-arterial space, while efflux of interstitial fluid to the CSF takes place by the paravenous space (Figure 6).

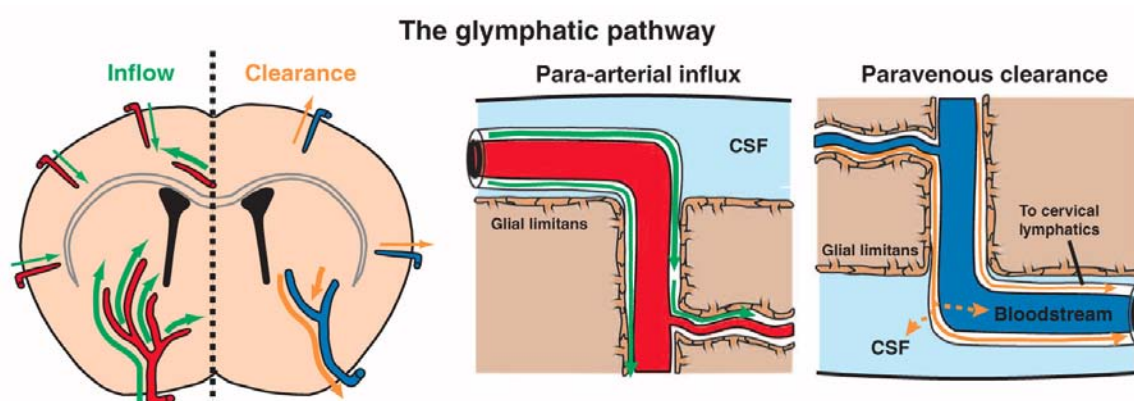


Figure 6: The glymphatic pathway. Green arrows depict para-arterial influx. Yellow arrows depict paravenous efflux. (From Iliff et al. (2012))

Therefore, in contrast to the delivery of AAV vectors into the cerebral parenchyma, the administration to the CSF allows a wider biodistribution because it bathes all the CNS structures.

To reach the CSF, three different administration routes are possible:

1. Intracerebroventricular injection (ICV): requires a cranial perforation to allow the vector administration into the lateral ventricles of the brain.
2. Injection into the cisterna magna (ICM): less invasive than ICV because the cisterna magna is located under the cerebellum and can be reached by puncture between the occipital bone and the atlas.
3. Intrathecal injection (IT): performed by puncture between two vertebrae usually at the lumbar region, is even less invasive than ICV and is being routinely performed in outpatient setting.

In neonate mice, ICV injection of AAV1 demonstrated efficient transduction of the brain, better than AAV2 and AAV5, mainly transducing neurons (Passini et al. (2003)). More recently, Chakrabarty et al. (2013) demonstrated that not only the serotype but also the timing of injection determined AAV transduction pattern in neonatal mouse brains after ICV delivery. Consistent with other studies, AAV8 and AAV9 were the serotypes presenting broader biodistribution in the brain. When injected at postnatal day 0, both serotypes displayed neuronal tropism together with some astrocytic transduction, whereas when injected at postnatal day 3 the preferential tropism was shifted and AAV8 and AAV9 mainly transduced astrocytes. Besides the tropism studies, successful therapeutic results were obtained by injection of AAV8 coding for the therapeutic gene in neonates of a MPS I mouse model (Wolf et al. (2011)).

Regarding ICV injection to adult mice, AAV1, AAV4 and AAV5 showed mainly ependymal cell transduction, being AAV4 and AAV5 ten times more efficient than AAV1 (Davidson et al. (2000)). In contrast, using AAV9 and AAVrh10, Wang et al. (2014) reported transduction throughout all the areas of the brain in adult mice, transducing neurons, astrocytes and oligodendrocytes. Moreover, Haurigot et al. (2013) described different degrees of transduction in the different areas of the adult dog brain after ICV administration of AAV9, targeting mainly neurons and scarcely astrocytes, suggesting that ICV administration may be applicable for CNS transduction in large animals, and possibly in humans.

Injection of AAV9 into the cisterna magna (ICM) in dogs also achieved similar transduction than via ICV (Haurigot et al. (2013)) . In fact, they used the ICM strategy for the treatment of a MPS IIIA mouse model achieving therapeutic benefits. Besides, the ICM delivery of AAV9 in NHP promoted significantly stronger transgene expression in brain compared to intravascular administration (Samaranch et al. (2012)), transducing mostly astrocytes but also some neurons. However, Gray et al. (2013) reported transduction of both neurons and astrocytes by AAV9 in brain and spinal cord using the same ICM delivery route in the same NHP species. A later work from Samaranch et al. (2013a) showed that AAV9 and AAV7 presented similar

transduction patterns after ICV administration in NHP: both transduced neurons and astrocytes in cortex and cerebellum, and were also able to efficiently transduce spinal cord motor neurons and dorsal root ganglia sensory neurons. In conclusion, the cisterna magna is also an efficient administration route to achieve CNS transduction. However, in humans, the proximity of the cisterna magna to vital centers makes ICM delivery a more risky and less common administration route, particularly in infant patients with skeletal deformities like in some MPSs (Haurigot et al. (2013)).

Finally, intrathecal (IT) administration of different AAV serotypes demonstrated the ability to transduce sensory neurons in the dorsal root ganglia (DRG) (AAV6 by Towne et al. (2009), AAV5 and AAV8 by Vulchanova et al. (2010), AAV9 by Schuster et al. (2014), AAVrh10 by Homs et al. (2014)), while IT-injected AAV9 and AAVrh10 are also able to transduce motor neurons in the spinal cord of mice (Snyder et al. (2011), Homs et al. (2014)). Concerning brain transduction after IT injection, Towne et al. (2009) described the presence of viral genomes in different areas of the mouse brain after IT injection of AAV6. Interestingly, IT delivery of AAV9 achieved the same efficient transduction in the spinal cord and brain than ICV administration in pigs (Bevan et al. (2011)). And the same was observed when comparing IT and ICM delivery in NHP (Gray et al. (2013)). In NHP, the efficiency of upper spinal cord and brain transduction was increased if the injection was performed with the animal placed horizontally but tilted with the head lower than the hips (Meyer et al. (2014)).

Since intrathecal administration of different AAV serotypes efficiently targets motor and/or sensory neurons, most of the intrathecal gene therapy approaches are designed for the treatment of pathologies affecting motor neurons such as amyotrophic lateral sclerosis (Wang et al. (2014)) and spinal muscular atrophy (Passini et al. (2014)), and also for sensory neuron affectations such as chronic neuropathic pain (Storek et al. (2008)), diabetic neuropathy (Homs et al. (2014)) and thermal hyperalgesia after peripheral nerve injury (Hirai et al. (2014)). Therefore, to our knowledge, the lumbar intrathecal delivery of AAV vectors with the aim to target the brain structures to treat neurodegenerative diseases has not yet been used in gene therapy approaches.

Apart from the wider biodistribution in the CNS reached by delivery of the AAV to the CSF, there is another feature of this administration route that must be taken into account. Even with the localized administration to the CSF, many of the studies delivering AAV vectors by ICM or IT routes report the detection of transgenic protein and/or vector RNA or DNA in somatic organs and serum, both in mice and also in NHP (Towne et al. (2009), Samaranch et al. (2012), Haurigot et al. (2013), Gray et al. (2013), Samaranch et al. (2013a), Meyer et al. (2014), Schuster et al. (2014)). Therefore, after direct administration to the CSF, and by an unknown mechanism, some vector is drained to blood and is able to transduce peripheral organs. This feature is not desirable for diseases restricted to the CNS, although it is of great interest in diseases affecting both the CNS and somatic organs such as MPS VII. In a MPS IIIA mouse model, a single administration of an AAV vector into the cisterna magna reached both the central and peripheral organs, leading to therapeutic effect using doses 1.5 logs lower than by intravenous delivery (Ruzo et al. (2012), Haurigot et al. (2013)).

In consequence, although the delivery of the AAV vector to the CSF avoids the initial contact of the vector with the host serum, the subsequent drainage to the bloodstream makes the preexisting immunity against AAV an important issue, as discussed in the next section of this work.

1.4. IMMUNOGENICITY CONCERNS OF GENE THERAPY WITH AAV VECTORS

Two different immunological issues are important in AAV gene therapy research: preexisting immunity and immune response to AAV treatment. Despite AAVs do not cause any symptomatic infection in humans, exposure to these viruses is common during the lifetime. And this exposure triggers anti-AAV immunity that can interfere in gene therapy strategies precluding success of the therapy. Besides, the administration of AAV vectors can trigger an immune response against the AAV capsid or against the transgenic protein that can also compromise the therapeutic effect.

1.4.1. Preexisting immunity against AAV capsid

An important factor to take into account when designing a gene therapy approach using AAV vectors is the impact of the host immune response against the viral capsid, because it can interfere in the successfulness of the therapy.

The humoral immune response against AAV capsids is induced in early childhood by non-pathogenic wild type AAV infections. These infections provoke the presence of neutralizing antibodies (NAbs) in the serum that are able to bind and neutralize AAV capsids. Preexisting immunity against AAV capsids is reported in all human populations analyzed by different groups, and prevalence of NAbs to various AAV serotypes is different. In general, NAbs against AAV2, followed by AAV1, are the most prevalent (30 to 60%), and NAbs against AAV7, AAV8 and AAV9 are less prevalent (15 to 30%) (Calcedo et al. (2009), Boutin et al. (2010), Thwaite et al. (2015)). Even so, prevalence of NAbs can vary depending on the geographical population studied, the gender of the individuals or the health status of the target population (Calcedo and Wilson (2013)). Therefore, preexisting immunity against AAV is an important issue that can compromise the use of AAV vectors for gene therapy, because NAbs can neutralize the AAV vector and preclude the transduction of the target tissue.

A strategy to circumvent the preexisting immunity against AAV could be the administration of very high doses of AAV vector, thus overcoming the NAb levels and allowing the transduction of the target tissue. However, it can cause cellular immunogenicity, as it will be discussed later on in this chapter. Another strategy is minimizing the contact of the AAV with NAbs, which can be done by alternative delivery routes. For example, in contrast to systemic organ targeting, when directly injecting the AAV vector into the eye an –immune-privileged organ– preexisting NAbs do not interfere in the transduction efficiency (Calcedo and Wilson (2013), Mingoizzi and High (2013)).

1.4.2. Immune response triggered by AAV vector delivery

Apart from the preexisting immunity concerns, AAV vector delivery can provoke different kinds of immune response: humoral immune response against the AAV

capsid, cellular immune response against the vector capsid, and cellular immune response against the transgene.

In gene therapy strategies, vector delivery can trigger a humoral immune response that must be taken into account. It is obvious that intravascular delivery of AAV vectors causes an increase in anti-AAV NAb in serum. On the contrary, the injection of small amounts of AAV vectors to the brain parenchyma in several clinical trials for Parkinson and other CNS diseases did not elicit any immune response (Mingozzi and High (2013)). In contrast to intra-parenchyma administration, AAV vector delivery to the CSF causes an increase in serum NAb titers in mice, dogs and NHP, a fact that may be related to the drain of the vector to the blood circulation. Nonetheless, anti-AAV Nabs in the CSF are maintained at low levels after AAV delivery, with a slight increase in some cases (Treleaven et al. (2012), Haurigot et al. (2013), Gray et al. (2013)).

Besides, the administration of AAV vectors also triggers a cellular immune response against the vector capsid. The first evidence of the cellular immunogenicity against AAV vectors was found in the first human trials for hemophilia (Manno et al. (2006)), when the factor IX protein levels in serum unexpectedly declined after four weeks. After many preclinical studies, the accepted hypothesis is that AAV capsid antigens are processed by transduced cells and presented on MHC class I, which triggers a T-cell immune response that depletes the transduced cells (Mingozzi and High (2013)). In the subsequent clinical trials for hemophilia, the administration of a short course of glucocorticoids could prevent the depletion of transduced cells and the loss of transgene expression (Nathwani et al. (2011b)). Besides, other strategies are being studied in order to circumvent the T-cell immunity to AAV in gene therapy strategies. Many of them attempt the reduction of the total capsid antigen dose by different means: using hyperactive variants of the therapeutic protein, using stronger promoter elements, engineering the AAV capsid to prevent its presentation onto MHC class I, etc. (Basner-Tschakarjan and Mingozzi (2014))

Therefore, the capsid dose is an important factor in the efficacy of the treatment, because the administration of high doses of AAV vector, with the aim to overcome the NAb levels and achieve transduction, could trigger T-cell responses to the AAV capsid

that would deplete the transduced cells and preclude the therapeutic outcome (Figure 7).

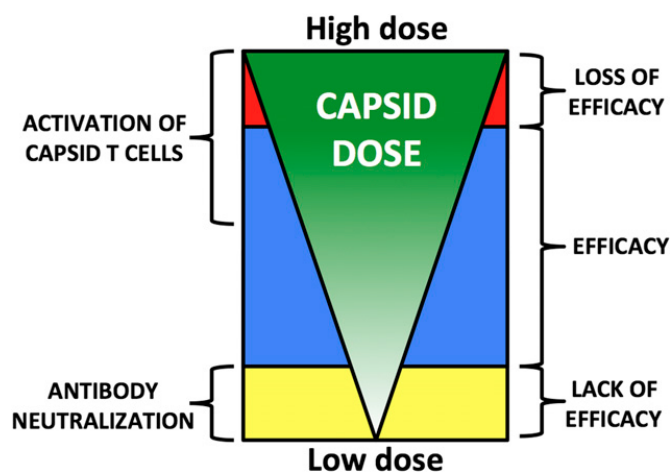


Figure 7: The capsid dose is an important factor in the efficacy of the treatment. Low doses of AAV capsid can be neutralized by NAbs and preclude transduction. High doses of AAV capsid can activate T-cell immune responses and lead to the clearance of the transduced cells. (From Mingozi and High (2013))

In addition to the immune response against the capsid, gene therapy can cause cellular immune response against the transgene product. In general, AAV vectors are relatively inefficient in transducing antigen-presenting cells (APC), thus the risk of immune response provoked by the transgene is low when compared to adenoviral vectors. Even so, it has been described in several studies and it is highly dependent on the AAV administration route: e.g. intramuscular delivery of AAV causes higher transgene-triggered immune response than liver-directed administration. When injected into the brain, no immune response has been described after AAV2 delivery in the clinical trials of Parkinson and other CNS diseases (Mingozi and High (2013)). However, in different animal models, the intra-parenchyma injection of AAV1, AAV5 and AAV9 coding for non-self proteins can elicit cell-mediated immune responses. Since these serotypes can transduce different cell types, they transduce APCs in the brain and trigger a cellular immune response that can cause neuronal loss (Ciron et al. (2006), Hadaczek et al. (2009), Ciesielska et al. (2013), Samaranch et al. (2013b)), which is also an important issue.

In summary, the preexisting immunity against AAV capsids and the subsequent immune response elicited by the AAV vector delivery are complex and important issues that must be taken into account for gene therapy strategies (**Figure 8**).

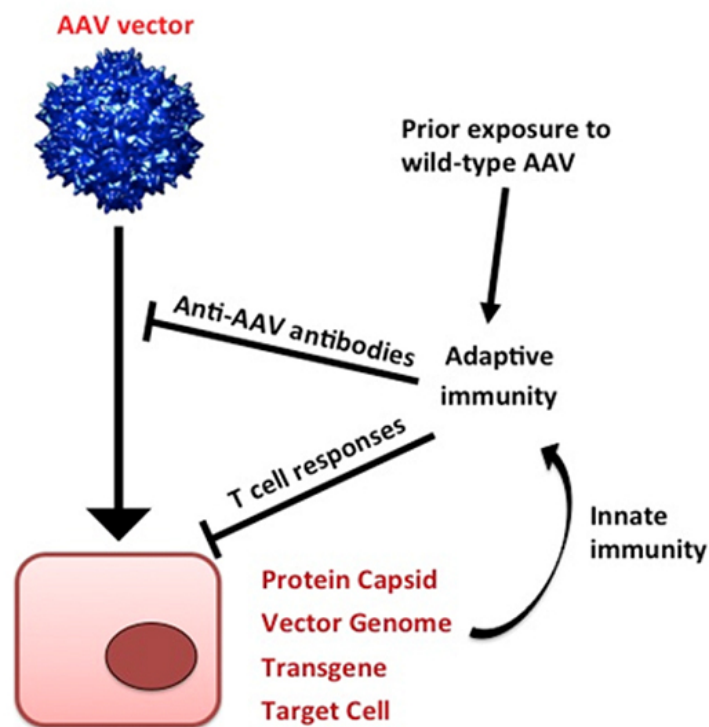


Figure 8: The complex interactions of AAV vectors with the immune system. Prior exposure to wild type AAV develops an adaptive immunity that can affect gene therapy strategies using AAV vectors: anti-AAV antibodies can preclude vector transduction of a target tissue, and T-cell mediated immunity to the AAV capsid can deplete the transduced cells. All components of gene transfer (vector capsid, vector genome, transgene product and target cell) are involved in shaping the interactions of AAV vectors with the immune system. (From Masat et al. (2013)).

2. LYSOSOMAL STORAGE DISEASES

Lysosomal storage diseases (LSDs) are a family of more than 60 disorders caused by inherited gene mutations that disturb lysosomal homeostasis. The incidence of LSD is estimated to be 1 in 7000 live births (Mehta and Winchester (2012)) although it might be greater because of possible undiagnosed or misdiagnosed cases. Most of LSDs affect infants and children. The different diseases present variable ages of onset and clinical courses, but in all the cases they are progressive and ultimately lead to premature death. Nearly two thirds of the described LSDs display extensive neurological impairment, including intellectual disability, dementia, seizures, motor system deficits, visual impairment and hearing loss. Other pathological features found in LSD are hydrops fetalis (see **Box 1**), facial dysmorphism, upper airway obstruction, cardiomyopathy, hepatosplenomegaly and skeletal abnormalities (Platt et al. (2012), Mehta and Winchester (2012)).

At the cellular level, the common feature of the different LSD is the initial accumulation of specific macromolecules or monomeric compounds into vesicular components of the endosomal-autophagic-lysosomal system, due to impaired lysosomal function.

Box 1: Hydrops fetalis

Hydrops fetalis is a serious condition in which abnormal amounts of fluid build up in two or more body areas of a fetus or newborn. Nonimmune hydrops fetalis occurs when a disease or medical condition disrupts the body's ability to manage fluid. Symptoms depend on the severity of the condition. Mild forms may cause liver swelling and change in skin colour (pallor). More severe forms may cause breathing problems, bruising or purplish bruise-like spots on the skin, heart failure, severe anemia, severe jaundice, total body swelling. Hydrops fetalis often results in death of the infant shortly before or after delivery. The risk is highest among the most premature babies and those who are severely ill at birth.

From National Library of Medicine (U.S.) (1998)

2.1. THE LYSOSOMAL SYSTEM

Lysosomes are ubiquitous acidic organelles that carry out the degradation of macromolecules, constituting the primary degradative compartment of the cell. Lysosomes receive the macromolecular substrates by fusion processes with

endosomes, phagosomes and autosomes. Phagolysosome formation is important for cellular pathogen defense, whereas autolysosomes mediate the degradation and turnover of cytoplasmic components, which is a key process for cell homeostasis, proliferation and death. Moreover, lysosome-associated membrane proteins permit the entrance of cytosolic proteins into the lysosome in the process called chaperone-mediated autophagy (Kaushik and Cuervo (2012)). Besides these catabolic functions, lysosomes are also involved in various physiological processes such as cholesterol homeostasis, plasma membrane repair by lysosomal exocytosis (McNeil and Kirchhausen (2005)), or lysosomal cell death (Aits and Jäättelä (2013)). Cell-type specific functions of the lysosomes include antigen processing in B-cells for MHC class II-dependent antigen presentation (Watts (2012)), and bone remodeling conducted by lysosome fusion with the plasma membrane in osteoclasts (Sun-Wada et al. (2003)). These complex functions make the lysosome a central and dynamic organelle, not only the final step of the degradative machinery of the cell (Saftig and Klumperman (2009)) (**Figure 9**).

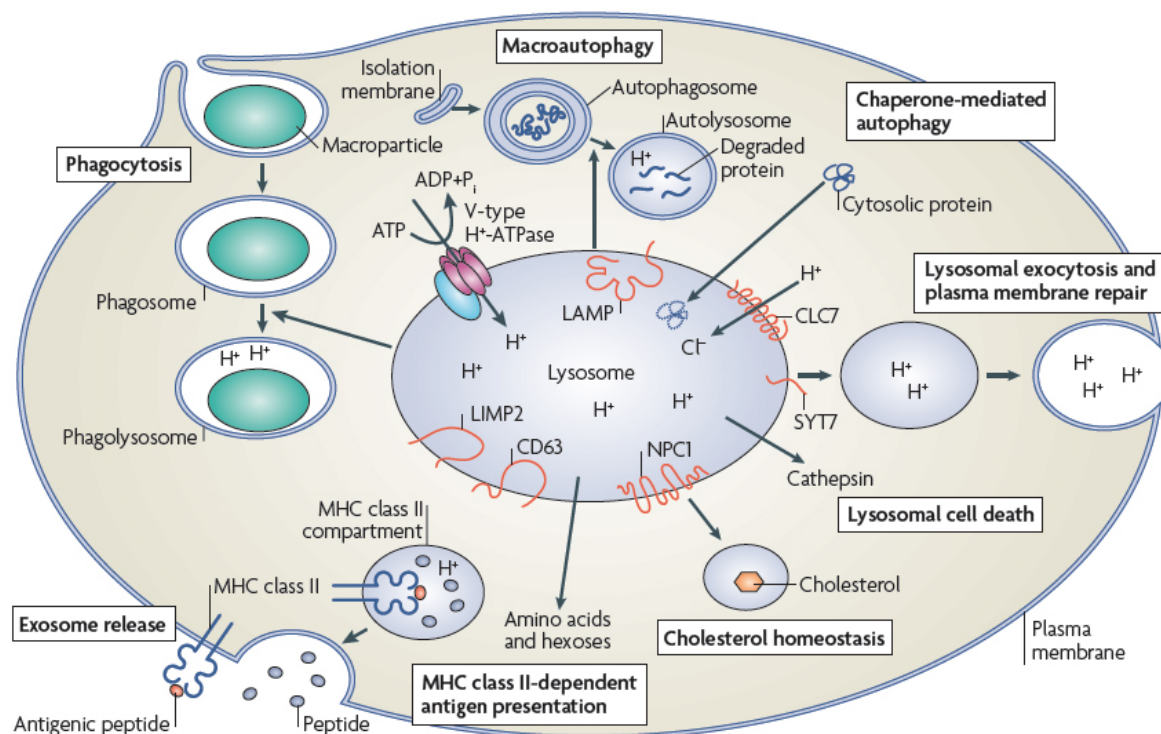


Figure 9: Major functions of lysosomes (From Saftig and Klumperman (2009))

Lysosomes contain two classes of proteins that are essential for their function: soluble lysosomal hydrolases and integral lysosomal membrane proteins (LMPs). There are approximately 50 known soluble lysosomal hydrolases that include proteases, glycosidases, nucleases, phosphatases, sulphatases and lipases. Each of them targets a specific substrate for degradation and together they constitute the total catabolic capacity of the lysosome. On the other hand, lysosomal membrane proteins have diverse functions, which include acidification of the lysosomal lumen, protein import from the cytosol, transport of degradation products to the cytoplasm, and membrane fusion processes. Lysosome-associated membrane protein1 (LAMP1) and LAMP2 are two of the most abundant LMPs (Saftig and Klumperman (2009)). In addition, lysosomes also contain non-enzymatic soluble proteins such as NPC2, a cholesterol binding protein, among others.

Soluble lysosomal hydrolases are synthesized in the endoplasmic reticulum (ER), where oligosaccharide chains are bound to selected aminoacidic residues. Then they are transferred to Golgi, where the majority of the hydrolases undergo post-translational modification that creates mannose 6-phosphate (M6P) residues. These M6P residues are responsible for the hydrolase trafficking to the *trans*-Golgi network (TGN), and subsequently to the endosomes, by specific binding to the mannose 6-phosphate receptors (MPR) present in the membrane of these organelles. The acidic pH in the endosomes induces the release of hydrolases from MPR into the endosomal lumen, from where they finally reach the fluid phase of the lysosome compartment by fusion processes. However, a proportion of newly synthesized lysosomal hydrolases escape binding to MPR and become secreted to the extracellular medium (5-20% in non-tumor cell lines). Once there, adjacent cells can internalize hydrolases, since MPR are also present at the plasma membrane. The MPR reaches outer part of the cells by membrane fusion processes, and it represents 3 to 10% of total cellular MPR (**Figure 10**) (Braulke and Bonifacino (2009)).

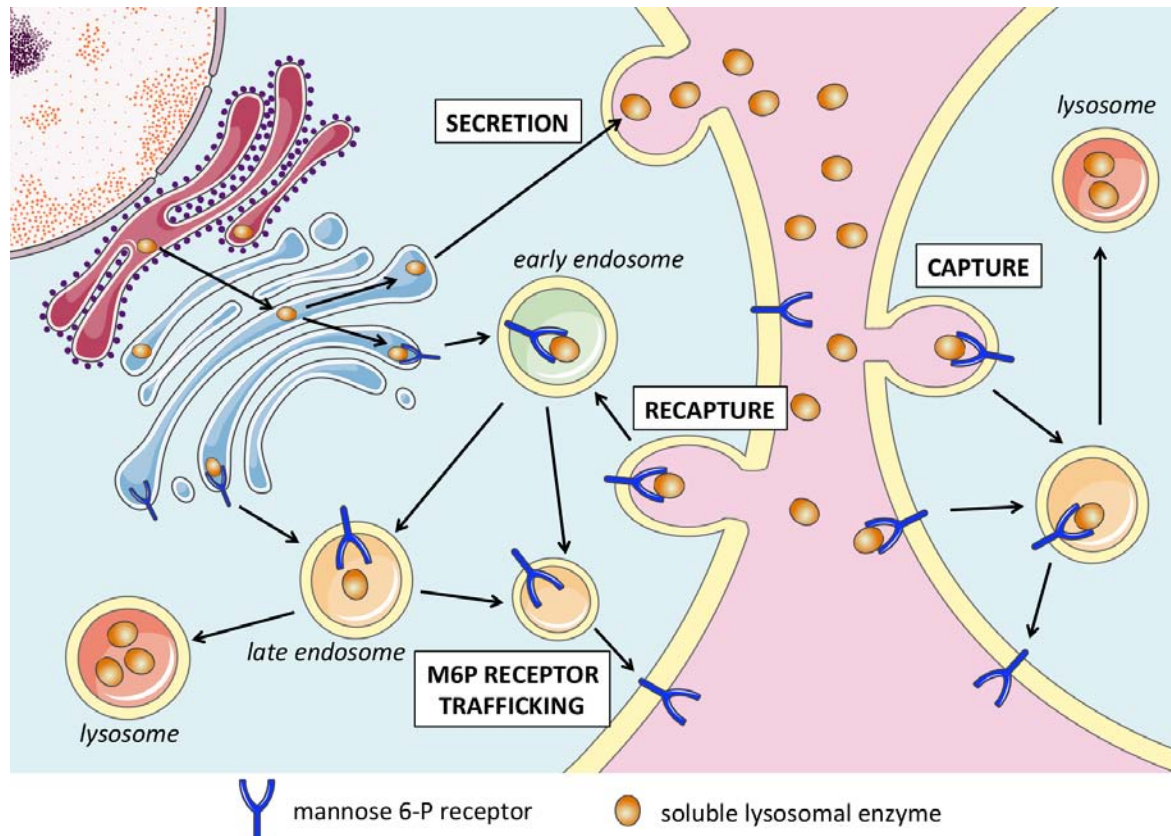


Figure 10: Intracellular and intercellular trafficking processes associated to mannose 6-phosphate receptors (MPR). MPR are present in the *trans*-Golgi network, in the endosomal compartment and also in the outer cell surface. Soluble lysosomal enzymes are bound to MPR and reach the lysosomal compartment, although a proportion is secreted to the extracellular medium and can be internalized by adjacent cells by the MPR present in the plasma membrane. (Redrawn and adapted from Bosch and Heard (2003))

2.2. CELLULAR FEATURES OF LYSOSOMAL STORAGE DISEASES

Lysosomal storage diseases are caused by genetic deficiencies in lysosomal function that lead to macromolecule storage in the endosome-autosome-lysosome compartment. Due to this storage, the majority of LSDs display severe neurological impairment, hepatosplenomegaly and skeletal alterations, among other symptoms. However, cellular and molecular mechanisms that lead to the pathological features are not yet fully understood.

LSDs are characterized by the lack of function of hydrolytic enzymes, lysosomal membrane proteins or non-enzymatic soluble lysosomal proteins. The lack of either hydrolase or permease activity causes the cellular accumulation of undegraded

macromolecules or monomers respectively, which are stored into endosomes, autophagosomes and lysosomes. The primary storage is the direct result of the enzymatic failure, with accumulation of biochemical components directly related to the lacking function. This primary storage causes diverse alterations in cell homeostasis, leading to a secondary accumulation of substrates and ultimately conducting to cell death by different pathways: the aberrant storage can lead to alterations of signaling pathways (e.g. GAGs can activate TLR4 and trigger the innate immune response, or can bind to growth factor receptors impairing signaling and compromising cell survival). Also, the intracellular calcium homeostasis can be affected, which can trigger an unfolded protein response that can lead to enhanced apoptosis. Moreover, lipid biosynthesis and trafficking can be altered, as well as receptor trafficking. Besides, altered autophagy has been demonstrated to have an important role in the pathogenesis of many LSD: the lysosomal storage may affect fusion efficiency between lysosomes and autophagosomes, causing a partial block of autophagy and a subsequent autophagy induction as a compensatory effect. Both situations, either highly blocked or highly induced autophagy, can cause deleterious effects. This altered autophagy is thought to be the main cause of secondary storage in LSD, which contains toxic proteins and aberrant mitochondria that could not be degraded by autophagy. All these alterations together provoke cellular damage and trigger inflammatory responses that conduct to cell death (Ballabio and Gieselmann (2009), Lieberman et al. (2012)).

2.3. NEUROLOGICAL FEATURES OF LYSOSOMAL STORAGE DISEASES

The neurological impairment is a main feature of many LSD and is characterized by neurodegeneration and neuroinflammation. Neurodegeneration can occur in multiple brain regions and it is typically region-specific, with differential affectation depending on the neuronal subtype. This phenomenon can be attributed to the three reasons: specific storage metabolites exerting differential effects on neuronal subtypes, varying proportions of macromolecules being synthesized in different neuronal populations, and differential neuronal vulnerability to storage. In addition to neurodegeneration, neuroinflammation exerts deleterious effects in CNS. Microgliosis

and astrogliosis are prevalent in LSD and directly contribute to CNS pathology, exacerbating the pathogenic processes. (Mehta and Winchester (2012))

2.4. CLASSIFICATION OF LYSOSOMAL STORAGE DISEASES

The classification of the lysosomal storage diseases is not completely straightforward, since there is significant overlap of pathological features and storage material between different LSD. The traditional classification of LSD was made on the basis of the biochemical nature of the stored material, e.g. lipidoses, glycogenoses, mucopolysaccharidoses, mucopolipidoses. However, several LSD did not fit into this classification due to the non-enzymatic nature of the mutation that causes them. Thus, the new classification of the LSD is based on the nature of the molecular defect in the lysosomal system. Following this classification, **Table 1** contains all the LSD types and a selection of the most common diseases, specifying the affected gene and protein, the storage material and the major pathological features.

Table 1: Classification of lysosomal storage diseases. Note that not all the existing LSD are listed in the table. In brackets it is reported the total number of diseases catalogued by OMIM in each group. *CS*, chondroitin sulfate; *DS*, dermatan sulfate; *HS*, heparan sulfate; *KS*, keratan sulfate; *oligos*, oligosaccharides. (Adapted from Mehta and Winchester (2012))

disease name	protein (gene)	storage material	peripheral affection	CNS affection
LYSOSOMAL ENZYME DEFECTS (43)				
Sphingolipidoses including sphingolipid activator defects (19)				
<i>GM1-gangliosidosis types I, II & III</i>	β -Galactosidase (GLB1)	GM1-ganglioside, KS, oligos, glycolipids	skeleton, heart	+
<i>Tay-Sachs disease (GM2-gangliosidosis)</i>	β -Hexosaminidase A (HEXA)	GM2-ganglioside, oligos, globoside, glycolipids	-	+
<i>Fabry disease</i>	α -Galactosidase (GLA)	galactosylated glycolipids	kidney, heart	-
<i>Gaucher disease types 1, 2 and 3</i>	β -Glucosidase (GBA)	glucosylceramide	spleen, liver, lung, skeleton, bone marrow	in types 2 and 3
<i>Metachromatic leukodystrophies</i>	Arylsulfatase A (ARSA), saposin B (PSAP)	sulfatides	PNS	+
<i>Krabbe disease (globoid cell leukodystrophy)</i>	β -Galactocerebrosidase (GALC)	galactosylceramide	PNS	+
<i>Niemann-Pick types A & B</i>	Sphingomyelinase (SPMD1)	sphingomyelin	liver, spleen. Type B also lung and skeleton	in type A

disease name	protein (gene)	storage material	peripheral affectation	CNS affectation
Mucopolysaccharidoses (13)				
<i>MPS I (Hurler, Hurler-Scheie, Scheie)</i>	α -L-iduronidase (IDUA)	DS, HS, oligos	spleen, liver, skeleton, heart in Hurler. Scheie Hurler-Scheie: milder	severe in Hurler. Mild in Hurler-Scheie
<i>MPS II (Hunter)</i>	Iduronate-2-sulfatase (IDS)	DS, HS, oligos	spleen, liver, skeleton, heart in severe form.	only in severe form
<i>MPS IIIA (Sanfilippo A)</i>	Heparan-N-sulfatase (SGSH)	HS, oligos	mild	+
<i>MPS IIIB (Sanfilippo B)</i>	α -N-acetylglucosaminidase (NAGLU)	HS, oligos	mild	+
<i>MPS IIIC (Sanfilippo C)</i>	Acetyl CoA: α -glucosamine N-acetyl transferase (HGSNAT)	HS, oligos	mild	+
<i>MPS IIID (Sanfilippo D)</i>	N-acetylglucosamine-6-sulfatase (GNS)	HS, oligos	mild	+
<i>MPS IVA (Morquio A)</i>	N-acetylgalactosamine-6-sulfatase (GALNS)	KS, oligos	skeleton, liver	-
<i>MPS IVB (Morquio B) allelic to GM1-gangliosidosis</i>	b-Galactosidase (GLB1)	KS, oligos	skeleton, liver	-
<i>MPS VI (Maroteaux-Lamy)</i>	N-acetylgalactosamine-4-sulfatase (ARSB)	DS, oligos	skeleton, heart, liver	-
<i>MPS VII (Sly)</i>	β -Glucuronidase (GUSB)	CS, DS, HS, oligos	skeleton, liver, spleen, heart	+
<i>MPS IX</i>	Hyaluronidase (HYAL1)	hyaluronan	joints	-
Glycoproteinoses (8)				
<i>Aspartylglucosaminuria</i>	aspartylglucosaminidase (AGA)	glycosyl-asparagines	skeleton, connective tissue	+
<i>α-Mannosidosis</i>	a-D-Mannosidase (MAN2B1)	oligos	skeleton, connective tissue	+
<i>β-Mannosidosis</i>	b-D-Mannosidase (MANBA)	oligos	skeleton, connective tissue	+
Other enzyme defects (3)				
<i>Pompe (glycogen storage disease type II)</i>	a-Glucosidase (GAA)	glycogen, oligos	skeletal muscle	-
DEFECTS IN POST-TRANSLATIONAL PROCESSING OF LYSOSOMAL ENZYMES (5)				
<i>Multiple sulfatase deficiency (mucopolysaccharidosis)</i>	Formylglycine generating enzyme (SUMF1)	sulfatides, GAGs, glycolipids	multisystemic	+
<i>Mucopolipidosis II (I-cell disease)</i>	N-acetylglucosamine-1-phosphotransferase a/b subunit (GNPTAB)	oligos, GAGs, lipids	liver, spleen, heart, skeleton	+
<i>Mucopolipidosis IIIA (pseudo-Hurler polydystrophy)</i>	N-acetylglucosamine-1-phosphotransferase a/b subunit (GNPTAB)	oligos, GAGs, lipids	heart, skeleton	-/+
LYSOSOMAL MEMBRANE AND TRANSPORT DEFECTS (13)				
<i>Cystinosis</i>	Cystinosin (cystine transporter) (CTNS)	cystine	kidney	-
<i>Danon disease</i>	Lysosome-associated membrane protein 2 (LAMP2)	cytoplasmic debris and glycogen	cardiac and skeletal muscle	+
<i>Niemann-Pick type C1</i>	Niemann-Pick type C1 protein, proton driven transporter (NPC1)	cholesterol and glycolipids	liver	+
<i>Niemann-Pick type C2</i>	Niemann-Pick type C2 protein, soluble lysosomal protein (NPC2)	cholesterol and glycolipids	liver	+

NEURONAL CEROID LIPOFUSCINOSES (11)

Group of LSD caused by mutations in genes related to the endosomal-lysosomal system whose normal function is unknown or unclear. The common pathophysiology involves the accumulation of autofluorescent storage material within the lysosome and widespread death of neurons. Clinical symptoms include visual failure, epilepsy and declines in motor and cognitive skills that lead to premature death.

DEFECTS IN LYSOSOME AND LYSOSOME-RELATED ORGANELLE BIOGENESIS (5)

Group of LSD caused by mutations in genes that affect the biogenesis of lysosome-related organelles that mainly affect melanosomes and platelet dense granules. Defective biogenesis of melanosomes causes pigmentation abnormalities and partial albinism. Impaired biogenesis of platelet dense granules leads to defective platelet function and bleeding tendency.

3. THE MUCOPOLYSACCHARIDOSES

The mucopolysaccharidoses (MPSs) are a family of LSD caused by the deficiency of lysosomal enzymes involved in the degradation of glycosaminoglycans (GAGs), formerly called mucopolysaccharides, which are accumulated in the endosomal-autosomal-lysosomal compartment. There are 11 known enzyme deficiencies that give rise to 7 distinct MPS with different phenotypes that will be presented later on. There is clinical similarity between different enzyme deficiencies. However, a wide spectrum of clinical severity exists among patients with the same enzyme deficiency.

3.1. GLYCOSAMINOGLYCANS AND THEIR DEGRADATION PATHWAYS

GAGs are linear polysaccharides comprised of disaccharide building blocks. These disaccharides consist of an amino sugar bound to either an uronic acid or a galactose (see **Table 2**) (Varki (2009)). They are present in abundance at the cell-extracellular matrix interface, bound to a protein core and forming proteoglycans. The GAG chains of the proteoglycans interact with numerous proteins present in the extracellular matrix (ECM), such as growth factors, morphogens, cytokines and enzymes (proteases and their inhibitors), modulating their function. Therefore, GAGs are involved in fundamental biological functions: developmental processes, angiogenesis, axonal growth anticoagulation, cancer progression and microbial pathogenesis (**Figure 11**).

The interactions of GAGs with growth factors, enzymes or cytokines are directly involved in initiating cell signaling events or inhibiting biochemical pathways. In these interactions, not only the interaction affinity is important: the positioning of the

protein-binding motifs along the GAG chain determine if an active signaling complex is assembled at the cell surface or an inactive complex is sequestered in the matrix. Moreover, the interactions of GAGs with morphogens are determinant factors during the process of morphogenesis, which depends on morphogen gradients and differential concentrations. GAGs and the different location of their protein-binding motifs are also implicated in the maintenance of morphogen gradients across a cell or tissue, thus determining the patterns of cell differentiation during tissue development. (Sasisekharan et al. (2006))

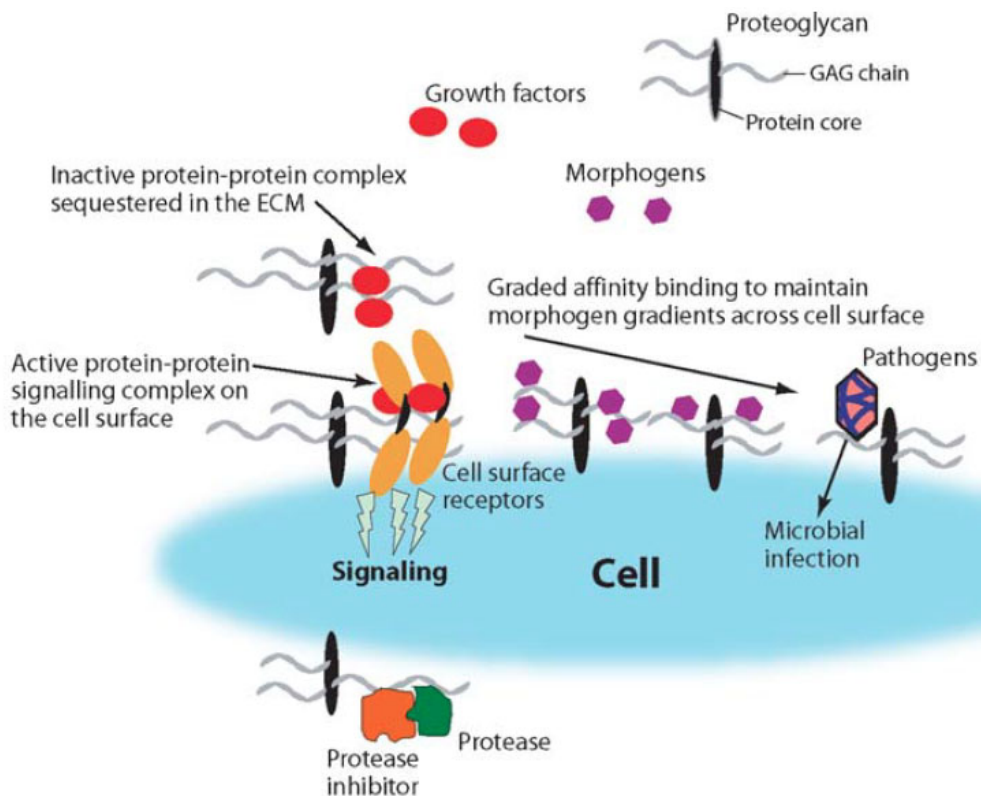


Figure 11: Biological interactions of glycosaminoglycans (From Sasisekharan et al. (2006))

Based on the backbone chemical structure, there are five classes of GAGs (**Table 2**):

Table 2: Types of glycosaminoglycans

GAG	Amino sugar	Uronic acid / galactose
Hyaluronan	N-acetylglucosamine	Glucuronic acid
Chondroitin sulfate	N-acetylgalactosamine	Glucuronic acid
Dermatan sulfate	N-acetylgalactosamine	Glucuronic acid and iduronic acid
Heparan sulfate	N-acetylglucosamine	Glucuronic acid and iduronic acid
Keratan sulfate	N-acetylglucosamine	Galactose

For their degradation, GAGs are internalized from the cell surface and each GAG type is degraded stepwise in a highly ordered way by unique or overlapping sets of enzymes in the lysosome. Chondroitin sulfate and dermatan sulfate have highly similar structures and their degradation pathways are mostly common (Varki (2009)). **Figure 12** summarizes the degradation pathways and the enzymes involved:

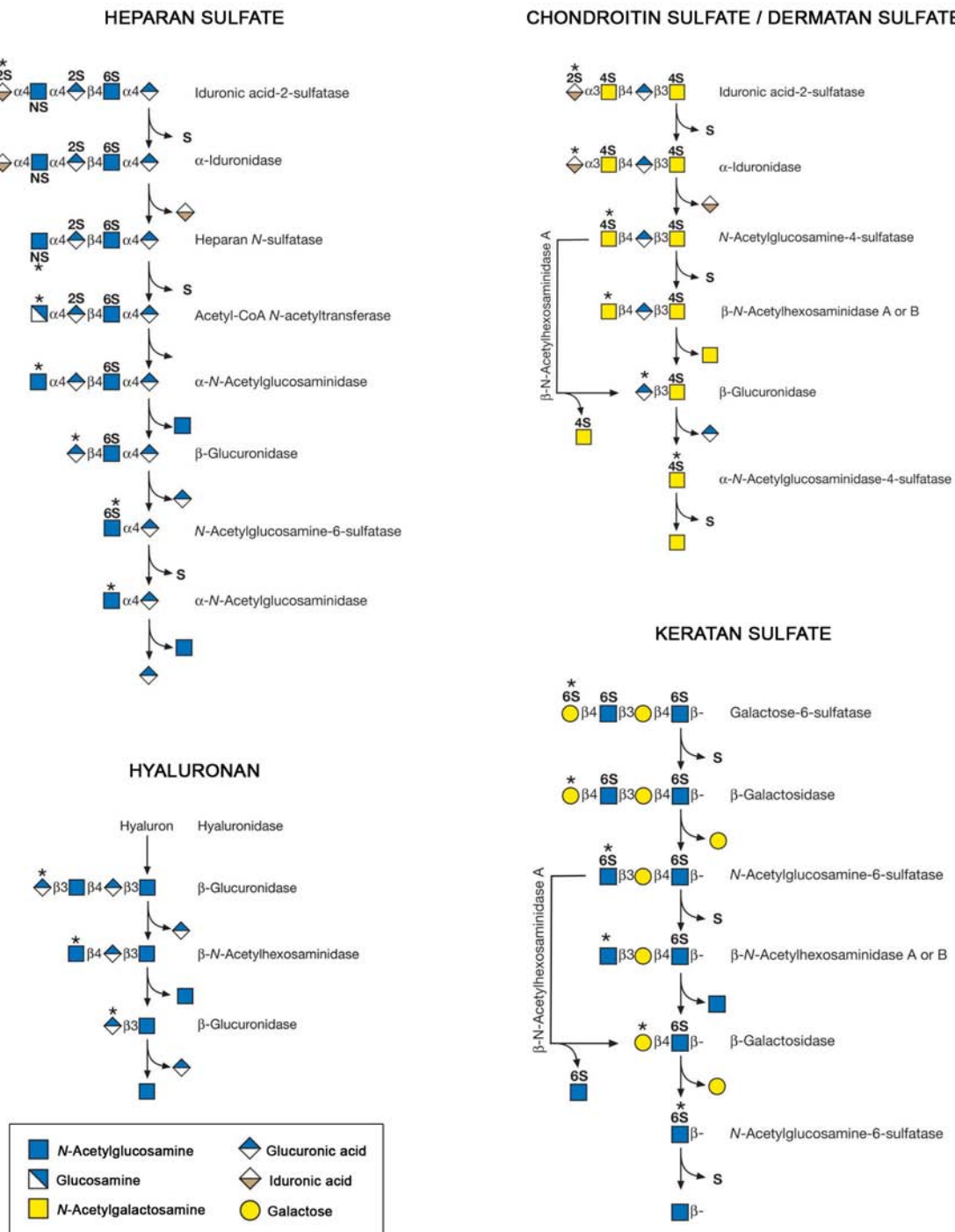


Figure 12: Degradation pathways of the glycosaminoglycans. (Adapted from Varki (2009))

3.2. CLINICAL DESCRIPTION OF THE MUCOPOLISACCHARIDOSES

As stated before, deficiencies in different enzymes involved in the degradation pathways of the GAGs are the cause of the different mucopolysaccharidoses. MPS are chronic, multisystemic and progressive diseases, inherited in a recessive manner. They share many clinical features among them, although in variable degrees: organomegaly, dysostosis multiplex (see **Box 2**) and abnormal facies are common features. Hearing, vision, respiratory, cardiovascular function and joint mobility are affected in some MPS. Neurological impairment is characteristic of several MPS but some do not present CNS affection. In general, a wide range of severity is found among patients of each MPS type. (Scriver et al. (2001))

Box 2: Dysostosis multiplex

Dysostosis Multiplex is a term used to describe the skeletal abnormalities observed in mucopolysaccharidoses, which include the following radiological features:

- *Skull*: macrocephaly with dolicocephaly, vertical frontal crest, abnormal J-shaped sella turcica, hickened cortical bone, facial anomalies, teeth widely spaced.
- *Thorax*: paddle-shaped or oar-shaped ribs, short and thickened clavicles.
- *Spine*: craniovertebral junction abnormalities (atlantoaxial instability, stenosis and compression of the spinal cord), gibbus in thoracolumbar spine, malformations of the vertebral bodies.
- *Pelvis*: rounded iliac wings, inferior tapering of the ileum, hip dysplasia, poorly developed acetabulum, underdevelopment of the medial portion of the proximal femoral epiphysis, coxa valga.
- *Long bones*: mildly hypoplastic epiphyses, proximal humeral notching, long and narrow femoral neck.
- *Knees*: genu valgum.
- *Hands and feet*: V-shaped deformity of the hypoplastic distal ulna and radius, hypoplastic and irregularly shaped carpal and tarsal bones, proximal pointed metacarpals and metatarsals, bullet-shaped phalanges.

From Palmucci et al. (2013)

The basic hallmarks of each MPS type are presented in the charts below. Unless otherwise stated, the information in the charts is summarized from Scriver et al. (2001) and the estimated prevalence data from “Orphanet Report Series: prevalence of rare diseases. Update May 2014”.

MPS I: Hurler, Scheie and Hurler-Scheie syndromes	
Prevalence	1:100,000
Enzyme deficiency	α -L-iduronidase
GAG deg. pathway	dermatan sulfate and heparan sulfate
Phenotype	<p><u>Severe phenotype: Hurler syndrome</u></p> <p><u>Onset</u>: a few months after birth. Death in childhood</p> <p><u>Symptoms</u>: developmental delay, short statures, cardiomyopathy, dysostosis multiplex, respiratory tract infections and progressive corneal clouding, among others.</p> <p><u>Mild phenotype: Scheie syndrome</u></p> <p><u>Onset</u>: usually after the age of 5</p> <p><u>Symptoms</u>: joint stiffness, aortic valve disease, corneal clouding, coarse face and some other somatic features. Normal intelligence and stature</p> <p><u>Intermediate phenotype: Hurler-Scheie syndrome</u></p> <p><u>Symptoms</u>: progressive somatic involvement including dysostosis multiplex, with little or no intellectual dysfunction.</p>

MPS II: Hunter syndrome	
Prevalence	0.6:100,000
Enzyme deficiency	iduronate-2-sulfatase
GAG deg. pathway	dermatan sulfate and heparan sulfate
Phenotype	<p><u>Severe phenotype</u></p> <p><u>Onset</u>: between 2 and 4 years of age. Death between 10 and 15 years of age.</p> <p><u>Symptoms</u>: Developmental delay, short statures, cardiomyopathy, dysostosis multiplex, respiratory tract infections and communicating hydrocephalus, among others. Slower progression than Hurler syndrome.</p> <p><u>Mild phenotype</u></p> <p><u>Symptoms</u>: Joint stiffness, aortic valve disease, corneal clouding, coarse face, dysostosis multiplex, hearing impairment, among others. Slower progression of somatic deterioration and normal intelligence. Longer lifespan, up to the fifth and sixth decades.</p>

MPS III: Sanfilippo syndrome	
Prevalence	0.87:100,000
Enzyme deficiency	<p><u>Sanfilippo A</u>: heparan-N-sulfatase</p> <p><u>Sanfilippo B</u>: α-N-acetylglucosaminidase</p> <p><u>Sanfilippo C</u>: acetyl CoA: α-glucosamine N-acetyl transferase</p> <p><u>Sanfilippo D</u>: N-acetylglucosamine-6-sulfatase</p>
GAG deg. pathway	heparan sulfate
Phenotype	<p><u>Onset</u>: between 2 and 6 years of age.</p> <p><u>Symptoms</u>: severe CNS degeneration but only mild somatic affectation. Hyperactivity, aggressive behavior, delayed development, delayed speech development, hearing loss. Severe neurologic degeneration in most patients by 6 to 10 years of age, causing deterioration of social and adaptive skills.</p>

MPS IV: Morquio syndrome	
Prevalence	0.4:100,000
Enzyme deficiency	<p><u>Morquio A</u>: N-acetylgalactosamine-6-sulfatase</p> <p><u>Morquio B</u>: β-galactosidase</p>
GAG deg. pathway	keratan sulfate
Phenotype	<p><u>Onset</u>: between 1 and 4 years of age.</p> <p><u>Symptoms</u>: preserved intelligence, mainly affects skeleton: Genu valgum, kyphosis, growth retardation, short trunk and neck, waddling gait, scoliosis, deformities of the vertebrae, osteoporosis, joint problems, atlantoaxial instability. Also hearing loss, mild corneal clouding, hepatomegaly, upper airway obstruction, cardiac valve lesions, small teeth and coarse facial features.</p>

MPS VI: Maroteaux-Lamy syndrome	
Prevalence	0.16:100,000
Enzyme deficiency	N-acetylgalactosamine-4-sulfatase
GAG deg. pathway	heparan sulfate
Phenotype	<p><u>Symptoms</u>: usually normal mental development. Enlarged head and deformed chest at birth. Growth arrest by 6 to 8 years of age. Corneal clouding, joint problems, carpal tunnel syndrome, hepatomegaly, cardiac valvular dysfunction, dysostosis multiplex. In some cases thickening of the dura mater leads to spinal cord compression.</p>

MPS VII: Sly syndrome	
Prevalence	0.01:100,000
Enzyme deficiency	β -glucuronidase
GAG deg. pathway	heparan sulfate, keratan sulfate, dermatan sulfate and hyaluronan
Phenotype	<u>Symptoms</u> : Heterogeneous phenotype, ranging from death in utero to life expectancy up to 20-30 years. Hydrops fetalis, hepatosplenomegaly, dysostosis multiplex and developmental delay. Mental retardation reported in many cases.

MPS IX: Hyaluronidase deficiency	
Prevalence	only four cases reported since 1996
Enzyme deficiency	hyaluronidase
GAG deg. pathway	hyaluronan
Phenotype	<u>Symptoms</u> : articular swelling in the four cases. Also growth retardation and mild dysmorphic craniofacial features in the first reported case. No evidence of neurological or visceral involvement. (Natowicz et al. (1996), Triggs-Raine et al. (1999), Imundo et al. (2011))

3.3. DIAGNOSIS OF THE MUCOPOLYSACCHARIDOSES

In the mucopolysaccharidoses, undegraded or partially degraded GAGs are excreted in urine, and this fact is used in the primary test for the diagnosis of the MPS. Since different enzymatic deficiencies lead to common excreted GAGs, urine analysis is not a differential diagnosis method, and subsequent enzymatic analyses are required in order to define the type of MPS. In addition, the genetic characterization of the mutation that leads to the functional deficiency can be done in each case. These genetic analyses can have prognostic value, after characterization of mutations and relation between each mutation and the symptoms and severity of the patients. Besides, the information can be used for carrier analysis and prenatal diagnosis within family members. In these cases, early diagnosis of MPS affected individuals may allow starting a treatment, if available, before clinical symptoms are evident. (Lehman et al. (2011))

3.4. CURRENT TREATMENTS FOR MUCOPOLYSACCHARIDOSES

Mucopolysaccharidoses are severe progressive diseases caused by the lack of an enzymatic activity that leads to glycosaminoglycan accumulation in tissues. Available treatments for MPS include symptom-based treatments, which are directed to the improvement of the MPS-related complications, and disease-based treatments, directed to restore the enzymatic function.

Many different treatments and interventions can be performed to improve the symptoms of MPS patients. Since affection is multisystemic, a multidisciplinary medical team is required for the management of the therapeutic interventions in bones, joints, heart, vision, hearing, respiratory tract, etc. Most of these complications require surgical intervention and anesthesia, which are more risky in MPS patients.

In contrast, disease-based treatments are designed to restore the enzymatic function in the organism by different means, thus expecting an arrest of the disease progression and/or an improvement of the symptoms. The main available disease-based treatments for MPS are hematopoietic stem cell transplantation and enzyme replacement therapy. In addition, gene therapy is an emerging alternative for MPS patients, with initial results of a clinical trial already published.

The rationale of these three therapeutic approaches is the cross-correction of enzyme-deficient cells by extracellular enzyme supply. As previously explained (see Figure 10), the trafficking of many lysosomal enzymes is dependent on the interaction with mannose 6-phosphate receptors (M6R), which are present in the intracellular compartments and also on the cell surface. Therefore, an external supply of enzyme can be internalized into endosomal and lysosomal compartments, thus leading to correction of defective cells. This is the therapeutic mechanism of the recombinant enzyme administered in the enzyme replacement therapies. In addition, a proportion of the lysosomal enzymes synthesized in the endoplasmic reticulum are secreted to the extracellular medium. Therefore, the presence of non-defective cells, achieved either by transplant or by in vivo gene therapy, allows cross-correction of adjacent defective cells by enzyme secretion and subsequent internalization.

Besides the treatments that attempt to restore the enzymatic function in the organism, an alternative treatment proposed for MPS is the substrate reduction therapy (SRT), which is currently used for other LSD.

Since MPS are progressive diseases, the early diagnosis and intervention is a key issue in the treatment outcome, a fact that is supported by the pathophysiological mechanisms of MPS. It is known that the initial cause of the disease is GAG accumulation, although the multisystemic affectation occurs afterwards, through multiple complex secondary pathways. Thus, early treatment could prevent or slow down GAG accumulation and reduce subsequent complications. (Valayannopoulos and Wijburg (2011), Noh and Lee (2014))

3.4.1. Hematopoietic stem cell transplant

The allogeneic hematopoietic stem cell transplant (HSCT) is the only current treatment available for MPS patients with severe phenotypes, particularly involving the CNS. It is the standard of care for severe MPS I Hurler patients, after considering particular benefits and risks, and it can achieve long-term metabolic correction, amelioration of neurocognitive and functional problems, and increased survival. The benefits of HSCT are provided by the engraftment of the transplanted cells as microglia in the CNS, Kupffer cells in the liver, and macrophages in spleen, lung and other organs. The engrafted cells lead to cross-correction of the neighboring cells by enzyme secretion, achieving improvements in the progression of the disease. The outcome of the transplants and the benefits obtained depend on many factors (e.g. the graft source, which can be either bone marrow cells or umbilical cord cells; the enzymatic levels of donor cells, which can be from a heterozygote related individual and provide only 50% of enzymatic activity; the level of engraftment achieved after transplant...). Importantly, early treatment after diagnosis is associated to better outcome of transplant, including increased neurocognitive preservation. Despite the benefits, HSCT presents many risks and significant rates of mortality and morbidity, as well as subsequent transplant-related complications, which must be considered.

The overall experience of HSCT in MPS types other than MPS I is limited and it is still under investigation. Clinical benefits have been reported in different MPS types, and results suggest that one of the most important factors in the transplant outcome is the timing of transplant (Prasad and Kurtzberg (2010)).

3.4.2. Enzyme replacement therapy

The enzyme replacement therapy (ERT) consists in the exogenous supply of the lacking enzyme by regular infusions of the recombinant enzyme weekly or every other week. ERT is a life-long therapy that is usually performed in hospital settings, and has some related risks and adverse events such as life-threatening anaphylaxis and other milder allergic reactions. A major limitation of intravenous ERT is that the recombinant protein is not able to cross the BBB, thus CNS affectation, if present, cannot receive any benefit.

Between 2003 and 2006, the recombinant enzymes for MPS I, II and VI were commercially delivered and patients started to be treated by intravenous infusions. Since then, ERT improved walking ability, endurance and pulmonary function of MPS I, II and VI patients, although not in all the cases. In contrast, cardiac valve function, airway disease, and joint and skeletal disease do not generally seem to improve with ERT if pathological changes are already present when ERT is started. With these results, and since ERT to young patients demonstrated to be safe, the early initiation of the treatment is recommended even in pre-symptomatic patients, because it can prevent the onset of the symptoms. This has been reported in many sibling case studies, with better treatment outcomes in younger MPS patients that could be diagnosed and treated soon after birth because of their older sibling's MPS previous diagnosis (Valayannopoulos and Wijburg (2011), Noh and Lee (2014), Muenzer (2014)).

In addition to MPS I, II and VI, a recombinant enzyme for MPS VIA has been recently approved in the USA and in the European Union (www.fda.gov; www.ema.europa.eu) and an initial clinical trial for intravenous ERT for MPS VII has already started (www.clinicaltrials.gov: NCT01856218).

As stated before, CNS affectation cannot be ameliorated by intravenous ERT because the recombinant enzyme is not able to cross the BBB, so an alternative administration route is needed. Currently, some clinical trials are testing the safety and efficacy of intrathecal ERT for MPS I, MPS II and MPS IIIA (www.clinicaltrials.gov: NCT00852358, NCT00920647 and NCT01155778, among others). This is especially relevant for MPS IIIA patients, which mainly present CNS affectation and have no available treatment. In this case, the first study is currently evaluating the long-term effects of the treatment in patients older than 3 years old (NCT01299727) and a new study will test the efficacy in patients younger than 2 years old (NCT02060526).

It is important to notice that most of MPS patients receiving ERT develop antibodies against the recombinant enzyme, because they have absent or very low residual enzyme activity. However, the antibodies do not seem to be neutralizing, so they do not negate the efficacy of the exogenous protein (Valayannopoulos and Wijburg (2011)).

3.4.3. Gene therapy

In the recent years, gene therapy has emerged as a promising therapeutic approach for MPS treatment after many studies in small animal models and some preclinical studies in large animal models (Ciron et al. (2006), Ellinwood et al. (2010), Haurigot et al. (2013), Murrey et al. (2014), Ferla et al. (2014)). In fact, the results of the first gene therapy clinical trial using AAV vectors were recently published (Tardieu et al. (2014)). This clinical trial for MPS IIIA enrolled 4 patients, three children between 5.5 and 6 years and one child younger than 3 years old. Patients received 12 intracranial injections of AAVrh10 carrying the therapeutic cassette for MPS IIIA, a protocol that demonstrated to be safe, with good tolerance and absence of adverse events or toxicity after one year. Preliminary efficacy assessment revealed stable brain atrophy in two patients, although it increased in the other two. Interestingly, the youngest patient is the most likely to display neurocognitive benefit after the treatment.

3.4.4. Substrate reduction therapy

The aim of the substrate reduction therapy (SRT) is to inhibit production of the stored products, the glycosaminoglycans. Unlike ERT, the small molecules used for SRT are expected to cross the BBB and improve CNS pathology. However, a clinical trial for MPS III that used Miglustat –a molecule that inhibits the synthesis of glucosylceramide– did not lead to any cognition or behavior improvement (Guffon et al. (2011)). Another compound used for SRT is Genistein, a plant isoflavone known to block the pathway that leads to the expression of GAG synthesizing enzymes. Initial data from mice were promising in reducing lysosomal storage in MPS IIIB brains (Malinowska et al. (2010)), although subsequent studies in MPS III patients revealed little or no improvement in behavioral and cognitive problems (Piotrowska et al. (2011), Delgadillo et al. (2011), Malinová et al. (2012), de Ruijter et al. (2012)). Therefore, a 15-fold higher dose was tested in MPS III patients, giving good safety although unclear efficacy results (Kim et al. (2013)). Currently, in order to establish the effectiveness of high dose Genistein for MPS III, a double blinded, placebo controlled clinical trial is ongoing (MPS III Genistein clinical trial in www.mpssociety.org.uk).

4. MUCOPOLYSACCHARIDOSIS TYPE VII (SLY SYNDROME)

4.1. DESCRIPTION AND CLINICAL SYMPTOMS

Named after Dr. William S. Sly who first described it (Sly et al. (1973)), MPS VII is an inherited disorder caused by the deficiency of the lysosomal enzyme β -glucuronidase, encoded by the GUSB gene. The inheritance is autosomal recessive and the estimated prevalence of the disease is 1 in 10 million births (Orphanet Report Series: prevalence of rare diseases).

There is a wide range of clinical severity among the different MPS VII cases described. The most common type of Sly syndrome may be the neonatal, characterized by non-immune hydrops fetalis, dysostosis multiplex, dysmorphic features, and other clinical and pathologic findings typical of a lysosomal storage disease. This neonatal form presents heterogeneity in the clinical manifestations that range from death *in utero*, to mild or no hydrops at birth (Van Dorpe et al. (1996)). Among the patients with the onset of the symptoms beyond the neonatal period there is also a wide spectrum of severity. The most severe forms affect infants or young children displaying hepatosplenomegaly, inguinal and/or umbilical hernias, moderate skeletal abnormalities, repeated episodes of pneumonia in the first years of life, short stature and developmental delay. Mental retardation is displayed in many cases (Bernsen et al. (1987)), and some patients present corneal clouding. Milder forms of later onset, after 4 years of age, are characterized by progressive skeletal involvement with normal intelligence and typically no corneal clouding (Scriver et al. (2001)). Life expectancy in severe MPSVII patients is months, whereas in milder forms it ranges from 20 to 30 years (Quiney et al. (2012)).

Currently, the only available treatments for MPS VII patients are interventions to improve the symptoms, as corneal transplantation (Bergwerk et al. (2000)) or other surgeries, and the hematopoietic stem cell transplant (Yamada et al. (1998)). However, few data are reported of the transplants and benefit is still under investigation. Recently, as stated before, a clinical trial of ERT for MPS VII has started (www.clinicaltrials.gov: NCT01856218).

4.2. HUMAN GENETICS OF MPS VII

Sly syndrome is caused by mutations in the GUSB gene, which is located in chromosome 7 (7q21.11) (CE et al. (1991); F et al. (1996)). It spans approximately 20 kb and it is composed of 12 exons and 11 introns. It is a highly conserved gene between species, and over 20 GUSB pseudogenes have been identified in the human genome database (Tomatsu et al. (2009)).

The product of the GUSB gene is the 651-aminoacid globular protein β -glucuronidase, which is further processed into a mature form by the proteolytic processing of the 22-aminoacid N-terminal sequence (Oshima et al. (1987)) and also by 4 N-linked glycosylations (Shipley et al. (1993)). The functional β -glucuronidase enzyme is a homotetramer (EC 3.2.1.31). Its crystallographic structure had been determined at a resolution of 2.6 Å on the first structural publication (Jain et al. (1996)) and later at a resolution of 1.7 Å (Hassan et al. (2013)) (**Figure 13**). β -Glucuronidase monomers contain a lysosomal targeting motif, which is homologous to other lysosomal proteins both in sequence and structure. This motif is recognized by the enzymes in the trans-Golgi network that catalyze the binding of the mannose-6-phosphate residues to the protein, which allows it to be transported to the lysosome. (Jain et al. (1996)).



Figure 13: Human β -glucuronidase protein structure, a homotetramer. (From RCSB Protein Data Bank)

β -Glucuronidase participates in the degradation pathway of heparan sulfate, dermatan sulfate, chondroitin sulfate and hyaluronan, catalyzing the reaction in **Figure 14**. Tomatsu et al. (2009) performed an exhaustive data analysis of human GUSB mutations and polymorphisms that are summarized in 49 different mutations identified in 103 mutant alleles from a total group of 56 patients. The mutations are

found along the whole gene and most of them are missense mutations (81 alleles, 78.6%), although nonsense, deletion and splice site mutations are also reported. Nearly two thirds of the different mutations occur less than four times in the mutant population, thus indicating an extensive molecular heterogeneity in GUSB mutations. In addition, the study establishes a correlation between genotype and phenotype for several GUSB mutations.

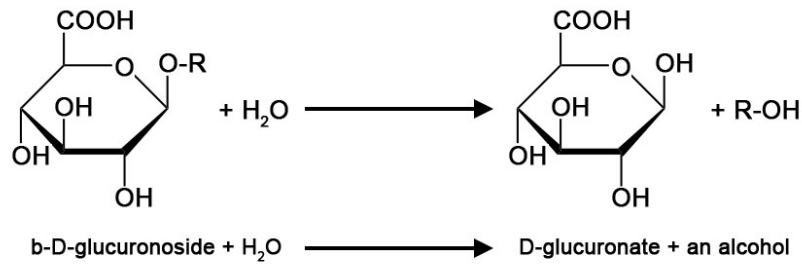


Figure 14: Enzymatic reaction catalyzed by β -glucuronidase

4.3 MPS VII ANIMAL MODELS

The first animal model of MPS VII described was a mouse with spontaneous β -glucuronidase deficiency that presented morphologic, genetic, biochemical and histological characteristics very similar to those of human MPS VII patients (Birkenmeier et al. (1989), Vogler et al. (1990)). Years later, two new MPS VII mouse models were generated by transgenesis on the spontaneous model, in order to have better models to test therapeutic strategies. These two new MPS VII mice present immune tolerance to human β -glucuronidase (Sly et al. (2001)) and to both human and mouse β -glucuronidase (Tomatsu et al. (2003)).

These three MPS VII mice are smaller and present shorter, stubby limbs and dysmorphic facies that are caused by skeletal abnormalities (**Figure 15**). They have shorter lifespan, and present vacuolar accumulation due to abnormal lysosomal storage in many tissues and organs: brain, heart, spleen, kidney, liver, eye, bone, articular cartilage, etc. This widespread abnormal lysosomal storage is present even *in utero* and in very young MPS VII mice (Vogler et al. (2005)).

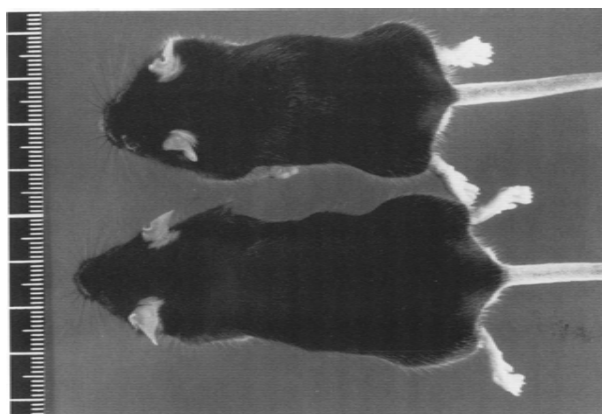


Figure 15: Dismorphic anatomy of the MPS VII mouse model. MPS VII mouse (top) and WT mouse (bottom). (From Vogler et al. (1990))

The behavior of MPS VII mice has been assessed in different studies to test the efficacy of diverse therapeutic approaches (Bastedo et al. (1994), O'Connor et al. (1998), Frisella et al. (2001), Sakurai et al. (2004), Liu et al. (2005), Fukuhara et al. (2006), Liu et al. (2007), Bielicki et al. (2010), Macsai et al. (2012), Derrick-Roberts et al. (2014), Ariza et al. (2014)). Each study used different behavioral tests, ages and conditions, which make their comparison difficult. However, taking together the different studies, it is evident that MPS VII mice display a less active phenotype than WT mice, as well as cognitive deficits.

It is noteworthy that MPS VII mice are not capable to breed, which is not due to any failure in gametogenesis but to impaired mobility and/or impaired cognitive function that hinder mating behavior. Therefore, to obtain MPS VII mice it is necessary to mate heterozygote mice. However, these matings give rise only to $\approx 18\%$ MPS VII pups, less than the expected 25%, a fact that is not due to prenatal death but to losses between birth and weaning (Soper et al. (1999)).

Besides the MPS VII mouse models displaying severe phenotype, a different spontaneous MPS VII mouse was described, which presents a loss of enzyme activity that leads to an attenuated MPS VII phenotype (Gwynn et al. (1998), Vogler et al. (2001)). In addition to the mouse models, two feline MPS VII models (Gitzelmann et al. (1994), Fyfe et al. (1999)) and two canine models (Haskins et al. (1984), Silverstein Dombrowski et al. (2004)) have been described, with pathologic characteristics similar to human MPS VII patients.

4.4. THERAPEUTIC STRATEGIES ON MPS VII MICE

The MPS VII mouse model was one of the first animal models available for a lysosomal storage disease. In consequence, many different therapeutic strategies have been tested in this model, which have been employed later on in other LSD during the last 25 years.

4.4.1. Cell transplantation and enzyme replacement therapy

Initially, syngeneic bone marrow transplantation (BMT) was assessed in the MPS VII model, both in adult and newborn mice (Birkenmeier et al. (1991), Sands et al. (1993)). The best results were obtained in newborns because brain and bone affectation were not corrected in adult mice. But BMT required myeloablation, which had severe side effects in newborns. To avoid myeloablation, Soper et al. (2001) performed transplantation of a high dose of congenic normal bone marrow into nonablated newborn MPS VII mice, getting increased lifespan, attenuation of some skeletal abnormalities, and biochemical and histological correction in many organs, and to a small extent in brain. Apart from BMT, and as a strategy to directly target the CNS pathology, several groups have employed transplantation of neural progenitor cells to the cerebral ventricles of newborn MPS VII mice, obtaining β -glucuronidase expression and lysosomal correction in the brain (Snyder et al. (1995)), which led to some behavioral amelioration two months after transplantation (Fukuhara et al. (2006)).

Many studies have been done in MPS VII mice using enzyme replacement therapy (ERT) as a therapeutic approach. However, since β -glucuronidase is a large protein (approximately 400 kDa) it is not able to cross the BBB. Therefore, the exogenous supply of recombinant enzyme by intravenous delivery is not capable to reach the CNS and provide benefit to its affectation.

In MPS VII mice, ERT administered by 6 weekly injections started after birth (Sands et al. (1994)) demonstrated to be effective in correcting liver, spleen and kidney lysosomal storage, and to some extent in brain and bone. The CNS correction is related to the administration soon after birth, since the BBB is not completely formed

in newborn mice. In addition, this treatment was capable to improve survival and growth (Vogler et al. (1996)), as well as hearing skills and some behavioral traits of MPS VII mice (O'Connor et al. (1998)), which could mate and breed, in contrast to non-treated MPS VII mice (Soper et al. (1999)). However, one year after the 6-week-long ERT treatment, nearly all the tissues presented lysosomal storage similar to the non-treated mice, suggesting that a lifelong continued treatment would be needed to maintain the therapeutic effect of ERT (Vogler et al. (1996)).

4.4.2. Gene therapy strategies

In the beginning of gene therapy research, some groups performed *ex vivo* gene transfer of the GUSB gene by retroviral vectors to either hematopoietic cells (Wolfe et al. (1992), Maréchal et al. (1993)) or skin fibroblasts (Moullier et al. (1993)) before transfer to MPS VII mice. They achieved transplant persistence at least for several months that led to lysosomal storage correction in somatic organs but not in brain or bone. Others have genetically modified fibroblasts (Taylor and Wolfe (1997)) or bone marrow cells (Sakurai et al. (2004)) before administering them to the CNS of MPS VII mice, achieving engraftment and different degrees of correction of the brain pathology, although without peripheral benefits. Even though, many more studies chose strategies of *in vivo* gene transfer to MPS VII mice by different administration routes.

Most of the *in vivo* gene therapy approaches in MPS VII mice involved either intravascular delivery or direct administration to the CNS, but some tested also the intramuscular delivery, mainly achieving only muscle transduction and some cross-correction in liver and spleen (Watson et al. (1998), Daly et al. (1999a)) Besides, other gene therapy strategies on MPS VII mice were directed to specifically treat the ophthalmic pathology, by intravitreal injection of AAV to treat the retina (Hennig et al. (2004)), or by delivery of a canine adenoviral vector to the cornea (Serratrice et al. (2014)).

4.4.2.1. Intravascular administration

Several groups delivered the GUSB gene by intravenous administration of different types of gene therapy vectors to the MPS VII mouse. As it was presented before in this work for AAV vectors, and in general, the intravenous delivery of viral vectors achieved better transduction of the CNS in newborn mice than in adult mice. This fact is because newborn mice still present a high degree of permeability of the BBB, which is lost during the days after birth (Stewart and Hayakawa (1987)).

The IV injection of adenoviral vectors to adult MPS VII mice mainly transduced liver cells, from where the enzyme was secreted to the bloodstream. After seven days, β -gluc activity was detected in spleen, kidney, lung and heart but not in brain. The secreted β -gluc led to lysosomal storage clearance in liver and spleen by cross-correction. However, due to T-cell activation by the vector, the transduced hepatocytes were eliminated and after 70 days the therapy was no longer effective (Ohashi et al. (1997), Kosuga et al. (2000)). In contrast, the IV injection of adenoviral vectors to newborn mice was able to target the brain, cornea, retina and cartilages, besides the peripheral organs also targeted in adult mice. In consequence, 140 days after treatment, MPS VII mice did not present vacuolated cells in the brain, while in the retina, cornea and knee joint the lysosomal accumulation was decreased. Moreover, the skeletal abnormalities in the cranial bones and the length of the tibia and fibula were corrected in the mice treated after birth (Kamata et al. (2003), Kanaji et al. (2003)).

The intravenous administration of a retroviral vector to adult MPS VII mice resulted, one month after injection, in 8% and 5.7% of WT β -gluc activity in liver and serum respectively, which led to a decrease in lysosomal storage in liver, spleen and bone marrow. However, both the improvements and the β -gluc expression were significantly reduced at 3.5 months (Gao et al. (2002)). In contrast, the IV injection of a retroviral vector to neonatal mice was more successful, because retroviral vectors are only able to transduce dividing cells and the hepatocyte division rate is higher in neonates than in adults. Therefore, this approach reached, 6 months after vector injection, a β -gluc activity in liver that was 8.7 and 127 times higher than WT in liver and serum respectively. This conducted to lysosomal storage clearance in many

organs such as liver, spleen, intestine, kidney and also brain. In fact, they detected some β -gluc gene expression in the brain, although the main source of β -gluc was the liver (Xu et al. (2002)). Moreover, this neonatal approach led to improvement in the skeletal abnormalities of the MPS VII, in body weight and in some abnormal histological features of bones and joints (Mango et al. (2004)). These studies in mice were translated to the canine MPS VII model and the results will be presented later on in this work.

Some studies have delivered lentiviral vectors intravenously in MPS VII mice. The injection to 6-week-old mice achieved 12% of WT β -gluc activity in serum, while liver presented β -gluc levels similar to WT, 6 months after treatment. This led to increased survival and reduced lysosomal storage in most somatic organs (Bielicki et al. (2010)). When this approach was compared to the injection to newborn mice it was shown that the neonatal injection led to higher β -gluc activity in liver but not in serum. Both treatment time points achieved similar success in reducing the lysosomal storage in somatic organs, while neonatal treatment also led to some lysosomal clearance in brain (Macasai et al. (2012)). In addition, both time points improved some bone parameter values. Moreover, some behavioral traits that are impaired in MPS VII mice were ameliorated by the treatment. A similar approach was conducted in an attenuated mouse model of MPS VII with similar results (Derrick-Roberts et al. (2014)). However, they also reported tumorigenesis after IV lentiviral administration.

AAV vectors have also been administered intravenously to MPS VII mice. After the treatment of adult mice with AAV2-GUSB, β -gluc activity was mainly detected in liver, heart and muscles, with 14% of WT activity in liver 13 weeks after treatment. This led to lysosomal storage clearance in liver at that stage, but no improvement in brain pathology (Watson et al. (1998)). In contrast, the treatment of newborn mice achieved β -gluc expression in liver and other somatic organs and also in brain, which was stable up to 1 year after treatment (Daly et al. (1999b), Daly et al. (2001)). These studies in neonates reported a decrease of lysosomal storage in liver and also in brain, improved weight, reduced skeletal abnormalities, increased physical activity, improved retinal and auditory functions and increased lifespan of AAV-treated MPS VII mice. However, some of these MPS VII mice developed hepatocellular carcinomas

at 18 months of age (Donsante et al. (2001)). Further work injecting the same AAV2 vector to WT and MPS VII mice demonstrated high rates of hepatocellular carcinoma development in both cases (57% and 33% respectively) that were caused by insertional mutagenesis of the AAV vector (Donsante et al. (2007)).

Other strategies performing IV administration of the therapeutic gene include the hydrodynamic plasmid delivery to liver (Richard et al. (2009)) and the antibody-targeted liposomes containing the GUSB plasmid (Zhang et al. (2007)). In the first approach, the enzyme is secreted from liver and reaches somatic organs and to a lesser extent the brain, leading to different degrees of lysosomal storage clearance. The second approach is directed to target the CNS, since liposomes are designed to cross the BBB, and it achieves great β -glucuronidase levels in brain and somatic organs. However, these non-viral gene therapy strategies would require repeated administrations because the transgene expression decreases with time.

After the overview of the studies performing systemic delivery of gene therapy vectors to MPS VII mice, we observe that the strategies performed in neonatal mice were capable to target both somatic organs and CNS and lead to global therapeutic effects. However, the studies that administered gene therapy vectors to adult MPS VII mice only achieved amelioration of the peripheral affectation of the lysosomal storage disease.

4.4.2.2. Direct administration to the CNS

Direct delivery of viral vectors to the CNS, either to brain parenchyma or to cerebral ventricles, is another *in vivo* gene therapy strategy that has been used in a number of works.

Adenoviral vectors injected into the cerebral ventricles only transduced ependymal and choroid cells (Ohashi et al. (1997)), while they achieved β -gluc expression in 56% of the brain when injected into the striatum, which led to histological correction in striatum and also in cortex after 3 weeks (Ghodsi et al. (1998)).

The injection of herpes simplex viral vectors to the somatosensory cortex achieved significant levels of β -gluc activity that reached WT levels in some brain areas. The axonal transport of this kind of vector permitted β -gluc expression in brain regions distal of the injection site and this approach led to reversal of lysosomal storage pathology in the β -gluc expressing areas 2 months after treatment (Berges et al. (2006)).

The injection of a lentiviral vector to the brain parenchyma of adult MPS VII mice revealed poor vector diffusion, thus multiple injections were required in order to achieve β -glucuronidase expression in 20% of the total brain volume 16 weeks after treatment. This widespread expression led to histological correction in 50 to 90% of the brain, depending on the areas (Bosch et al. (2000b)). In addition, ICV delivery of a lentiviral vector gave rise to widespread enzyme expression in the brain, reaching 2.5 to 51% of WT levels in different areas, which achieved broad lysosomal storage clearance that correlated with some behavioral improvement (Bielicki et al. (2010)). Besides, a canine adenoviral vector, a vector that undergoes axonal transport, was administered to the striatum of MPS VII mice and was able to correct lysosomal storage by around 80-90%, leading to behavioral improvement (Ariza et al. (2014)).

Finally, AAV vectors have also been administered to the CNS of the MPS VII mice, being AAV2 the serotype first used for therapeutic approaches. Bosch et al. (2000a) administered 1×10^7 vg of AAV2-GUSB to the striatum of MPS VII mice unilaterally, with a single injection. They described that the vector particles did not diffuse throughout the brain parenchyma and mainly transduced cells near the needle track, a transduction that was stable from 6 to 16 weeks post-treatment. β -Glucuronidase expressed by those cells could reach distant areas of the brain, including the non-injected hemisphere, thus leading to β -gluc levels similar to HTZ in the ipsilateral hemisphere and 24% of HTZ in the contralateral one. This approach achieved complete lysosomal storage clearance in the cortex and striatum areas where β -gluc activity was detected, and a significant decrease in vesicle accumulation was seen in the areas where β -gluc activity could not be detected. Moreover, Heuer et al. (2002) described that the intracranial injection of AAV2 to adult mice could reverse the neurodegeneration markers in cortex and hippocampus of MPS VII mice, which are

revealed by ubiquitin inclusions, neurofilament inclusions and reactive astrogliosis. A similar improvement in the lysosomal storage than that achieved in adult mice was also seen when an AAV2 vector was administered to neonatal MPS VII mice, by delivery to the frontal cortex and hippocampus, bilaterally (Frisella et al. (2001)). Total brain β -gluc activity levels were similar to WT at 18 weeks of age, although the vector was mainly expressed near the injection sites. At 10 weeks of age, MPS VII treated mice displayed improved cognitive function.

After AAV2, other AAV serotypes were administered intracranially to MPS VII mice. Liu et al. (2007) reported similar β -gluc activity and lysosomal storage correction with bilateral intrastriatal delivery of 6×10^9 vg of AAV5 in adult mice than Bosch et al. (2000a). They also observed some recovery of the cognitive deficit of MPS VII mice 6 weeks after treatment. Besides, AAV serotypes 7, 8, 9 and rh10 were tested in adult MPS VII mouse brains by Cearley and Wolfe (2006). They injected 4×10^{10} vg in each mouse brain, which is a much higher dose than the previous studies, divided in four injection sites in one hemisphere (cortex, striatum, hippocampus and thalamus). They reported that AAV9 and AAVrh10 were the serotypes achieving broader β -gluc activity distribution throughout the brain, reaching the non-injected hemisphere and also the cervical spinal cord. Using this approach with AAV9, they report lysosomal storage clearance throughout the injected hemisphere and also in many regions of the non-injected hemisphere, but not in cerebellum. Moreover, Cearley and Wolfe (2007) demonstrated, using GUSB-coding vectors, that AAV1, AAV9 and AAVrh10 could undergo axonal transport and achieve transduction of many distal brain regions after a single injection of 1.2×10^{10} vg in the ventral tegmental area (VTA), which presents many afferent and efferent projections. And using AAV9-GUSB they demonstrated a complete correction of the lysosomal storage in MPS VII mouse brain two months after injection in the VTA.

The injection of AAV vectors to the cerebral ventricles of neonate mice was tested using AAV2, AAV1 and AAV5 (Passini and Wolfe (2001), Passini et al. (2003)). AAV5 only transduced ependymal cells and the choroid plexus, whereas AAV1 and AAV2 were able to transduce the brain parenchyma. With the delivery of $\approx 10^{10}$ vg in the cerebral ventricles, AAV1 was the serotype that achieved the broadest distribution of

β -gluc expression throughout the brain parenchyma, reaching many brain regions. This expression was sustained up to 1 year after administration and it led to total reversal of storage lesions in the brain, including cerebellum and regions with low β -gluc activity. Moreover, ICV injection of 3.3×10^{10} vg of AAV1 to fetal brain of MPS VII mice achieved high, broad and widespread β -glucuronidase expression in the entire brain and in a great part of the spinal cord. Some β -gluc activity was detected in peripheral organs, with 10% of HTZ β -gluc levels in liver, although any vector DNA was present in those organs (Karolewski and Wolfe (2006)). This approach, analyzed at one year, prevented lysosomal storage in brain and spinal cord and increased MPS VII mice life span: only 35% of MPS VII mice survived up to one year, while 80% of treated mice were alive, which was comparable to normal mice. Although it could have been interesting, these authors did not perform any behavioral tests to the fetal-treated mice. Besides, the ICV administration to adult MPS VII mice was performed using 10^{10} vg of AAV4 and it only transduced ependymal cells (Liu et al. (2005)). These transduced cells acted as a source of enzyme secretion, being able to reach the brain parenchyma and achieve 28% β -gluc activity levels of HTZ mice in cortex, and 12% in cerebellum. Six weeks after AAV delivery, MPS VII mice displayed some improvement in the cognitive deficits that correlated to reduced lysosomal storage in many brain regions.

In summary, gene therapy strategies that administer the vector into the CNS of either adult or newborn MPS VII mice report β -glucuronidase activity and lysosomal storage clearance in the CNS. In some studies, this CNS histological correction correlates to behavioral improvement. However, any of them report β -glucuronidase activity or therapeutic effects on somatic organs after CNS delivery.

One strategy that tried to circumvent this limitation was the simultaneous administration of an adenoviral vector both intravenously and into the striatum of adult MPS VII mice (Stein et al. (1999)). This study reported $\approx 80\%$ of WT β -gluc activity in liver after 16 weeks and high β -gluc activity in the ipsilateral hemisphere that led to lysosomal storage correction in liver and brain. They achieved better

lysosomal clearance in somatic organs when mice were transiently immunosuppressed, while it did not affect CNS correction. It is noteworthy that the levels of β -gluc activity achieved in somatic organs were better than the previously reported by Ohashi et al. (1997) by intravenous administration of an adenoviral vector. Stein et al. (1999) claim that the simultaneous injection in the CNS may affect the immune response to intravenously injected adenoviral vector.

OBJECTIVES

OBJECTIVES

The main purpose of this work is to design and evaluate the outcome of a gene therapy strategy for mucopolysaccharidosis type VII using AAV vectors in the MPS VII mouse model.

Like MPS VII patients, the mouse model displays both central nervous system and peripheral symptoms such as hepatomegaly and dysostosis multiplex. Thus, the gene therapy approach needs to target the CNS and peripheral organs. This requires choosing a gene transfer vector and an administration route that allow efficient transduction. To increase biosafety, it is important to choose a vector with low immunogenicity, and also to administer the lowest possible dose. Finally, it is important to establish the best conditions concerning the age of mice at the beginning of the treatment, the dose of vector and the duration of the experimental treatment.

Therefore, the main purpose of this work will be addressed by the following objectives:

1. To evaluate the transduction efficiency, the tropism and the immune response elicited by AAV9 and AAVrh10 after intravenous administration in wild type mice.
2. To analyze brain and somatic transduction after AAVrh10 intrathecal administration in wild type mice.
3. To evaluate and compare different vector doses and time of treatment onset, performing intrathecal delivery of AAVrh10-GUSB to MPS VII mice.
4. To characterize the therapeutic outcome of the treatment of MPS VII mice by intrathecal administration of AAVrh10-GUSB, assessing biochemical, histological, behavioral and survival parameters.

