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Review

GM2 Gangliosidosis: Clinical Features and Current Therapies

Andrés Felipe Leal[1], Eliana Benincore-Flórez[1], Daniela Solano-Galarza[1], Rafael Guillermo Garzón Jaramillo[1], Olga Yaneth Echeverri-Peña[1], Diego A. Suarez[1,2], Carlos Javier Alméciga-Díaz[1]*, Angela Johana Espejo-Mojica[1]*

[1] Institute for the Study of Inborn Errors of Metabolism, Faculty of Science, Pontificia Universidad Javeriana, Bogotá D.C., 110231, Colombia; lealb.af@javeriana.edu.co (A.F.L.), elianabenincore@javeriana.edu.co (E.B.F), aura.solano@javeriana.edu.co (D.S.G.), rafael.garzon@javeriana.edu.co (R.G.), oyecheve@javeriana.edu.co (O.Y.E.), suarezd.i@javeriana.edu.co (D.A.S.)

[2] Faculty of Medicine, Universidad Nacional de Colombia, Bogotá D.C., Colombia; dasuarezg@unal.edu.co (D.A.S.)

* Correspondence: cjalmeciga@javeriana.edu.co; Tel.: +57-1-3208320 (Ext 4140) (C.J.A-D.). aespejo@javeriana.edu.co; Tel.: +57-1-3208320 (Ext 4099) (A.J.E.M)

Abstract: GM2 gangliosidosis are a group of pathologies characterized by GM2 ganglioside accumulation into the lysosome due to mutations on the genes encoding for the β-hexosaminidases subunits or the GM2 activator protein. Three GM2 gangliosidosis have been described: Tay-Sachs disease, Sandhoff disease, and AB variant. Central nervous system dysfunction is the main characteristic of GM2 gangliosidosis patients that include neurodevelopment alterations, neuroinflammation, and neuronal apoptosis. Currently, there is not approved therapy for GM2 gangliosidosis, but different therapeutic strategies have been studied including hematopoietic stem cell transplantation, enzyme replacement therapy, substrate reduction therapy, pharmacological chaperones, and gene therapy. The blood-brain barrier represents a challenge for the development of therapeutic agents for these disorders. In this sense, alternative routes of administration (e.g. intrathecal or intracerebroventricular) have been evaluated, as well as the design of fusion peptides that allow the protein transport from the brain capillaries to the central nervous system. In this review, we outline the current knowledge about clinical and physiopathological findings of GM2 gangliosidosis, as well as the ongoing proposals to overcome some limitations of the traditional alternatives by using novel strategies such as molecular Trojan horses or advanced tools of genome editing.

Keywords: Lysosomal Storage Disorders; GM2 gangliosidosis; Tay-Sachs disease; Sandhoff disease; β-Hexosaminidases; Therapeutic alternatives

1. Introduction

Gangliosides are a group of glycosphingolipids mainly located in the neuronal cell membrane and that are responsible of several pivotal biological functions for the correct functioning of the central nervous system (CNS) [1]. About 5% of all gangliosides into the brain correspond to GM2 gangliosides [2, 3]. In normal conditions, the GM2 gangliosides are catabolized by the lysosomal hydrolases known as β-hexosaminidases (Hex, EC 3.2.1.52) through the hydrolysis of the N-acetylgalactosamine residues present on the structure of the GM2 ganglioside [4]. Hex are a subset of isozymes formed by the dimerization of the α and β subunits as follows: HexA (αβ), HexB (ββ) and HexS (αα). In addition, GM2 gangliosides degradation involves the GM2 activator protein (GM2-AP), which present the gangliosides to α subunit of HexA [1]. Mutations in the genes encoding for the α (*HEXA*), β (*HEXB*) or GM2-AP (*GM2A*) proteins, promote an impaired lysosomal degradation of the GM2 ganglioside, as well as other glycolipids, causing their accumulation into the lysosome [4].

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Mutations in *HEXA*, *HEXB,* and *GM2A*, and the subsequent GM2 ganglioside accumulation, lead to the GM2 gangliosidosis Tay-Sachs (TSD), Sandhoff (SD), and AB variant diseases, respectively [4]. Upon the GM2 ganglioside accumulation, several cytotoxic effects take place mainly in neurons, which frequently cause neuronal death [5]. Individuals with GM2 gangliosidosis have a progressive neurological impairment including motor deficits, progressive weakness, hypotonia, decreased responsiveness, vision deterioration, and seizures, among others [6]. SD individuals present systemic manifestations as organomegalies, unlike TSD patients [7, 8]. The diagnosis for these disorders begins with recognition of the clinical characteristics of these disorders, which is follows by the measurement of the enzymatic activity that can be confirmed by mutation analysis [9, 10].

Both the understanding of physiopathology and the development of therapies for GM2 gangliosidosis have benefited from the different animal models available for these diseases. These animal models include mice, cats, and sheep, which mimics some of the biochemical and physiological characteristics of GM2 gangliosidosis [11]. An ideal TSD mouse model was recently developed through a combined deficiency of HexA and Neu3, which mimics the neuropathological and clinical abnormalities of classical early-onset TSD patients and may provide a valuable tool for treatments development for this condition [12].

Several therapeutic approaches have been evaluated for GM2 gangliosidosis, including enzyme replacement therapy, hematopoietic stem cell transplantation, pharmacological chaperones, substrate reduction therapy, and gene therapy. Nevertheless, currently there is not an approved therapy for these disorders. The efficacy of the therapeutic approaches is affected, among others things, by the blood brain barrier (BBB) that limits the access of intravenous therapeutic agents to the CNS [13], and that has led to the design of novel therapeutic strategies to overcome this limitation. In this regard, novel strategies using chimeric recombinant enzymes, a direct brain injection, or the development of vehicles to target proteins to the brain have shown promising advantages respect conventional administration strategies [14]. Likewise, the development of novel gene editing tools as CRISPR/Cas9 has supposed a new horizon to the treatment of the lysosomal storage disorders including the GM2 gangliosidosis [15]. In this paper, we provide a critical review about physiopathology features and diagnosis of these diseases as well as the major up-to-date data about the alternative therapies for GM2 gangliosidosis.

2. Gangliosides: Structure and physiological role.

Gangliosides are complex glycolipids composed of a ceramide linked to a glycan with at least one sialic acid [2]. Currently, over 180 gangliosides have been identified in vertebrates [2, 16]. In humans, GM3 ganglioside is predominantly in peripheral tissues such as liver, adipose tissue, aorta, and platelets [17]; whereas GM1, GD1a, GD1b, GT1b, and GQ1b are the major gangliosides in human brain (~95%) [18]. The remaining 5% of brain gangliosides corresponds to other gangliosides among which GM2 is found [2, 3, 19]. Gangliosides are distributed in caveolae-rich microdomains of the plasma membrane [16, 20, 21], where they perform crucial functions such as membrane organization [21], neuronal differentiation [20, 22], cell adhesion [23], signal transduction [24], inflammation [3], and neurite outgrowth [22, 25], among others.

The *novo* biosynthesis of gangliosides starts with the formation of ceramide in the cytoplasmic side of rough endoplasmic reticulum (RER) and that ends in the *trans*-Golgi network with through the sequential addition of carbohydrates in a process catalyzed by several glycosyltransferases to generate lactosylceramide [1, 26]. Subsequently, the LacCer-α-2–3 sialyltransferase add sialic acid to form GM3 ganglioside [26]. This GM3 ganglioside acts as precursor for more complex gangliosides like GM2, by the action of the β-1,4-N-acetylgalactosaminyl transferase (GM2/GD2 synthase), which transfers the N-acetylgalactosamine residue to the GM3 structure [4, 26, 27]. GM1 and GD1a gangliosides differ from GM2 by the number and type of monosaccharides presents, as well as by the number of sialic acid residues.

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3. β-Hexosaminidases: Synthesis, transport, and catalytic functions.

β-hexosaminidases are dimeric lysosomal enzymes composed by the α and/or β subunits to form HexA (αβ), HexB (ββ), and HexS (αα) isoforms [4]. Genes of the α (*HEXA*) and β (*HEXB*) subunits are located in chromosome 15q23 and 5q13.3, respectively [4]. Fourteen exons and thirteen introns are described for both genes which share a 60% of identity, suggesting a common ancestor [28]. Early studies using pulse and chase analysis showed that Hex are synthesized as long precursors of 67 (α) and 63 (β) kDa that are proteolytic processed to 54 and 52 kDa peptides, respectively [29, 30]. The β subunit suffers further proteolysis to obtain a mature form of 29 kDa peptide and other smaller peptides that remain linked by disulfide bonds [29]. Two major cleavages points for α precursors have been identified [31]: 1) alanine 22 that allows removing the signal peptide (22 a.a.) into the ER and 2) lysine 86 that is followed by lysosomal exopeptidase-mediated trimming of three amino acids to give the mature form of α subunit into the lysosome [28, 31]. For β precursors, cleavages points are found in valine 42 that removes signal peptide (42 a.a.) and alanine 45 [32]. Furthermore, the mature subunit is nicked internally in the valine 48, threonine 122, and lysine 315, which remain joined through disulfide bonds [32].

Post-translational modifications of Hex as N-glycosylations and phosphorylations are carried out during the ER-Golgi traffic [33]. For the α chains, asparagine (Asn) 115, 157, and 295 have been identified as putative N-glycosylation sites [34]; whereas Asn 84, 142, 190, and 327 have been described for β chains [34, 35]. Additionally, early studies suggested that N-glycosylations present on Asn84, Asn115, and Asn295 must be phosphorylated to be recognized by the mannose-6 phosphate receptor (M6PR) [33]. After these modifications take place, the subunits are dimerized to obtain the active enzymes [33]. Although it is not clear in which organelle Hex are dimerized; some authors suggest that this process is carried out into the *trans*-Golgi network (TGN) before being targeted to the endosome-lysosome pathway [36-38]. These enzymes can also reach the extracellular compartment through sorting from TGN and which can be taken up by neighbouring cells or be re-internalized through fluid-membrane endocytosis [4]. The uptake of these enzymes by neighbouring cells constitutes the cross-correction mechanism that is the base of the main therapeutic strategies for lysosomal storage disorders. In addition, functional HexA has also been identified *in vitro* in the plasma membrane of cultured fibroblasts, as well as the activity of this membrane-bound enzyme towards the GM2 ganglioside [39]. However, the *in vivo* physiological role of this enzyme and the transport mechanism from TGN it is not completely understood yet.

Once the Hex are dimerized, active sites of both HexA and HexB can hydrolyze the N-acetylgalactosamine present in GM2 ganglioside and globoside, respectively [4, 33]. The initial hydrolysis of galactose in the GM1 ganglioside structure is necessary for the catalytic activity of the HexA on the GM2 ganglioside (Figure 1) [38]. For the HexA, two active sites have been described, one on each α and β subunits (Figure 2) [33, 40]. Glutamate 323 and 355 in the α and β subunits, respectively, act as general residues that allow the protonation of the glycosidic oxygen atom; whereas aspartate 322 and 354 in the α and β subunit, respectively, contribute to the necessary stabilization during the nucleophilic attack to the N-acetylgalactosamine [33, 40]. In addition, it has been proposed that arginine 424 of the α subunit form hydrogen bonds with the carboxylate group of the substrate sialic acid; whereas aspartate 452 in the β subunit would repeal this sialic acid [33, 40]. This fact may explain the differences in the affinities of the natural and artificial substrates for HexA and HexB [41]. In contrast to globoside degradation by HexB, the GM2 ganglioside degradation requires a previous step that is mediated by the GM2-AP, which is encoded by the gene *GM2A* that is localized in chromosome 5q31.2 [42]. GM2-AP is considered as a lipid transporter protein that removes the GM2 ganglioside from the endosome membranes, which are derived from the plasma membrane internalized during caveolae-mediated endocytosis [1]. *In vitro* approaches have predicted that GM2-AP simultaneous interactions between the GM2 ganglioside and the α subunit of HexA are necessary for the hydrolysis of N-acetylgalactosamine residue [1, 43].

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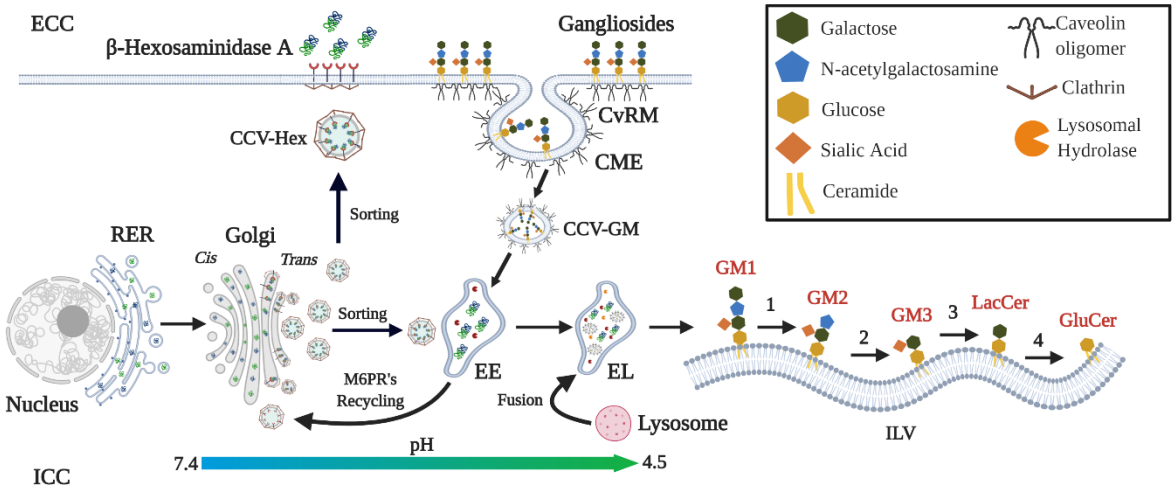

Figure 1. M6PR-dependent transport of β-Hexosaminidase A and ganglioside degradation. α and β subunits of Hex are synthesized in the rough endoplasmic reticulum (RER) and transported to the *Cis*-Golgi network. In this compartment, Hex is subject to N-glycosylations and phosphorylations from *Cis*-Golgi network to the *Trans*-Golgi network [33]. Monomers are dimerized in the *Trans*-Golgi network and coupled to mannose-6 phosphate receptors (M6PR) [33, 37]. New vesicles are sorted to both early endosomes (EE) and to the secretory pathway, where can be uptake by neighbour cells through M6PR [37, 44]. Hexosaminidases are dissociated from the M6PR in the EE; which allows the M6PR recycling to the *Trans*-Golgi network by both clathrin-dependent and independent mechanisms [37]. On the other side, gangliosides are placed in caveolae-rich microdomains (CvRM), and in the turnover of the plasma membrane undergo caveolae-mediated endocytosis (CME) [1, 38]. New caveosomes containing gangliosides (CCV-GM) reach the EE and further fusion events result in a late endosome, which can be fused with the lysosome to give rise to the endo-lysosome (EL, pH: 4.5) [37]. Gangliosides degradation starts with the hydrolysis of the galactose of the GM1 ganglioside to generate GM2 ganglioside which are harboured on intralysosomal vesicles (ILV)[1]. GM2 interacts with HexA through a GM2AP-mediated mechanism to removes the N-acetylgalactosamine resides [38]. Additional reactions implied in the ganglioside degradation to glucosylceramide (GluCer) are shown. The enzymes of each reaction are as follow: **1 and 4:** β-Galactosidase/GM2AP, **2:** β-Hexosaminidase A/GM2AP, and **3:** Neuraminidase. **LacCer:** Lactoceramide. **ECC:** Extracellular compartment. **ICC:** Intracellular compartment.

4. Mutations of β-Hexosaminidases A and B, and GM2 activator protein.

Several mutations in *HEXA*, *HEXB*, and *GM2A* genes lead to the development of TSD (OMIM #272800), SD (OMIM #268800), or GM2-activator protein deficiency (AB variant; OMIM #272750), respectively [45-47]. According to *The Human Gene Mutation Database*, currently, 181, 103, and 9 mutations have been reported for *HEXA*, *HEXB* and *GM2A* genes, respectively, including missense/nonsense, splicing, small deletion and indels, and gross deletions (Figure 3) [48-50]. Although the type and frequency of mutations have been linked to the demographic origin of the patients, for *HEXA* the most representative mutation is the transition c.533G>A that changes arginine by histidine (p.R178H) and affects the catalytic site of the α subunit and alters its function and stability [51-54]. In the case of *HEXB*; a frequent mutation is c.445+1G>A, which occurs in a conserved intronic site that promotes a complete loss of a canonical splice donor site [55, 56]. Finally, mutations in *GM2A* are extremely rare and only 9 mutations have been described on 11 patients [46, 50]. Figure 3 show some of the most common mutations on *HEXA*, *HEXB*, and *GM2A* genes.

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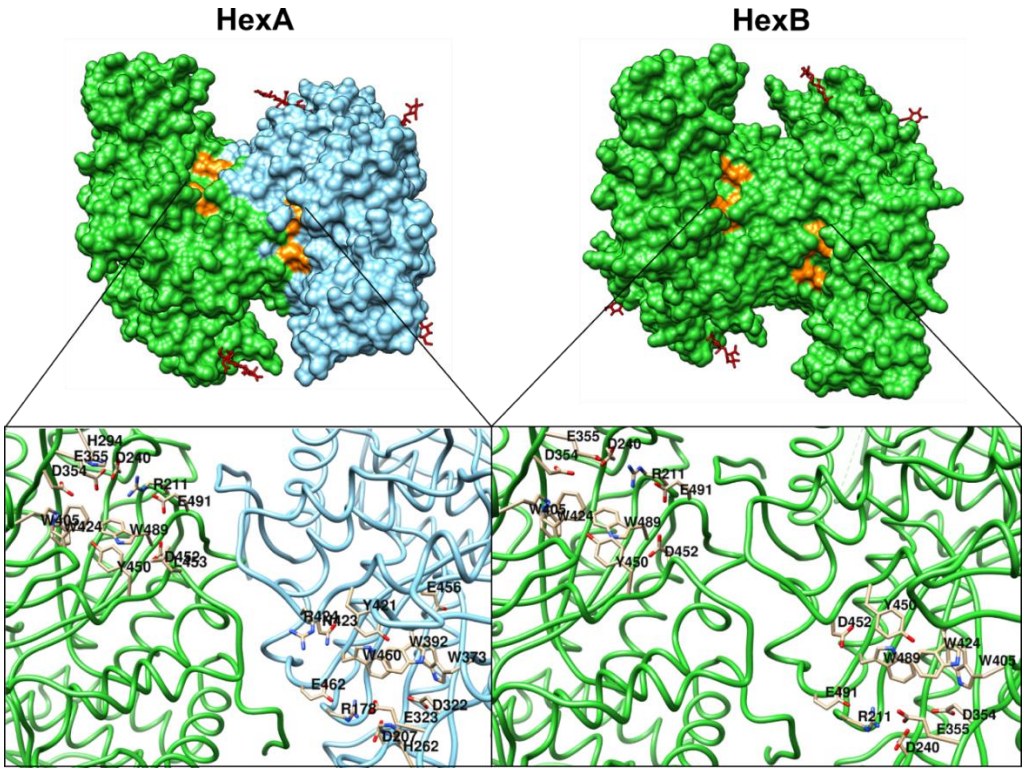

Figure 2. Structure of HexA and HexB. HexA (PDB 2gjx) isolated from human placenta, while HexB (PDB 1o7a) was recombinantly expressed in insect cells. α- and β-subunits are colored in light blue and green, respectively. N-glycans and active sites are colored in red and orange, respectively. The residues present in the active site of each subunit are also shown.

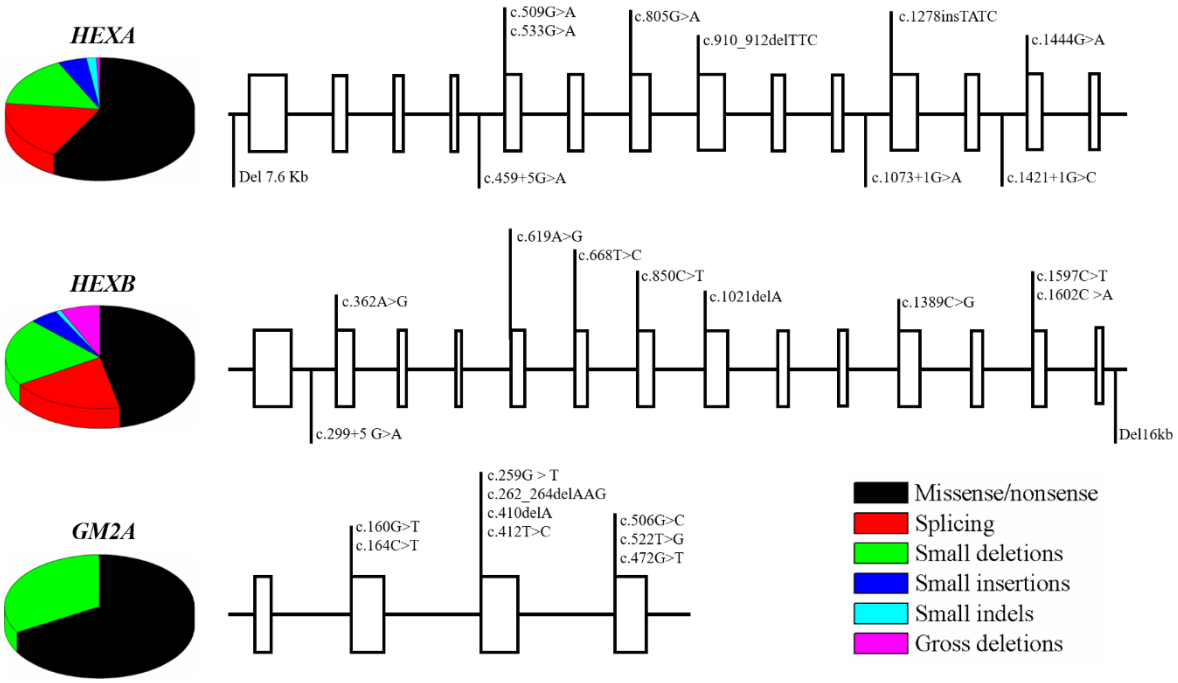

Figure 3. Common mutations on *HEXA*, *HEXB*, and *GM2A genes*. The figure shows some of the most common mutations identified on *HEXA*, *HEXB*, and *GM2A*, as well as their distribution throughout the gene. Mutations can be found either on exons (boxes), introns, and the 5' and 3'UTRs. 14 exons and 13 introns are represented to *HEXA* and *HEXB*, whereas 4 exons and 3 introns are shown for *GM2A*. This figure was made according to the reviewed in [46, 50, 54-58].

Table 1. Main neurological features in GM2 gangliosidosis

Disease	Affected gene	Affected protein	Accumulated substrate*	Common findings			Ref
				Onset	Symptoms	Neuroimaging	
TSD	*HEXA*	HexA	GM2 Ganglioside	**Infantile Acute**	Seizures, axial hypotonia, cherry-red spot, regression in developmental milestones, exaggerated startle response	Bilateral thalamic involvement, brain atrophy, hypomyelination	[57, 59, 60]
SD	*HEXB*	HexA, HexB	GM2 Ganglioside, Globoside	**Juvenile Subacute**	Ataxia, myoclonus, motor regression, psychotic episodes, intellectual disability, progressive clumsiness	Cerebellar atrophy	[52, 61]
AB variant	*GM2A*	GM2AP	GM2 Ganglioside	**Adult Chronic**	Dysphagia, muscle atrophy, cerebellar ataxia, dysarthric speech, manic depression, muscle weakness, psychotic episodes	Severe cerebellar atrophy, hypodensity of the thalamus	[4, 6, 58]

TSD: Tay-Sachs disease **SD:** Sandhoff disease. ***Note:** β-Hexosaminidases can hydrolyse molecules with N-acetyl-hexosamines residues such as glycosaminoglycans (GAGs). Accumulation of partially degraded GAGs has also been reported. For more detail please see [62, 63].

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5. Clinical presentations and biochemical correlations of GM2 gangliosidosis

Although TSD, SD, and AB variant are a consequence of mutations in different genes, neurological compromise is similar among these three diseases [1]. Classical findings are associated to the clinical onset as follow: *acute*: seizures, hypotonia, regression in developmental milestones [60], *subacute*: motor regression, psychotic episodes, intellectual disability [52] and *chronic*: dysphagia, cerebellar ataxia, muscle weakness and manic depression [6]. In Table 1 we summarized these neurological findings. In addition, individuals with SD, unlike TSD patients, can present systemic manifestations including cardiomegaly, hepatosplenomegaly, macroglossia, and skeletal abnormalities [7, 8].

5.1 Diagnosis of GM2 gangliosidosis.

The diagnostic approach of GM2 gangliosidosis patients begins with recognition of the clinical characteristics of these entities (Table 1) [7-9, 61]. Diagnosis is also favored by neuroimaging characterized by hyperdensity of basal ganglia, which can be accompanied by other changes in white matter and sometimes prominent, but non-specific, cerebellar atrophy [9].

The specific diagnosis requires the enzymatic determination of both HexA and HexB isoenzymes by using artificial substrates. For instance, the use of 4-methlyumbelliferyl-N-acetylglucosaminide (MUG) allows to measure the activity of both HexA and HexB isoenzymes, by taking advantage of HexA thermostability, while to specifically measure HexA activity a sulfated substrate (MUGS) is required [9]. It is important to note that while the test can be performed on plasma, amniotic liquid, and dried blood spots (DBS) [10], the diagnosis gold standard is the assay of the catalytic activity in leukocytes, fibroblasts, or chorionic villi in the case of prenatal diagnosis [10]. Enzymatic determination in GM2 gangliosidosis has some limitations, as it does not allow identification of asymptomatic carriers and the diagnosis of patients with deficiency on the GM2 activator protein, who require molecular verification of the gene defect [9]. Molecular diagnosis by sequencing the *HEXA*, *HEXB*, and *GM2A* genes allows to confirm the diagnosis of all GM2 gangliosidosis subtypes.

The heterogeneity of onset correlates inversely with the residual catabolic activity of Hex [1, 64]. Whereas patients with the acute presentation have absent or very low (< 5%) enzyme activity, patients with subacute or chronic onset may have enzyme activities between 5 and 10% [4, 65, 66]. In this sense, it has been proposed that a 10% of wild-type enzyme activity may avoid the disease, which has been based on early studies describing GM2 ganglioside degradation with activities between 10 and, 15% [67], and pseudo-deficiencies reported in healthy individuals [1, 4]. Nevertheless, some pathogenic mutations in both TSD and SD may lead enzyme activities around 15% of wild type levels [57, 59]. In this sense, it has been proposed that a therapeutic benefit of any of the treatment alternatives, developed for GM2 gangliosidosis, may be obtained with enzyme activities higher than 15% of the wild type activity. Recent findings in this interesting topic will be discussed in detail later.

5. Physiopathology of GM2 Gangliosidosis

As a consequence of mutations on subunits of Hex or GM2 activator protein described above, GM2 gangliosides are accumulated into lysosomes [1, 4]. Although it is not completely understood yet, early evidence using animal models of SD showed the presence of autoantibodies against GM2 gangliosides in serum and CNS [68], suggesting that its accumulation could promote the disruption of the lysosome and the release of GM2 ganglioside, which has been also described for the mucopolysaccharidosis (MPS) [69]. Since the lysosome has a pivotal function in the cell for the degradation of most macromolecules as well as organelles through autophagy [70-72], it is just obvious though that its impaired function must affect the general cellular functions with an impact on the global tissue physiology. Here we described current findings, which are summarized in the Figure 4.

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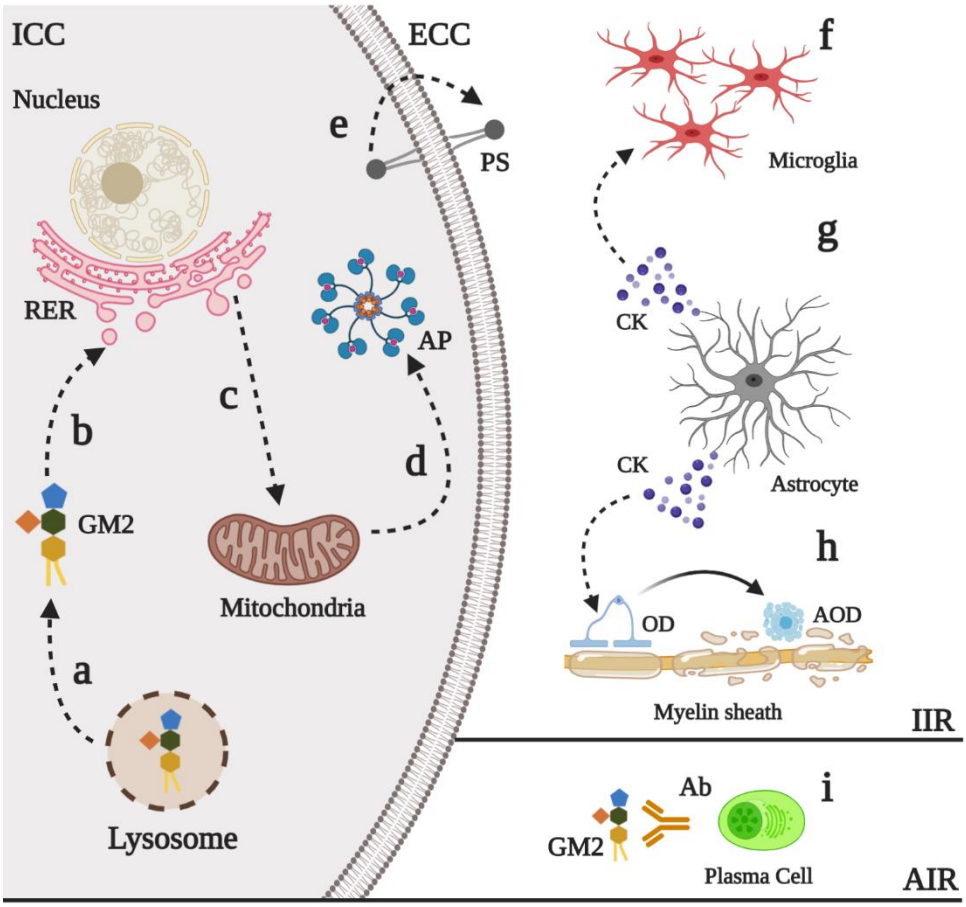

Figure 4. Physiopathological events in the GM2 gangliosidosis. Innate (IIR) and adaptative (AIR) immune response have been described in the GM2 gangliosidosis. Upon the impaired of lysosomal degradation of the GM2 ganglioside, this can be released into the cytoplasm (**a**) where its sialic acid can interact with the rough endoplasmic reticulum (RER) (**b**). Sustained stress into RER induces the activation of proapoptotic proteins like CHOP (**c**) that promotes mitochondrial-mediated apoptosis (**d**) In early apoptosis states, the phosphatidylserine (PS) is externalized (**e**), which promotes the recruitment of several proinflammatory cells such as microglia (**f**). Astrogliosis has also been reported in the GM2 gangliosidosis (**g**). The release of several astrocyte-derived cytokines (CK), such as CCL2 and CXCL10, increases the recruitment of microglia and the apoptosis in myelinating oligodendrocytes (AOD), which induces neuron demyelination (**h**). Finally, auto antibodies (Ab) against GM2 ganglioside seem to contribute to the physiopathology of the GM2 gangliosidosis (**i**), although the precise mechanism of its release has not described. **ECC:** Extracellular compartment, **ICC:** Intracellular compartment, AP: Apoptosome, OD: Oligodendrocyte.

5.1 Neurodevelopment Process.

The use of a cerebral organoid model of SD has been an interesting approximation to discover novel implications of GM2 accumulation [73]. In this context, recent findings have described that impaired Hex activity promotes an increase in the size of the cerebral organoids generated from patient-derived iPS cells, which is corrected after transduction with adeno-associated virus (AAV) carrying the *HEXA* and *HEXB* cDNAs [74]. In concordance to the above and with sophisticated assays using *HEXB* deficient zebrafish embryos, Kuil *et al*., 2019 found an increase in the lysosomal speckles in radial glia and a discrete decrease in the microglia, accompanied by abnormal locomotor activity in larvae 5 days postfertilization [75]. Nevertheless, an increase in the apoptosis rate was not reported in these studies [74, 75]. Together, these results are evidence of the cellular and functional consequences of GM2 ganglioside accumulation in the maturing nervous system, and support the

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fact that these early processes could have an impact on the acute symptoms of the SD, that has been previously identified [76].

5.2 Neural Death and Neuroinflammation.

Neural death has been proposed as an important mechanism in the physiopathology of the GM2 gangliosidosis [4]. Early studies of brain and spinal-cord from autopsy samples of TSD and SD patients revealed an increase in the *in-situ* DNA end-labeling, suggesting that apoptosis could contribute to the neurodegenerative process in these patients [77]. Similar findings were later described in SD animal models [78] and TSD mouse model (*HEXA*$^{-/-}$/*NEU*$^{-/-}$) [12], showing a marked reduction in neuronal density [79]. Although the precise pathway that explains the increase of neural apoptosis has not been completely resolved, recently findings have shown that GM2 ganglioside can induce endoplasmic reticulum stress [5, 80]. In addition, it has been proposed that GM2 ganglioside, but not asialo-GM2 ganglioside, promotes neurite atrophy and cell dead trough PERK-mediated apoptosis with downstream CHOP activation [5], which is an inductor of mitochondrial apoptosis [81]. These results suggest that the sialic acid on the GM2 ganglioside may have proapoptotic properties. Nevertheless, the use of a PERK inhibitor did not completely abolish the apoptosis, suggesting that further mechanisms in the neuronal death in GM2 gangliosidosis could be involved [5].

Given the typical mechanism of early apoptosis such as phosphatidylserine externalization on the surface of the neuron [82, 83], it has been suggested that the characteristic microgliosis and infiltration observed in patients and animal models, could be the response to the neuronal death [4, 78, 84]. Early studies conducted by Jeyakumar *et al*., 2003 using a SD (*HEXB*$^{-/-}$) mouse model, showed that as the disease progress and symptoms such as head tremor, motor disfunction, and hind limb paralysis appear, there is an increase in the immunoreactivity for the Mayor Histocompatibility Complex type II in brain stem and thalamus, as well as an increase in the proinflammatory cytokines TNFα and IL1β [85]. These findings suggest that innate immune activation of the CNS could be produced, at least in part, in response to the neuronal death as was previously proposed. Although authors reported similar findings on the TSD (*HEXA*$^{-/-}$) mouse model, cytokines levels were not increased, which could be associated to the bypass of neuraminidase allowing the GM2 ganglioside degradation, and avoiding the typical neurological manifestations [11, 12].

Despite microglial activation and proliferation have been extensively reported in models of both TSD and SD [84, 86], astrogliosis plays also an important role in GM2 gangliosidosis even in asymptomatic states of the disease [87, 88]. Astrocytes are complex cells localized in gray and white matter, blood vessels, and wrapping the synaptic space in quiescent, active or reactive states [89]. In this regard, Ogawa *et al*., 2017 showed that astrocytes are activated by an FcRγ-dependent mechanism [88]. Upon activation, astrocyte secretes the chemokines CCL2 and CXCL10 which is consistent with a robust microglial activation as wells as an invasion of peripheral immune cells. It could allow the degeneration of myelinating oligodendrocytes and its death, promoting active demyelination [89, 90], which is frequently observed in infantile forms of GM2 gangliosidosis [4, 91, 92]. Together, these findings highlight the pivotal interplay between microglia and astrocytes in the inflammatory response observed in the GM2 gangliosidosis, which could be the functional consequence of neuronal injury due to GM2 ganglioside accumulation contributing to the neurodegenerative process.

6. Current proposals for the treatment of GM2 Gangliosidosis

Several approaches have been tested for the development of specific treatments for GM2 gangliosidosis, which are summarize in Figure 5 [93-95]. These strategies range from traditional enzyme replacement therapy alternatives to novel biotechnological tools such as CRISPR/Cas9 and prime editing, with promising results in both *in vitro* and *in vivo* models. Although some of these developments have been translated to clinical trials [96-99], there is not an approved and effective

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treatment for TSD, SD, or AB variant yet. In this section, we review the current proposals and advances in therapeutics for GM2 gangliosidosis.

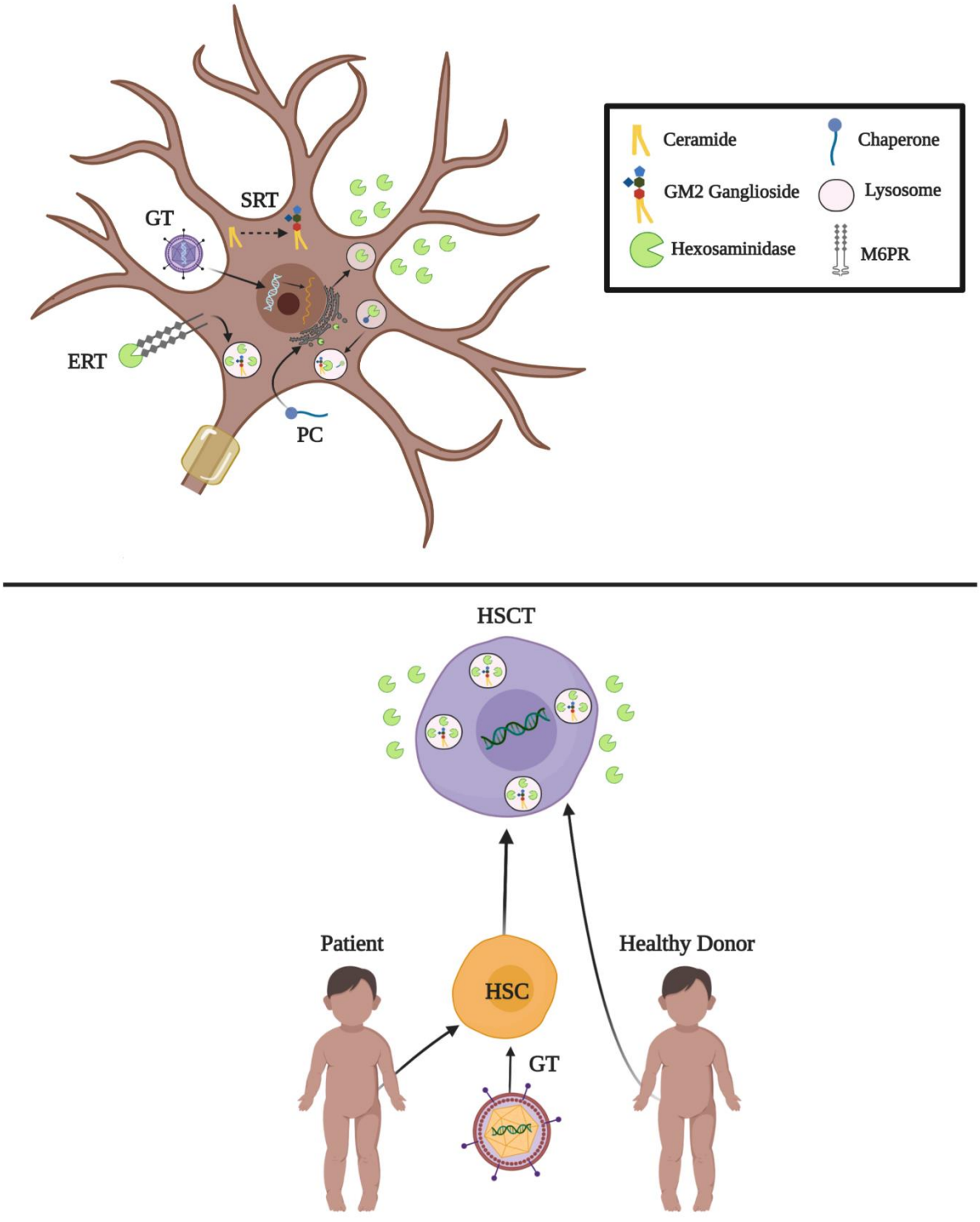

Figure 5. Therapeutic alternatives for GM2 Gangliosidosis. The figure shows current proposals for *in vivo* (upper) and *ex vivo* (lower) approaches. Extracellular Hex represents exocytosis of the enzyme upon its translation, which supports the cross-correction hypothesis. **GT:** Gene Therapy. **SRT:** Substrate Reduction Therapy. **PC:** Pharmacological Chaperones. **ERT:** Enzyme Replacement Therapy. **HSCT:** Hematopoietic Stem Cell Transplantation. **HSC:** Hematopoietic Stem Cell.

6.1 Enzyme replacement therapy.

Enzyme replacement therapy (ERT) is a therapeutic alternative conceived in 1964 by Christian de Duve in which the lysosomal enzymes can be uptake through endocytosis and end up into lysosomes [14]. The development of ERT for LSDs was boosted by the description of the cross-correction mechanism in pivotal experiments using co-culture of fibroblast from Hurler (MPS I) and Hunter (MPS II) diseases [4, 100]. Currently, it is well known that some lysosomal enzymes, such as Hex, have M6P residues at the terminal end of the N-glycosylations, which allows their cellular uptake through a M6PR-dependent manner [4, 33, 93]. ERT has been approved for several LSDs including Gaucher, Fabry, and Pompe diseases, late infantile neuronal ceroid lipofuscinosis type II, acid lipase deficiency, alpha-mannosidosis, and MPS type I, II, IVA, VI, and VII [93, 101].

In the case of GM2 gangliosidosis, early studies carried out by Johnson *et al*., 1973 using intravenous administration of HexA in a patient with SD showed that the enzyme was presented in liver but not in the cerebrospinal fluid or brain parenchyma, suggesting that enzyme was unable to cross the BBB [102], due to the absence of M6P receptors in endothelial cells [14]. To overcome these limitations, promising strategies to enable that exogenous lysosomal enzymes can cross the BBB include the development of fusion proteins, also known as molecular Trojan horses [14]. These fused proteins are recombinant chimeric enzymes fused to a monoclonal antibody (MAb) that recognize either the human insulin receptor (HIR) or the transferrin receptor (TfR), and which allow the passage of the BBB through a receptor-mediated endocytosis [103]. In this field, several studies using non-human primates have shown that intravenous administration of HIRMAb fused to different lysosomal enzymes as α-iduronidase [104], iduronate-2-sulphatase [105], sulphamidase [106], α-N-acetylglucosaminidase [107] can cross the BBB without side effects. Although these approaches have been evaluated in animal models of GM2 gangliosidosis, recently Boado et al., 2019 showed that an HexA fused to HIRMAb has a similar activity to a non-fused enzyme (2.464 ±109 mU/mg vs 2.557 ±187 mU/mg, respectively), suggesting that use of molecular Trojan horses could be an alternative to treat GM2 gangliosidosis [103].

The use of several routes like direct injection in cerebrospinal fluid and intrathecal or intracerebroventricular (ICV) injections have shown potential therapeutic effects for some LSDs with CNS compromise [14, 108]. In this sense, the most common administration route tested in GM2 gangliosidosis has been the ICV. Using this approach, in early studies conducted by Matsuoka et al. in 2010, it was compared the therapeutic effect of two HexA: wild type (WTHexA) and a modified HexA with an additional N-glycan (NgHexA) on SD mice. The study showed that SD mice treated with NgHexA presented an increase of 1.5- and 2.5-fold on the activity levels in brain and cerebellum after 24 h post-injection, respectively. In addition, all brain regions showed a significant increase on the Hex activity 6 days post-injection and the reduction of GM2 gangliosides into the brain of NgHexA (32%) and WTHexA (7%) SD treated mice [109]. Together, these results suggest that the addition of M6P-type-N-glycan into recombinant HexA may have a positive effect on the enzyme uptake, biodistribution, and reduction of substrates throughout the brain. Similarly, Tsuji *et al*., 2011 found that by increasing the M6P residues on the N-glycans of a recombinant HexA produced in the yeast *Ogataea minuta* (Om4HexA), improved the therapeutic effect of the modified enzyme on *HexB*$^{-/-}$ mice compared to the unmodified HexA enzyme (OmHexA), after ICV administration [110]. Treatment with Om4HexA led a higher reduction of accumulated GM2 and GA2, compared with OmHexA treated mice. Interestingly, the authors reported a decrease of MIP-1α chemokine in the hindbrain region (~60%) when animals were treated with Om4HexA. Also, a single administration of both enzymes OmHexA and Om4HexA increase the lifespan by 7.8% and 12.9%, respectively [110].

Meanwhile, in novel experiments, Matsuoka *et al*., in 2011 designed a chimeric HexB enzyme that contains the β-subunit with six point mutations (p.R312G, p.Q313S, p.N314E, p.K315P, p.D452N, and p.L453R) and a partial sequence from α-subunit, which allow to this enzyme to bind to charged substrates as well as to GM2AP. Using this approach, the authors found that ICV administration of the chimeric enzyme on SD mice allowed a significant reduction of GM2 ganglioside in the brain (77%) and cerebellum (57%), compared to untreated SD mice. Likewise, a 2-fold increase in Hex activity and a marked reduction of GM2 ganglioside was observed in liver; which could have a

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significant impact on the disease [111], since, unlike to TSD, hepatosplenomegaly can be found in SD patients [92].

Although most of the enzymes used for ERT are produced in Chinese hamster ovary cells (CHO), since they can produce proteins with similar human N-glycosylation patterns [101], several alternatives like *O. minuta* and *Pichia pastoris* have been evaluated [110, 112, 113]. In this sense, recombinant HexA and HexB produced on the methylotrophic yeast *Pichia pastoris* GS115 have shown a high stability on a wide pH range and in human serum [112]. Recently, it was found that these enzymes can be internalized by both HEK293 cells and healthy fibroblasts in an M6PR-dependent mechanism without further modifications to expose M6P residues [114], as was previously reported for the same enzyme but using the yeast *O. minuta* [110]. Recombinant HexA produced in *P. pastoris* normalized lipid accumulation in neural stem cells derived from TSD iPSCs, showing the potential of this enzyme, and *P. pastoris*, in the development of an ERT for GM2 gangliosidosis [115]. Recent efforts to improve the delivery of this recombinant Hex to BBB, involved their conjugation with nanoparticles [116].

6.2 Hematopoietic Stem Cell Transplantation.

Since Hex have N-glycans M6P residues [33] and they can be exported to the extracellular space [37, 44], a cross correction could be possible through of its capture by neighbor cells in an M6PR-mediated mechanism [4, 117]. As a consequence, the administration of a cells source with pluripotential and self-renewal capacities, like hematopoietic stem cell (HSC), could provide sufficient amounts of the deficient enzyme in a natural o engineered-dependent manner [117, 118]. Allogenic HSC transplantation (HSCT) can be performed from bone marrow (BM), umbilical cordon (UC), or peripheral blood after a myeloablative regimen [119]. This strategy has been performed in other LSDs, such as MPS I [120, 121], MPS II [122], and Gaucher type 1 and 2 [123].

For GM2 gangliosidosis few approaches have been performed and a limited number of patients have been subject to HSCT. In this regard, Jacobs *et al*., 2005, used allogeneic BM transplantation to treat a 3 years-old asymptomatic child with subacute TSD. The results showed an increase in leukocytes HexA activity but without prevention of neurodegenerative events of the disease [124]. Later, in a single-center study, five children with infantile TSD were enrolled and subjected to unrelated UC transplantation. After treatment, the survival was extended in 2 cases with an arrest on the neurodegenerative process but without improvement in motor skills [125].

Meanwhile, Ornaghi *et al*., 2020 showed that the transduction of HSC isolated of healthy donors with bicistronic lentiviral vectors carrying both α- and β-subunits allowed the increase of up to 2-fold in total Hex activity [126]. Similar findings were reported for the enzyme activity on neural stem cells and murine HSC transduced with the LV; whereas physiological functions of the stem cell such as proliferation, self-renewal, or multipotency, remain unchanged, suggesting that *ex vivo* gene therapy could be an interesting option for the treatment of GM2 gangliosidosis. Despite the human leukocyte antigen (HLA) compatibility is frequently realized at immunogenetics laboratories as a crucial prerequisite for HSCT [127, 128], a common challenge in allogeneic HSCT is the graft-versus-host disease (GVHD) [127]. In fact, studies in patients with inborn errors of metabolic have shown up to 10% of acute GVHD [125]. In order to overcome these limitations, recent developments using hypoimmunogenic human stem cells have opened a new horizon for the HSCT by avoiding the events of GVHD through evasion of both cellular and humoral immune response [129, 130]. However, this approach has not been applied to GM2 gangliosidosis yet. Likewise, an attractive alternative for the treatment of GM2 gangliosidosis could be the use of autologous HSCT, in which the mutations are corrected thorough different gene therapy approaches in hematopoietic precursors isolated from the patients [118, 131].

6.3 Pharmacological chaperones.

Pharmacological chaperones (PC) are small molecules that can influence the correct folding and assembly of native proteins with abnormal configurations codified by mutated genes, improving their physical stability as well as intracellular traffic [132]. Misfolded proteins are subject to

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premature degradation, through a retrotranslocation from ER to the cytoplasm, after ubiquitination in the N-terminal, to be finally degraded into the proteasome [133]. As a consequence, the protein cannot reach its final cellular location, *i.e.* lysosome, leading to a pathological condition. PC bind with high affinity and selectivity to a misfolded protein in the neutral pH of the ER and promotes the correct folding of the protein [134, 135]. The PC-protein interaction is dissociated into the lysosome as a consequence of the acidic pH, as well as by presence of the natural substrate, which competes with the PC for the active site [134, 135]. Since PC may act as competitive inhibitors [132], it has been proposed the use of allosteric sites to identify non-inhibitory PC [134]. In addition, PCs have a mutation-dependent activity, which limits the number of patients that may respond to the treatment [134, 135]. PC must not be confused with chemical chaperones like dimethyl sulfoxide (DMSO), which can also bind to and stabilize some proteins but with less selectivity and greater toxicity [136].

In a screening of 1040 FDA-approved drugs, Maegawa *et al*., 2007 found that pyrimethamine (PYR) was the most promising PC for HexA with an IC_{50} between 5 and 13 μM at pH 4.3 [137]. PYR is a typical drug used in the treatment of cerebral toxoplasmosis and complicated malaria, which targets folic acid synthesis and can cross the BBB [138]. PYR is a competitive inhibitor of HexA (Ki: 13 μM at pH 4.5) that induced an up to 3-fold increase in enzyme activity on TSD fibroblasts [137]. Effectiveness of PYR as a PC depends on the binding to the active site, which is mediated by hydrogen bonds and Van der Waals forces [139]. Consequently, mutations that affect the catalytic active site would not allow the binding of the PC. PYR was evaluated in a phase I/II clinical trial, in which eight late-onset patients were orally treated with scaling doses of PYR up to a maximum dose of 100 mg per day [98]. HexA activity was increased in leukocytes up to 4-fold, compared to baseline levels, with doses lower than 50 mg/day [98]. Patients experienced side effects such as hypersensitivity with doses higher than 75 mg/day; and neurological side effects, which in one case led to seizures after the drug was increased from 50 to 70 mg/day. Osher *et al*., 2015, in an extended pilot study, evaluated the effect of low doses of PYR (~2.7mg) in four late-onset TSD patients, in which an increase of 2.24-fold on the HexA activity was achieved. Although these levels were gradually reduced during the continuous administration of PYR; upon the instauration of cyclic doses of PYR (~33 weeks) the HexA activity was enhanced again [140]. Nevertheless, only one patient remained stable whereas three individuals showed neurological deterioration.

To identify potential PCs for GM2 gangliosidosis, an *in silico* analysis of iminosugar inhibitors that bind to the active site of HexA was carried out [141]. Throughout molecular docking and dynamics simulations, the pyrrolidine DMDP amide was identified as the strongest competitive inhibitor of HexA. Using TSD fibroblasts patients, DMDP amide improved the intracellular activity of HexA up to 14.8-fold compared to untreated cells in a dose-dependent manner, reaching up to 43% of wild-type levels [141]. These results showed that iminosugars could be an interesting therapeutic alternative for GM2 gangliosidosis, as has been previously described for other LSDs with a neurological compromise like Gaucher and Fabry diseases [142, 143].

Another potential strategy to increase the folding of mutant Hex is the use of progranulin (PGRN), which is a glycoprotein that is secreted by epithelial, neuronal, and immune cells, and is involved in a variety of physiological processes and diseases such as early embryogenesis, cell proliferation, immune and neurodegenerative diseases, inflammation processes, wound healing and tissue repair [144, 145]. Jian *et al*., 2016 showed that the heat shock protein 70 (HSP70) associates with the lysosomal enzyme β-glucocerebrosidase (GCase) in the ER/Golgi apparatus by a PGRN-dependent manner, avoiding the aggregation of the GCase [145]. Since PGRN could be considered as a co-chaperone, it was evaluated to increase the HexA activity in fibroblasts from TSD patients. The results showed that the G and E domains of the PGRN bind to HexA, increase its enzyme activity and lysosomal delivery, and promotes the GM2 reduction in TSD fibroblasts [144].

6.4 Substrate reduction therapy.

Substrate reduction therapy (SRT) is a therapeutic strategy based on the partial inhibition of enzyme involves in the synthesis of the accumulating substrate [146]. One of the molecules that have been evaluated for this purpose is N-butyldeoxynojirimycin (NB-DNJ, also termed Miglustat or

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Zavesca) [147], an iminosugar that inhibits the glucosylceramide synthase (GCS), which catabolize the first step of glycosphingolipid synthesis like glucosylceramide (accumulated in Gaucher disease [148], sphingomyelin (accumulated Niemann-Pick type C) [149], GM1 (accumulated in Gangliosidosis GM1) [150] and GM2 gangliosides (GM2 gangliosidosis) [151]. Miglustat is an oral drug able to cross the BBB, slowing the accumulation of gangliosides in neurons, and delaying the progression of neurological symptoms [146]. Therefore, miglustat has been evaluated in murine models of SD [152] and TSD [153] with promissory results regarding ganglioside storage reduction, the decrease of neurological symptoms, and the extension of life span. Subsequent studies that evaluated the effect of miglustat in five patients with juvenile GM2 gangliosidosis in advanced disease stage over a period of 24 months, however, they did not show improvement on the neurological impairment [154]. Likewise, Masciullo *et al*., 2010 showed that a 3-years treatment with miglustat on an adult with chronic SD, did not arrest the neurodegeneration [155]. In this sense, implementation of miglustat in early disease stages should be assessed, to know the therapy efficacy during early intervention.

Under the assumption that the administered dose of miglustat was one of the possible causes of its poor satisfactory results, Ashe *et al*., 2011 evaluated the molecule Genz-529468, which has an IC_{50} 250-fold greater than miglustat [156]. Genz-529468 was administrated to SD mice and the results were compared with miglustat-treated mice. The effect of each drug on brain GM2 levels was opposite. In this sense, whereas Genz-529468 increased GM2 levels to about 120% of untreated mice; miglustat decreased the GM2 levels to about 90% of untreated mice. However, levels of other brain glycosphingolipids, specially GL1, were dramatically increased with both inhibitors. The impact of this increase was not evaluated. The authors also reported similar results for both drugs in terms of the delayed loss of motor function and extended of the lifespan. In addition, mice treated with miglustat and Genz-529468 showed lower microglia activation and astrogliosis, and delay of neuronal apoptosis. These findings suggest that the use of inhibitors of GCS could improve the clinical outcomes due, at least in part, to their an anti-inflammatory properties [4, 157].

Finally, Arthur *et al* 2013 reported the evaluation of EtDO-PIP2, a GCS inhibitor, on juvenile SD mice [158]. A previous study showed that this drug reaches the CNS and reduce glucosylceramide in wild type mice [159]. The intraperitoneal administration of EtDO-PIP2 to SD mice reduced the total content of brain and liver gangliosides, suggesting that it could be an interesting alternative for the treatment of ganglioside storage diseases with CNS manifestations.

6.5 Gene therapy.

Since GM2 gangliosidosis is characterized by mutations in *HEXA*, *HEXB*, or *GM2A* genes [4]. As a consequence, delivering a functional gene should correct the genetic defect and lead to a normal physiological development [160]. In recent years, several strategies based on gene therapy have been developed for LSDs, including GM2 gangliosidosis [160-163], which will be discussed below.

Vectors for the delivery of therapeutic genes have been a wide research topic. Usually, gene therapy uses recombinant viral vectors to deliver transgenes into specific tissues, from which the adeno-associated virus (AAV)-derived vectors have gained great attention during the last years [4]. AAV are small icosahedral virus that contain a single-strand DNA [164]. Unlike retrovirus and lentivirus, AAV vectors remains as episomal DNA structures avoiding insertional mutagenesis [165, 166]. To the date, there are several AAV serotypes with tropisms that can change between hosts [164]. Although several serotypes of AAV can be used, AAV8 has shown tropism to CNS with promising evidence for the delivery of Hex in SD cats [167]. Since its biochemical properties mostly of the capsid, AAV can induce an immune response in the host [168]. Therefore, new viral vectors have been engineered to modify the viral capsid and reduce the immune response that can limit the efficacy of the therapy [169, 170].

Consistent with the above, several papers describe a variety of studies of gene therapy applied to the brain of small and larger animal models of GM2 gangliosidosis, mostly using AAV vectors [171]. In this regard, Cachón *et al*., 2012 evaluated the effect of the intracranial coadministration of AAV vectors carrying the human α and β subunits of HexA on a SD mouse model. The results

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showed a wide distribution of the hexosaminidases throughout the perivascular space into the brain and spinal cord of the SD mice, as well as the rescue of the classical symptoms of GM2 gangliosidosis up to 2 years post-treatment [172]. Similarly, Bradbury *et al*., 2013 using SD cats showed that a single bilateral thalamic injection of an AAVrh8 vector expressing feline Hex subunits (α and/or β), led to a 2-fold lifespan increase [173]. The treatment shows improvement on motor functions compared to untreated cats as well as a slight immune response against AAVrh8; contrary to the strong humoral and cellular immune response that was observed when AAV1 and cDNA of human subunits were used [173]. The cats who received the AAVrh8 carrying the feline Hex subunits, did not show clinical or histopathological features of toxicity. Together, these results highlighted the importance of species-specific cDNAs and support the effectiveness of gene therapy in a SD validated model.

In 2015, McCurdy *et al*. also reported the successful translation of a therapeutic approach from GM1 mice [174] to GM2 presymptomatic cats. They evaluated the therapeutic effect of bilateral injections in the thalamus and deep cerebellar nuclei (DCN) of AAV vectors expressing feline Hex subunits (α and β), to treat naturally occurring feline GM2 gangliosidosis. The authors observed an increase on Hex activity up to 19-fold in CNS compared to untreated SD cats after 16 weeks post-treatment. Likewise, clearance of GM2 ganglioside was reported in the brain (89-99%) and cervical intumescence of the spinal cord (72%). Improvement on clinical features, such as reduced ataxia and subtle tremors, were observed in the GM2-treated cats too [175].

Bilateral injection into the thalamus alone or in combination with ICV injection of AAVrh8 vectors encoding feline Hex subunits (α and β) has been tested in SD cats. Using these approaches, reduction of GM2 ganglioside was observed in the cortex (96%), cerebellum (89%), thalamus (87%) and cervical spinal cord (95%), as well as a significant increase in the Hex activity (3.7-56.6-fold) [176]. Moreover, treated SD cats showed an increase on the myelin-enriched cerebrosides and a decrease in the microglial activation, suggesting an attenuation of the neuroinflammation after the treatment [84, 176] and supporting the amelioration of an important physiopathology feature of GM2 patients [78, 85, 89]. Similar findings were reported by Gray-Edwards *et al*., 2018 when used AAVrh8 vectors carrying the α and/or β subunits to evaluate the therapeutic effect of an intracranial injection in a TSD sheep model, which mimics the late-onset infantile phenotype [177, 178]. The TSD sheep that received both subunits, achieved wild type or even supraphysiological levels of HexA activity. Decrease in disease progress was observed in all treated sheep. Also, reduction of accumulated gangliosides to normal levels was reached in most areas of the brain, except in cerebellum and spinal cord where the storage of gangliosides did not change. Neuroinflammation was also attenuated after treatment [86].

To extend the promising results described above to larger brain animals, as an important step towards human clinical trials, Golebiowski *et al*. used intracranial injection to administer, to unaffected non-human primates, AAVrh8 vectors encoding the cynomolgus macaques Hex subunits (α and β). Supraphysiological levels of Hex subunits were achieved in CNS. Most of the animals subjected to treatment showed altered neurological function reflected in ataxia, general weakness, and lethargy as well as histopathological findings suggestive of neurotoxicity associated with eosinophilic inclusions in neurons, white matter loss, and necrotic areas in the thalamus [179]. These findings suggest different responses not only against AAVrh8 but also against supraphysiological levels of Hex in non-human primates, which contrasts to the results observed in other species such as feline or sheep, where slight or absent toxicity has been reported [86, 173]. These aspects could be better resolved with similar preclinical testing but using validated non-human primates' models for GM2 gangliosidosis to know the real therapeutic effect on species close to human.

Despite the interesting findings described with AAV vectors, its limited DNA packaging capacity (~4.6 Kb) has suppose the need to develop new AAV-therapeutic approaches that avoid the use of multi-vector injections. In addition, it is also important to develop novel approaches that allows the correction of CNS impairment. In this sense, Tropak *et al*. designed a self-complementary AAV9.47 vector encoding a hybrid μ subunit (scAAV9.47-HEXM) [180], which combine the α-subunit active site, the stable β-subunit interface, and unique areas of each subunit necessary for the interaction with the GM2AP [41]. Using both *in vitro* and *in vivo* models of TSD and SD, it was found that HexM was able to catabolize the GM2 ganglioside in a GM2AP-dependent manner with its

subsequent reduction in thalamus, hypothalamus, and hippocampus [180]. This effect correlated with an improvement on the behavioral tests, which reached similar values to that of heterozygotes animals.

Finally, novel approaches using bicistronic vectors to reach high levels of Hex in the correct stoichiometric rate have been developed [181]. With this strategy, a proof-of-concept to evaluate the therapeutic potential of the bicistronic ssAAV9-HexBP2A-HexA vector, which has a short P2A linker, the cDNA of *HEXA* and *HEXB* genes under the control of a chicken beta-actin promoter [182]. A single administration of the vector into neonatal SD mice allowed a 56% extension of the lifespan compared with untreated animals. In addition, higher enzyme activity values and lower levels of GM2 gangliosides were obtained in brain and serum of treated SD mice compared with untreated mice [182]. Similar observations have been reported for novel bicistronic lentiviral vectors, carrying murine or human cDNAs encoding for the α- and β-subunits linked by a P2A self-cleaving peptide. This bicistronic lentiviral vectors allowed an increase of Hex activity up to 5-fold of the normal values in murine neurons and human stem progenitor cells, as well as a 30-60% reduction of GM2 storage. Similarly, in treated SD fibroblasts the total Hex and HexA activity increased in a 8:1 ratio, respectively [126].

6.6 CRISPR/Cas9-based gene therapy

Genome editing is a promising strategy for correcting mutations that cause a pathology [161, 183, 184]. Three major genome editing tools have been developed: zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and more recently, clustered regularly interspaced short palindrome repeats/Cas9 (CRISPR/Cas9) [183, 185]. CRISPR/Cas9 uses an RNA-guide nuclease (sgRNA-Cas9) to induce double-strand breaks (DSB) into a specific locus of the genome [186, 187]. DSB can be repaired either through non-homologous end joining (NHEJ) or homologous direct repair (HDR) pathways [15]. On the first case, upon the DSB and on absence of a DNA template, the cellular repair machinery recruits several effectors such as Ku70/80, DNA-PKcs, Artemis, and DNA ligase IV that promote the binding of non-homologous ends in an error-prone mechanism. Through this mechanism is possible to induce deletions or insertions leading to insertional inactivation [188]. This approach is used to knock-out target genes, and has been used to generate *in vitro* and *in vivo* models of GM2 gangliosidosis [41, 74, 75]

DSB can be also repaired through the HDR pathway mechanism by using an exogenous DNA fragment as template (donor DNA), to mediates the insertion of the therapeutic DNA fragment on a specific locus (Figure 6) [185, 188, 189]. Although the punctual correction of each mutation could be interesting, in pathologies with hundreds of mutations, like GM2 gangliosidosis, this approach is not suitable. As a consequence, the knock-in strategy using safe harbors to introduce the cDNA into the genome draws more attention [190, 191]. Ou *et al*., 2020 recently used the albumin locus for the intravenous administration of two AAV8 vectors: one carrying a promoterless HexM cDNA, and other one carrying the Cas9 gene and the gRNA [192]. This approach allowed the constitutive expression of the HexM under the control of the albumin promoter. Four-months post-treatment, the enzyme activity increased in plasma, heart, liver, spleen, and brain, respect to untreated SD mice. This increase in the HexM activity positively correlated with a decrease of the GM2 ganglioside in liver, heart, and spleen. On the other hand, neither a decrease of GM2 ganglioside in the brain nor positive changes on behavioural tests (fear conditioning and pole test) were observed as a result of the treatment [192]. Despite the above, a significant improvement in the rotarod test, which measure motor function, was reported in SD mice treated respect to untreated animals suggesting a slight therapeutic effect on the CNS. Overall, these results show that although this strategy must be optimized, the knock-in of HexM gene into hepatocytes is an alternative to reach supraphysiological levels of the enzyme, particularly in the brain, where typical strategies such as ERT fails due to the inability to cross the BBB [13].

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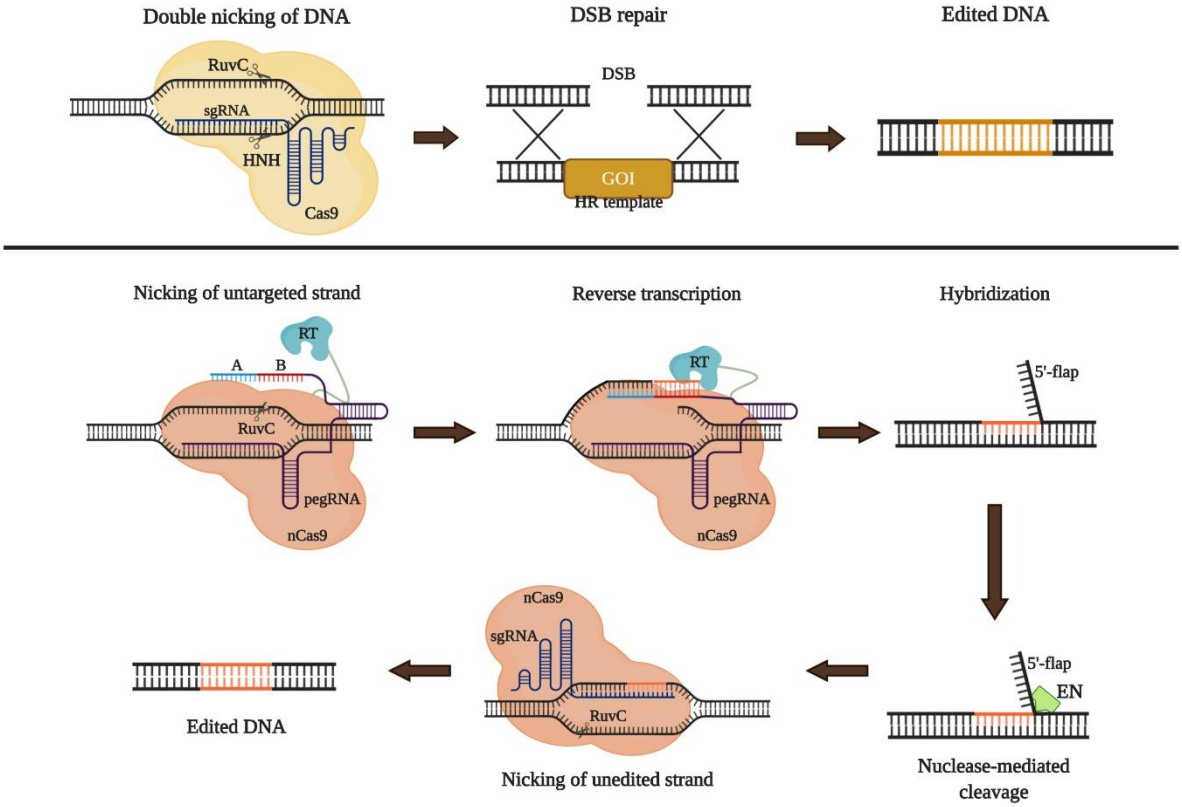

Figure 6. Approaches for genome editing using CRISPR/Cas9. The upper panel shows the classical strategy of knock-in using a ribonucleoprotein complex (sgRNA-guide Cas9) to guide to Cas9 to the target DNA and cut the double-strand (**DSB**). After the DSB, repair machinery is activated. In the presence of a donor sequence (**HR template**), homologous recombination is favored. To promote the recombination event of a gene of interest (**GOI**), the HR template must be flanked by homologous recombination arms which are complementary to the 5′ and 3′-ends of the sequence into the gene that will be subject of edition. Typically, between 100-150bp and 400-800bp are suitable for small (< 50bp) and large (>100) insertions, respectively [193, 194]. In the lower panel, *Prime Editing* (PE) is represented. PE uses a nickase Cas9 (nCas9-H840A) fused to reverse transcriptase (**RT**) and a guide RNA (**pegRNA**) which is engineered with a sequence in the 5′-end.20 nucleotides guide to nCas9 to the target DNA and a sequence in the 3-end with a primer-binding site (**A**) as well as an RT template (**B**) that could be between 7 to 12 nucleotides [195]. Upon reverse transcription, newly synthesized strand hybridizes to the unedited strand (US) forming a mismatch and a **5′-flap** strand which is removed by exonucleases (**EN**) like EXO1 [196]. The mismatch is resolved with the introduction of a new nCas9 coupled to a simple sgRNA which guide to nCas9 to the edited strand (ES), about 50 bp from the pegRNA-mediated nick, to cut the US and use the sequence of the ES as a template for repair de simple cut [195]. In both cases upper and lower panels, newly edited DNA is successfully obtained with different efficiencies.

Despite HDR is the main approach used in CRISPR/Cas9-mediated genome editing, novel strategy without DBS or donor DNA, termed prime editing, was recently described [96, 197]. This tool uses a nickase Cas9 (H840A) fused to reverse transcriptase (RT) and a short engineered RNA sequence (prime editing guide RNA-pegRNA) [195]. The pegRNA is designed to function as a guide of Cas9 to the target DNA and serve as the template for a RT-mediated retrotranscription, avoiding the need of a DNA donor [197, 198]. An additional sgRNA-Cas9 is necessary to nicking the unedited strand and promotes the final correction using the edited strand as a template, in a process that occurs only after the edition and avoiding the generation of DSB [195, 198] (Figure 6). Targeted insertions, deletions, and all 12 possible base to base conversions are feasible with this novel strategy [195]. Using prime editing, Anzalone *et al*., 2019 recreate, in HEK293T cells, a 4-bp insertion in *HEXA* gene

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(1278+TATC), which is associated with TSD with high efficiency (31%) and low indels (0.8%). Once mutations were generated, the cells were correct using the same strategy of prime editing. For this, 43 pegRNA and three sgRNA to induce nicking of the unedited strand were tested. Nineteen of all pegRNAs showed edition efficiencies higher than 20% and lower indels 0.32% [195], suggesting that this novel strategy could be a new therapeutic approach for the treatment of GM2 gangliosidosis without DSB or donor template.

7. Conclusions and Perspectives

During the last years significant advances have been done to understand the physiopathology and natural history of GM2 gangliosidosis. For instance, the natural history programs have allowed to identify the shared and specific manifestations of each disease (TSD and SD) and phenotype (infantile/acute, juvenile/subacute, and adult/chronic), which represent valuable information to improve the diagnosis and patients follow up. However, important efforts still need to be done in order to include a wider number of patients with different genetic backgrounds, since most of the studies have been carried out in specific populations (e.g. clinicaltrials.gov NCT01869270, NCT02851862, NCT00668187, NCT03333200, and NCT00029965).

On the other hand, although gangliosides remain as the main compounds responsible for neuron homeostasis alteration, neuroinflammation and demyelination have also shown to play an important role in disease progression. In addition, the presence of anti-ganglioside autoantibodies, progressive accumulation of α-synuclein, and impaired autophagy, are elements that have been also associated with the disease process [4]. In this sense, as observed in other LSDs [199], it is possible that a single therapeutic strategy would not be enough to treat GM2 gangliosidosis and that the co-administration of different therapies may be required. Noteworthy, different therapeutic strategies including ERT, gene therapy, pharmacological chaperones, HSCT, and substrate reduction therapy have shown promising results and some of them have reached clinical phases (Table 2). In addition, the National Tay-Sachs, and Allied Diseases (https://ntsad.org/) announced the beginning of the gene therapy clinical trials for GM2 gangliosidosis. Although the use of CRISPR/Cas9 is still on its initial stages, the first pre-clinical studies have shown the potential of this tool in the design of novel gene therapy strategies.

Finally, epigenetics should be also considered in GM2 gangliosidosis. Although the role of epigenetic mechanisms in lysosomal diseases has not been well stablished, it has been proposed that these mechanisms may contribute to the clinical heterogeneity observed in these disorders [200]. However, to the best of our knowledge, no studies on this field has been performed for GM2 gangliosidosis. The understanding of the epigenetic alterations observed in GM2 gangliosidosis patients may represent an opportunity to the develop of novel treatment alternatives [201].

Table 2. Clinical trials for GM2 gangliosidosis reported at Clinicaltrials.gov by June 2020

Therapy	NCT Number	Intervention	Status	Phase	Country
Pharmacological Chaperone	NCT00679744	Pyrimethamine	Withdrawn	Phase 1	USA
	NCT01102686	Pyrimethamine	Completed	Phase 1/2	Canada
Substrate Reduction Therapy	NCT00418847	Miglustat	Completed	Phase 2	Canada
	NCT03822013	Miglustat	Recruiting	Phase 3	Iran
	NCT00672022	Miglustat	Completed	Phase 3	USA
	NCT04221451	Venglustat	Recruiting	Phase 3	USA
	NCT02030015	Miglustat and ketogenic diet	Recruiting	Phase 4	USA

HSCT	NCT01372228	Enriched hematopoietic stem cell infusion	Active, not recruiting	Phase 1/2	USA
	NCT00176904	Chemotherapy and hematopoietic cell transplantation	Completed	Phase 1/2	USA
	NCT01626092	Chemotherapy, total body irradiation with marrow boosting and Hematopoietic stem cell transplantation	Completed	Phase 1/2	USA
	NCT00383448	Chemotherapy, total body irradiation and Hematopoietic stem cell transplantation	Completed	Phase 2	USA
Umbilical Cord Blood Transplantation (UBC)	NCT02254863	UBC-derived oligodendrocyte-like cells	Recruiting	Phase 1	USA
	NCT01003912	Fetal UCB transplantation	Withdrawn	Phase 1	USA
	NCT00654433	UBC cells expressing high levels of the intracellular enzyme aldehyde dehydrogenase	Terminated	Phase 3	USA
Cerebellar ataxia treatment	NCT03759665	N-Acetyl-L-Leucine	Recruiting	Phase 2	USA

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Abbreviations

AAV	Adeno-associated virus
Ab	Antibodies
AIR	Adaptative immune response
AOD	Apoptotic oligodendrocytes
AP	Apoptosis
BBB	Blood brain barrier
Cas9	CRISPR associated protein 9
CK	Cytokines
CNS	Central Nervous System

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DMSO	Dimethyl sulfoxide
DOAJ	Directory of open access journals
DSB	Double-strand break
ECC	Extracellular compartment
EE	Early endosome
ERT	Enzyme Replacement Therapy
FDA	Food and Drug Administration
GAG	Glycosaminoglycans
GCS	Glucosylceramide synthase
GluCer	Glucosylceramide
GM2-AP	GM2-activator protein
GT	Gene therapy
GVHD	Graft-versus-host disease
HDR	Homologous direct repair
Hex	Hexosaminidase
HLA	human leukocyte antigen
HSCT	Hematopoietic Stem Cell Transplantation
HSP70	Heat shock protein 70
ICC	Intracellular compartment
IIR	Innate immune response
iPS	Induced pluripotent stem
LacCer	Lactoceramide
LD	Linear dichroism
LSD	Lysosomal Storage Disorders
M6PR	Mannose-6 phosphate receptor
MDPI	Multidisciplinary Digital Publishing Institute
MPS	Mucopolysaccharidosis
MUG	4-methlyumbelliferyl-N-acetylglucosaminide
MUGS	4-methylumbelliferyl-beta-D-N-acetyl-glucosamine-6-sulfate
NHEJ	Non-homologous end joining
OD	Oligodendrocytes
PC	Pharmacological Chaperones
PGRN	Progranulin
PS	Phosphatidylserine
PYR	Pyrimethamine
RER	Rough endoplasmic reticulum
SD	Sandhoff disease
SRT	Substrate Reduction Therapy
TGN	Trans-Golgi network
TSD	Tay-Sachs disease

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