# Genetics and Therapies for GM2 Gangliosidosis

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**ABSTRACT**

Tay-Sachs disease, caused by impaired β-N-acetylhexosaminidase activity, was the first GM2 gangliosidosis to be studied and one of the most severe and earliest lysosomal diseases to be described. The condition, associated with the pathological build-up of GM2 ganglioside, has acquired almost iconic status and serves as a paradigm in the study of lysosomal storage diseases. Inherited as a classical autosomal recessive disorder, this global disease of the nervous system induces developmental arrest with regression of attained milestones; neurodegeneration progresses rapidly to cause premature death in young children. There is no effective treatment beyond palliative care, and while the genetic basis of GM2 gangliosidosis is well established, the molecular and cellular events, from disease-causing mutations and glycosphingolipid storage to disease manifestations, remain to be fully delineated. Several therapeutic approaches has been attempted in patients, including enzymatic augmentation, bone marrow transplantation, enzyme enhancement, and substrate reduction therapy. Hitherto, none of these stratagems has materially altered the course of the disease. Authentic animal models of GM2 gangliodidosis have facilitated in-depth evaluation of innovative applications such as gene transfer, which in contrast to other interventions, shows great promise. This review outlines current knowledge pertaining the pathobiology as well as potential innovative treatments for the GM2 gangliosidoses.

**Keywords**: GM2 gangliosidosis, Tay-Sachs disease, Sandhoff disease, lysosomal storage disease, neurodegeneration, therapies

## INTRODUCTION

The GM2 (monosialoganglioside2) gangliosidoses are caused by mutations in the α- and β-subunits of β-hexosaminidase (EC 3.2.1.52), named Tay-Sachs disease (TSD; OMIM 272800) and Sandhoff-Jatzkewitz disease (or Sandhoff disease, SD; OMIM 268800) respectively, and mutations in the GM2 activator protein, GM2 activator protein deficiency (OMIM 272750). These are emblematic neuronopathic lysosomal storage diseases (LSDs), that are relentlessly fatal and clinically indistinguishable but for subtle visceral and skeletal features in SD. Based on the time of onset, these diseases are often classified into infantile, juvenile and adult forms. Broadly speaking, the acuteness and severity of the disease hinges on the activity of the residual enzyme. The GM2 gangliosidoses have in common the inability to degrade GM2 ganglioside, an intermediate

sphingolipid in the synthesis and degradation of higher brain gangliosides, which, being particularly abundant in the nervous system, can accumulate in large quantities. Tay-Sachs and Sandhoff-Jatzkewitz disease bear the eponyms of the physicians/scientists who first described/reported them.

## FIRST DESCRIPTIONS

The British ophthalmologist Waren Tay, Assistant Surgeon to the London hospital, Whitechapel and working at several hospitals in East London in the late 19$^{th}$ century carried out an examination of the eye in a one-year-old infant who suffered from weakness in the neck and limbs. Using the primitive ophthalmoscopes of the day, he found an unusual discoloration of the maculae, now known as "cherry-red spot". The child died six months after Tay's first examination and he reported the case in 1881[1]; unfortunately, a post-mortem examination was unavailing as to the cause of the neurological disease. In 1884, Tay described further children, similarly affected in the same family. Noting the clustering of this disease in families, Tay considered this to be a congenital disorder.

Bernard Sachs, a neurologist in New York City, unaware of Tay's reports, published in 1896 a description of a disorder in infants with characteristics strikingly similar to those reported by Tay - the familial nature of the condition, early blindness, arrest of cognitive development, progressive weakness, and death in early childhood - and gave it the name "amaurotic familial idiocy" [2]. The following year, having studied several post-mortem samples, Sachs provided a description of the cardinal neuropathological features of the disease, namely, the grossly enlarged nature of most neurones in the brain, which contained a "detritus-like mass", realising that this appearance represented a pathognomic manifestation of the disease [3]. Later, based on descriptions from the medical literature of more than 130 affected children Slome proposed the inheritance of the disease to be autosomal recessive [4].

## NATURE AND LOCATION OF THE STORAGE MATERIAL

The composition of the major lipids accumulating in TSD was elucidated by the German biochemist, Ernst Klenk in the 1940s: Klenk coined the term ganglioside for the previously unknown group of neuraminic acid-containing glycosphingolipids (GSLs) that are abundant in ganglion cells. The saccharide and sialic acid sequences in the principal ganglioside in TSD, GM2, was deduced by Svennerholm [5], and its exact structure elucidated by Makita and Yamakawa in the early 1960s [6].

The introduction of electron microscopy revolutionised morphological evaluation of cellular structure. It allowed for the first time, visualisation of the characteristic "membranous cytoplasmic bodies" (MCBs) in the neurones of post-mortem brain tissue from a patient with Tay-Sachs disease [7] [8], and revealed in florid detail the "detritus-like mass" to which Sachs had alluded as the pathological hallmark of the disease. Correy and Terry also showed that these bodies could be partially purified by centrifugation and the fractions enriched for MCBs contained abundant GM2 ganglioside.

It is notable that Christian de Duve and colleagues in the 1950's had adopted an independent and entirely distinct approach to the exploration of cellular structure and function - in effect using the centrifuge ultimately

to characterize the biochemical properties of what he designated subcellular "lysosomal particles" by means of tissue fractionation. De Duve discovered that acid phosphatase and other hydrolases co-sedimented with these dense components (or, as termed later, organelles). While these sedimentable enzyme activities catalysed the hydrolysis of many diverse substrates, it was noted that all possessed a pH optimum in the range 4 -5.5 - immediately suggesting a digestive function for the newly identified particles [9]. With the introduction of lead phosphate and other substrates yielding electron-dense products of the phosphatase reaction before fixation and electron microscopy, it was possible to demonstrate acid phosphatase activity in MCBs, thus indicating their lysosomal nature [10] [11]. Brady proposed a catalytic impairment in the hydrolysis of the terminal sugar [N-acetylglactosamine] in the accumulating GM2 ganglioside as the biochemical defect in TSD, resembling enzymatic defects already identified in related diseases such as Gaucher and Niemann-Pick diseases [11].

Early descriptions of the lysosome as the digestive machinery of the cell still stand, but in its brief history, our view of this organelle has undergone an extraordinary evolution. Beyond its catalytic properties, the lysosome is central for autophagy (a term coined by de Duve) to be taken to completion, and participates in plasma membrane repair, endocytosis and exocytosis, energy metabolism and signalling. In particular cells, certain lysosome-related organelles, such as the melanosome and platelet granules are able to carry out specialised functions. Externalised lysosomal enzyme undergoing bulk release into the fluid phase of sub-osteoclastic space or immune synapse generated by NK lymphocytes carry out other essential but highly differentiated functions. Moreover, lysosomal biogenesis and function is exquisitely regulated, with transcription factor EB (TFEB) as the master regulator; not only does TFEB regulate the lysosome but also controls autophagy [12]. Transcription factor EB orchestrates the co-expression of genes encoding lysosomal proteins as a response to specific cellular cues. TFEB translocates to the nucleus, where it activates transcription of its target genes in what appears to be an adaptive response to the presence of stored metabolites, in cells from patients with lysosomal storage diseases; remarkably, under normal conditions, TFEB does not drive basal transcription of its targets [13].

## FUNCTION OF GLYCOSPHINGOLIPIDS

Glycosphingolipids (GSLs) are a large family of complex molecules comprising many thousands of biological variants which localize primarily to the plasma membrane, but they are also components of several cellular organelles and the nuclear membrane [14]. At the cell surface, glycosphingoplipids cluster in microdomains [15, 16], with functions involved in cell-cell recognition [17], signal transduction [18, 19], and internalization of cargo [20], to name but a few. As inferred from their qualitative and quantitative patterns of expression, it is now clear that GSLs participate in dynamic neurodevelopmental processes [21], including neuritogenesis, axonogenesis, and synaptogenesis [22-25]. Glycosphingolipids show striking molecular diversity as expected for complex biological molecules, implicated in a multiplicity of functions, including provision of the water-barrier layer in the skin of all land-based vertebrates; but the extent of their rôle in nature remains to be fully characterized. This represents a considerable challenge, partly due to the large number of GSLs, their heterogeneity and interrelationships [26]. Experiments employing genetic engineering to disrupt specific pathways of GSL biosynthesis in mice and the subsequent analysis of the resulting phenotype has unveiled some of their key biological functions.

The GM2/GD2 synthase (β -1, 4-N –acetylgalactosaminyl transferase), encoded by *B4galnt1*, transfers N-acetylgalactosamine to lactosylceramide, and initiates the synthesis of the ganglio-series sphingolipids, including the sialic acid-containing gangliosides [27]. Two groups working independently, created mice with deletions in *B4galnt1*, and, as expected, *B4galnt1*$^{-/-}$ mice lacked complex gangliosides, and instead expressed GM3 and GD3 [28, 29]. Unexpectedly, brain development in *B4galnt1*$^{-/-}$ mice was not compromised. However, analysis of the two animal models led to different interpretations: Takamiya and co-workers described their mouse model as having subtle neurological dysfunction at birth, with neurodegeneration only developing with age. In contrast, those mice reported by Sheikh and colleagues suffered dysmyelination of central axons and demyelination of peripheral nerves, leading to axonal degeneration and impairment of motor function. Axonal membrane gangliosides are thought to act as ligands for the oligodendrocyte protein MAG (myelin associated glycoprotein), which favours axon-glia interactions [30]. This idea is suggested by the finding that *B4Galnt1*$^{-/-}$ mice have diminished MAG expression in their brain and conversely, MAG-deficient mice develop a phenotype resembling *B4Galnt1* $^{-/-}$ [31]. Overall, the experimental results obtained in these experimental mice support the view that complex gangliosides are essential for myelin function and stability [32].

**ELEMENTS OF THE β-HEXOSAMINIDASE SYSTEM**

A seminal finding by Robinson and Stirling in 1967 revealed the existence of two forms of *N*-acetyl-β-glucosaminidase, which could be resolved by starch gel electrophoresis and later separated by chromatography on DEAE-cellulose, an acidic form A and a basic form B. In agreement with de Duve's findings, both isoforms of the enzyme, now known as ß-hexosaminidase, are localised to the lysosomal fraction [33]. Using similar analytical methods, Okada and O'Brien showed that leukocytes from patients with Tay-Sachs disease lacked the activity of the A isoform, while heterozygotes had reduced activity, consistent with the expected gene dosage effect for a simple Mendelian autosomal recessive trait [34]. This discovery had important implications for families afflicted by the disease, through targeted screening programmes in the at-risk Ashkenazi community at large and, subsequently, for clinical diagnosis. Prenatal diagnosis of fetal TSD from amniotic cells, and carrier status for individuals wishing to be tested was soon introduced, so that within two years, community education and screening programs were made widely available. These programmes, pioneered by Michael Kaback, have been critical for reducing the incidence of the disease principally in Jews, in whom the carrier frequency of 1 in 27 individuals predicts a natural incidence of ≈ 1 in 2900 births – now a greater than 90% reduction has been reported for the USA, and has been emulated in many other countries [35, 36].

Sandhoff and colleagues were puzzled to examine material from an infant with severe neurodegeneration and GM2 gangliosidosis but with a total absence of both forms of β-hexosaminidase in brain tissue; in contrast, ß-hexosaminidase activity in tissues from TSD patients show an increase in total β-hexosaminidase activity [37]. This disorder is now known as Sandhoff-Jatzkewitz disease. The explanation for these unexpected enzymological findings emerged from the studies by Srivastava and Beutler, who employed immunochemical techniques to distinguish between the A and B forms and concluded that the two isoforms of β-hexosaminidase share a common subunit, Hex A being a heteropolymer $(\alpha\beta)_n$ and Hex B a homopolymer $(\beta\beta)_n$ [38]. The model also predicted the existence of a Hex S isozyme, a homopolymer of the α subunit $(\alpha\alpha)_n$. If correct, the absence

of subunit β would obliterate the activity of Hex A and Hex B, whereas if subunit α was missing, Hex A and Hex S activities would be deficient. The model of Srivastava and Beutler was subsequently verified by insightful hybridization and cell fusion experiments in which TSD and SD fibroblasts were able mutually to complement each other, resulting in the formation of Hex A [39], later supported by mapping of Hex A and Hex B expression to chromosomes 15 and 5 respectively [40]. Additional complexities of the β-hexosaminidase system emerged some years later when Conzelmann and Sandhoff identified another exceptional infant with GM2 gangliosidosis who was found to have activities of Hex A and Hex B within the healthy reference range when assayed in the presence of artificial substrates. Detailed investigations, including the use of the natural substrate, GM2 ganglioside, led to the discovery of a specific activating factor, later shown to be a small protein that is specifically required for the lysosomal breakdown of GM2 ganglioside. The existence of this activator protein accounts for the apparently normal activities of both β-hexosaminidase isozymes often reported in these very rare cases of GM2 gangliosidosis [41] (Fig. **1**).

## β-HEXOSAMINIDASE BIOSYNTHESIS AND UPTAKE

Experiments in the laboratory of Elizabeth Neufeld elegantly allowed elucidation of the β-hexosaminidase biosynthesis pathway. D'Azzo and colleagues investigated the cell biology of β-hexosaminidases by means of radiolabelled pulse-chase studies using $^{32}$P-labelled inorganic phosphate and [$^{3}$H]-L-Leucine in fibroblasts cultured from healthy subjects and patients with GM2 gangliosidosis; immunoprecipitation with antisera specific for the Hex isozymes and their subunits permited the molecular forms of the proteins to be followed during biosynthesis. Their discovery, "that proteins destined for the lysosome undergo modifications on the way to and at their final destination in the lysosome", was of great significance. They described the principal steps of this process as: (1) β-hexosaminidase isozymes first appear in a precursor form; (2) mannose 6-phosphate (M6P) motifs are attached to these precursors, and ( 3) after traversing the Golgi and on reaching the lysosome, the precursors are converted into mature forms of smaller molecular mass [42, 43]. Previous studies from the laboratories of William Sly, Stuart Kornfeld and Elizabeth Neufeld had shown that the M6Ps are recognition tags necessary for correct targeting of nascent proteins to the lysosome.

Experimental evidence indicates that in the complex case of heterodimeric Hex A, subunits α and β are synthesised independently and when in the endoplasmic reticulum (ER): 1) they lose their signal peptide; 2) acquire N-linked oligosaccharides and intra-chain disulphide bonds; and 3) M6P moieties are attached. It is only after the acquisition of the M6P tags that α and β subunits associate in the ER. It is believed that in a step facilitated by M6P receptors, the precursor isozymes travel through the Golgi. They reach the lysosomes through intermediate acidified compartments where the enzyme dissociates from the receptors - presumably, allowing the receptors to recycle back to the Golgi for further use. As with other soluble acid hydrolases destined for the lysosome, a significant fraction (≈ 20%) of the M6P-tagged protein is secreted into the extracellular space and can be detected in the medium of cultured cells. The secreted enzymes are catabolically active and able to re-enter the secreting and indeed neighbouring cells - a process of secretion-recapture that allows mutual cross-correction of lysosomal enzyme deficiencies in cultured cells. The ability to supply exogenous glycoproteins that are appropriately decorated with mannose 6-phosphate and other moieties for

delivery to the lysosome is a phenomenon of great importance for therapeutic exploitation in lysosomal diseases (Fig. **1**). Binding and internalization is mediated largely through M6P receptors present on the cell surface; the protein cargo eventually reaches the lysosome after intracellular trafficking through intermediate compartments in the endosomal-lysosomal pathway. Of note, based on their biosynthetic labelling studies, D'Azzo and colleagues were of the view that association with beta-chains is necessary not only for acquisition of catalytic activity but also for transport of alpha-chains to lysosomes. The proteolytic cleavage sites in the hexosaminidases and resulting processed products were identified after the amino acid sequences of the subunits could be inferred from characterization of their cDNAs [44-47]. It transpires that proteolytic cleavage of the assembled β-hexosaminidase subunits (which remain associated by disulphide bonds after processing) is not necessary for their enzymatic activity [48, 49].

## CHARACTERIZATION OF *HEXA*, *HEXB*, AND *GM2AP*

Cloning of the β-hexosaminidase genes encoding α- (*HEXA*) and β-subunit (*HEXB*), and the GM2 activator protein (*GM2AP*) required considerable effort, partly because of the low abundance of their mRNAs. Myerowitz and Proia isolated the first α-subunit cDNA clone in 1984 [50], and using a different approach Korneluk and colleagues published similar results a year later [51]. Two α-subunit mRNA species of 2.1 kb and 2.6 kb were identified; importantly, these were not detectable in fibroblasts obtained from patients with classical infantile Tay-Sachs disease [52]. The total coding sequence, which includes a 22-amino acid signal peptide at the N-terminus, consists of 528 amino acids; and the genomic structure, scattered over a 35 kb sequence on chromosome 15q24.2, comprises 14 exons [53]. O'Dowd and co-workers detected a β-subunit mRNA of 2.2 kb, absent in cells obtained from a child with Sandhoff diease [54]. Information provided by the O'Dowd's paper allowed Proia and colleagues to isolate a 1.7 kb cDNA from liver. The total β-subunit coding sequence consists of 556 amino acids, with a 42-amino acid signal peptide at the N-terminus. Of note, *HEXB* also consists of 14 exons distributed over a 40 kb sequence localised to chromosome 5q12. Moreover, the intron/exon boundaries are conserved between the two genes in 12 out of 13 introns [55]. Korneluk aligned the primary sequence of pre-α and pre-β polypeptides, revealing 55% and 57% similarity in the nucleotide and amino acid sequences, respectively. This level of sequence similarity suggested a common ancestral gene for the two subunits [56].

Schröder and colleagues succeeded in cloning the GM2 activator protein gene (*GM2A*), consisting of 4 exons and 3 introns spread across a 16 kb sequence [57]; this maps to human chromosome 5q31.2.

In spite of the similarities between subunits α and β, functionally different active sites permit the multi-isozyme system to hydrolyse diverse substrates containing β-linked N-acetylhexosaminyl residues. The active sites of both subunits, in dimeric form, are able to hydrolyse many of the same neutral natural and artificial substrates - oligosaccharide moieties from proteins and neutral glycolipids, as well as from certain mucopolysaccharides - showing redundancy in the system. In contrast, it is the catalytic site in subunit α that hydrolyses negatively charged substrates. Degradation of GM2 ganglioside can only be attained by heterodimeric Hex A *in vivo*. In association with the GM2 activator protein, Hex A is specifically required to catalyse removal of the terminal non-reducing *N*-acetylgalactosamine residue from GM2 ganglioside - the activator protein serves as a lipid transporter that interacts with both the carbohydrate and lipid portions of the ganglioside and presents it to Hex A for hydrolysis [58-61]. The crystallographic structures of Hex A [62], Hex

B [63], and GM2 activator protein [64] provide useful fine detail about the effects of pathogenic mutations in these proteins and the mechanisms of catalysis. Structural analysis moreover suggests that dimerization is essential for activity in each subunit and is consistent with the lack hitherto of any evidence that monomeric subunits possess enzymatic activity. [65]. Characterization of the *HEXA, HEXB* and *GM2A* genes enabled identification of the molecular defects that cause GM2 gangliosidosis. These advances in molecular genetics also definitively confirmed that while mutations in the α- and β-subunit genes cause TSD (B variant) and SD (0 variant) respectively, defects in the GM2 activator protein cause the so-called AB variant form of GM2 gangliosidosis [66, 67] (Fig. **1**). Consequently, personalised medicine for disease diagnosis and genetic counselling is now possible because disease-causing mutations can be pinpointed with precision and, in a few cases, the clinical phenotype predicted.

**HETEROGENEITY OF THE DISEASE**

For historical reasons, when Sachs first came across TSD, the catchment area of his patients in New York City included many Jewish families of Eastern European ancestry, and he logically concluded TSD to be a Jewish disease. However, we now know the disease is panethnic, including blacks [68] and Asians [69]. Moreover, contrary to early indications that TSD is a disease due to a single cause, with the advent of DNA sequencing, it became clear that numerous mutations cause infantile disease. An early example came from mutation analysis carried out in French Canadians and Ashkenazi Jews, against all preconceptions the disease was due to distinct mutations in the two populations [70]. Unexpectedly, TSD within Ashkenazi Jews can also be caused by several mutations [71]; seven *HEXA* mutations have been found in Moroccan Jews with Tay-Sachs disease, a Sephardic group - five mutations are apparently exclusive to the Moroccans but two also occur in the Ashkenazim [72].

Carrier frequency can vary greatly between different populations; for example, TSD is estimated at 1 in 14, 1 in 30 and 1 in 300 for Eastern Quebecois in Canada, Ashkenazi Jews and the ‘general’ population, respectively [73, 66]. These early revelations pointed to the existence of different mutations being responsible for the same disease, which paralleled findings in other genetic disorders. Similarly, SD is no respecter of ethnic group [74-76], and the number of mutations in subunit β are numerous and distributed widely over the entire gene [77].

Over 100 distinct mutations have been reported for *HEXA* alone [78] (see also an online database at www .hexdb.mcgill.ca), which include deletions, splicing aberrations, nonsense and missense mutations; leading to deficiencies in transcription, translation, inappropriate protein folding and/or dimerization and catalytic dysfunction [79]. The most common and severe forms of GM2 gangliosidosis are often found to have a complete deficiency in Hex A activity. While total Hex activity is close to normal in TSD - attributable to active Hex B - only 2-4% of normal total Hex activity is detectable in SD, despite normal amounts of α-subunit mRNA. Low affinity of the α-subunits for each other explains the detectable but small quantities of Hex S. The less severe juvenile (subacute) and adult (chronic) onset forms are a consequence of mutations that allow for preservation of varying degrees of Hex A function, around 1-5% of normal, often the result of inadequate folding or dimerization. Most known missense mutations result in misfolded proteins; detected by the ER quality control system they are degraded by the ER-associated degradation pathway (ERAD), followed by

ubiquitination and removal by the proteasome [80-82]. Five to 10% of normal Hex A activity is estimated to be compatible with a disease-free life [79, 83]. In GM2A protein deficiency, Hex A and Hex B function is maintained against some substrates, but not against GM2 ganglioside - the critical threshold of normal hydrolysis being estimated at around 5-10% of normal activity, and disease manifestations are predicted to occur below this figure [84]. While these discussions provide guidance, caution is needed in relation to estimating true residual enzyme activities - especially using information obtained from assays conducted in diagnostic laboratories. In reality, determining the true activity of Hex A against its natural substrate GM2 ganglioside (itself a biological substrate of chemical diversity owing to variation in sphingosine structures and acyl chain-lengths in the body) in lysosomes of nervous tissue *in situ* is extremely challenging. While substrates based on simple or more complex analogues of various composition can be used, in the past, the preferred solution has been to utilize radiolabelled natural GM2 ganglioside(s) – now prohibitively expensive and cumbersome to use.

An intimate understanding of Hex A function, revealed by its crystal structure, was anticipated to facilitate prediction of disease phenotype for individual mutations. This expectation has not been fully realised; this is because spatial location of a mutation does not readily correlate with disease severity, except for those altering the active site. Moreover, many patients are compound heterozygotes, often harbouring a unique or unusual combination of mutations, and uncharacterized disease-modifying genes within families, together with epigenetic and environmental factors are suspected to influence the course of the disease.

## NATURAL HISTORY

It is a remarkable fact that the prevalence of GM2 gangliosidosis in some at-risk populations is now lower than in the general population due to enzymatic and genetic screening. Although the authors are aware of tragic examples where targeted screening has failed, particularly in couples of mixed ancestry who request reassurance before planned pregnancies, community-based carrier screening and counselling in traditional at-risk communities of high endogamy has been extraordinarily effective. [85, 86]. Reported estimates of the incidence of TSD and SD in the population at large are 1 in 222,000 and 1 in 422,000 live births, respectively [87].

A few recent studies have formally reviewed survival and rate of functional decline in GM2 gangliosidosis. The study by Bley and colleagues focused on the infantile forms of TSD and SD. It included data from patient surveys (TSD, n= 72; SD, n= 20) recruited by the National Tay Sachs & Allied Diseases Association (NTSAD), NTSAD retrospective database (n= 103) in the USA, and literature reports (n= 121). Most infants were of mixed ethnic origin, Jewish or unknown. Diagnosis of disease was at a mean of just over one year of age, and the most common initial symptoms were developmental arrest (83%), abnormal startle response (65%), and reduced muscle tone (60%). Seizures were common and particularly prominent during the late stages of the disease. Importantly, early development appeared to progress normally in most cases - supporting the generally held notion that developmental arrest, and not delay, is more typical of infantile GM2 gangliosidosis. About half these patients died by the age of 3½ years [88].

Maegawa and colleagues reported on 21 new patients with juvenile/subacute GM2 gangliosidosis: of these, 13 were from Canada and 8 from Brazil (TSD, n= 15; SD, n= 6) representing 15 unrelated families of diverse ethnicity. Among the 15 patients with TSD and 6 with SD, respectively 11 and 5 different mutations were identified. The mean age of onset (first symptom) was 5.3 ± 4.1 years (range: 1.5–15 years). About half these patients died in the first decade of life, and only 25% survived to their late teens. Disease manifestations in this group of patients are more variable than in the acute infantile form, but gait and speech difficulties were experienced by all patients as the disease progressed, impaired coordination (95%) and cognitive impairment (80%) were almost invariable; psychiatric disturbances were noted in two patients. Cerebellar atrophy, followed by generalized cerebral atrophy was the most common neuro-radiological finding. Very similar trends resulted from the analysis of a group of 134 patients previously reported in the literature (1968-2006) (TSD, n= 96; SD, n= 27) [89]. Differences in symptoms between TSD and SD patients were also apparent; for example, sleep problems, muscle wasting, and diarrhoea/constipation were more prevalent in SD suggestive of autonomic neuropathy.

Smith et al. studied 73 cases of GM2 gangliosidosis in the UK (TSD, n= 40; SD, n= 31; GM2AP deficiency, n= 2) during the period 1997-2010, using data provided by the British Paediatric Surveillance Unit. The GM2 gangliosidoses represented 6% (73/1164) of all diagnosed cases of progressive intellectual and neurological deterioration. Most (85%) were sporadic cases with no family history but notably, children of Pakistani ancestry predominated, in particular the juvenile form of SD (10/11). As with other populations, infantile forms predominated (75%). The mean age at symptom onset was 6.2 and 4.7 months for infantile TSD and SD, respectively, and 26.2 and 34.7 months for the corresponding juvenile variants. As with the earlier findings by Bley and colleagues, the mean survival for the infantile forms was about 3 years, the clinical manifestations were not appreciably different from those reported in the literature [90].

Markedly attenuated late-onset forms of Tay-Sachs and Sandhoff diseases have been increasingly recognised. Although initially first reported as Late-onset Tay-Sachs disease (LOTS) principally in Ashkenazi Jews - associated with homozygosity for the missense *HEXA* mutation, G269S - both subtypes of GM2 gangliosidosis may cause late-onset disease. Presentation is highly variable and the disease is frequently mistaken for other well-known neurodegenerative disorders such as amyotrophic lateral sclerosis (motor neurone disease) and cerebellar ataxia; peripheral neuropathy may be a presenting feature –especially in Sandhoff disease. Common presenting symptoms include clumsiness and ataxia combined with leg muscle weakness with onset in the late adolescent period. Cognitive ability is often long-preserved allowing retention of employment, but up to 40% of patients develop psychiatric manifestations including bipolar disorder or psychosis. It is unclear whether this form of disease shortens life, but most affected patients at least lose their physical independence, require assisted ambulation and support [91].

With several new treatments on the horizon for the GM2 gangliosidoses, accurate description of clinical manifestations to chart the natural history of the disease and validated disease severity scores, will be important for the evaluation of potential therapeutic efficacy and in the development of inclusion/exclusion criteria in clinical trials. Given the relentless nature of the disease early therapeutic intervention will be of the essence for optimal outcomes. Moreover, early diagnosis facilitates management of the condition and increases awareness in affected families and at-risk relatives, making informed reproductive choices possible.

## DIAGNOSIS

GM2 gangliosidosis in an infant with a neurodegenerative disease is usually characterized by developmental arrest and features indicating combined cerebral and cerebellar disease (poor axial tone with progressive limb spasticity and is typically raised by certain clinical signs such as poor visual fixation and a prominent startle reflex. The presence of perifoveal pallor and the cherry-red or pigmented macular spot is not completely specific but is a near-pathognomic sign of this disease.

In suspected cases, the mainstay of diagnosis relies on enzymatic assay of β-hexosaminidase activities, usually carried out in plasma and/or peripheral blood white cells using two fluorogenic artificial substrates - MUG (4-methylumbelliferyl-beta-D-N-acetylglucosamine) and its sulphated congener, MUGS (4-methylumbelliferyl-beta-D-N-acetylglucosamine-6-sulphate)]. The latter preferentially hydrolysed by Hex A and Hex S. Fibroblast lysates or extracts of other tissues can also be used but in most cases the reference parameters for healthy subjects are less statistically robust. Assay of control lysosomal enzyme activities should be determined in parallel. These studies should be conducted in an experienced biochemical genetics laboratory that has ideally been prepared for timely receipt of the samples. Enzymatic diagnosis is not fool-proof and for carrier detection, as well as rigorous diagnosis of disease, molecular analysis of the *HEXA* and *HEXB* genes is increasingly carried out in addition. This is to avoid discrepancies arising from artefacts and the presence of pseudodeficiency alleles or mutations such as R288H in *HEXA* that lead to a modest or undetectable deficiency of enzyme activity with the artificial substrates but a Hex A enzyme that fails to be activated by the GM2 activator protein. Molecular analysis of the *GM2*A gene is necessary for diagnosis of the cognate activator deficiency [92].

## ANIMAL MODELS

Studies of GM2 gangliosidosis using cell lines or primary cells in culture have been instrumental in our understanding of the biology underlying GM2 gangliosidosis, and have played a key rôle in mechanistic understanding. However, to study pathogenesis and test prospective therapies, authentic animal models of disease are highly desirable. Naturally occurring as well as genetically engineered animal models are available for experimentation. Mutations in *hexb* have been identified in several breeds of cats [93], and in *hexa* in sheep [94], dogs [95, 96] and flamingos [97]. Clearly, among these models of disease, some are more amenable to a research setting, and we refer the reader to the excellent review by Lawson and Martin on the utility of these animals [98].

In the 1990s, two groups working independently, the laboratory of Proia [99] and that of Gravel [100], generated mouse strains with disrupted *hexa* and *hexb* genes as models of TSD and SD, respectively. To their surprise, the TSD mice did not mimic human infantile TSD, and although the animals showed some storage of GM2 ganglioside, clinical manifestations were not immediately apparent. In contrast, SD mice developed a fatal neurodegenerative disease and died at age 4-5 months. These investigators reasoned that the mild disease in TSD mice could be explained by the existence of a biochemical bypass mediated by a sialidase, now considered

to be Neu3 [101] which if conserved in humans fails to function effectively. Mouse and cat models of the GM2 protein activator deficiency are also available [102, 103].

As illustrated by the disparate phenotypes between murine and human TSD, species-specific differences may exist, and interpretation of results from animal models should be taken with caution when extrapolating to human disease. Nevertheless, these animal models are powerful research tools and their importance cannot be overestimated for translational studies. The advantages of using mouse models of disease for testing novel therapies are: 1) readouts of successful or failed approaches are relatively rapid; 2) large numbers of animals are produced in a short time, which are easily housed in a small animal facility; and 3) high throughput analysis of tissue is of a manageable scale for small laboratories. However, the mouse brain is small, about 1/1000 that of a human infant, making accurate injections of small structures difficult, but those of the cat and sheep better approximate to the infant human brain in complexity, organization, and size (1/10 and 1/3 respectively). Cat and sheep are long-lived animals and well suited for surgical interventions and clinical evaluation over many years [98]. Nevertheless, there are disadvantages associated with the use of large animal models. These include: 1) difficult ethical justification associated with the experimental use of more sentient animals - particularly for early evaluation of novel therapies; 2) high maintenance cost; 3) specialist care by highly qualified veterinarians; 4) time required to generate animals numbers to achieve meaningful statistical power; and 5) the large volumes of tissue that is required for analysis and to obtain a systematic and thorough investigation of interventional outcomes.

## PATHOLOGY

There are few reports in the literature on the histopathological features of human GM2 gangliosidosis that are more comprehensive than those by Aronson, on his observations of infantile TSD. He found that at autopsy, the brains were heavier than normal, probably due to gliosis; but in contrast to the obvious microscopic abnormalities, they showed no gross pathological features macroscopically, except for oversimplification of surface fissures and broadened sulci. Neuronal depletion was only prominent in the cerebral cortex, and demyelination in the cerebral white matter. Aronson considered that arrest and inhibition of myelin formation rather than loss of existing myelin was most likely, because no evidence of recent myelin breakdown was apparent; however, numerous activated microglia and changes of mild astrogliosis were present in areas of grey matter. All levels of the neuraxis showed the characteristic neuronal alterations and gliosis and, in addition, endothelial cells were distended with pathological lipid. Aronson was struck by the abrupt onset of the illness and despite the severe neuropathological destruction, the degree of function remaining until late in the course of the disease, when a threshold appears to be reached and normal neurodevelopment stops [104, 105].

It remains a matter of intense debate, which changes represent the primary expression of the neuropathology and which are, in effect, secondary changes consequential on the original defect. While the accumulation of gangliosides is generally seen as the primary insult to neurons, demyelination and inflammation for example are often considered a mere consequence of neuronal deterioration. However, this has been strongly contested, and there is evidence to suggest that both, demyelination and inflammation, actively participate in the disease process and its destructive effects on neurological function. Despite more than a century of study, the path, from ganglioside accumulation to progressive neurodegeneration and death, is not yet fully characterized

or unravelled in molecular terms; better understanding of the cellular and molecular mechanisms of disease may still open up new possibilities for therapeutic intervention.

**Storage of GM2 ganglioside and related glycolipids**

The widespread presence of distended neurones containing numerous modified lysosomes - membranous cytoplasmic bodies - is the most conspicuous histological alteration in GM2 gangliosidosis. These bodies are in effect pathological lysosomes containing GM2 ganglioside and related glycosphingolipids, which can be detected immunohistochemically with monoclonal antibodies and gives a reaction for glycans with the periodic acid-Schiff reagent (Fig. **2**).

Pathological elements implicated in the disease process include:

1) The presence of the unacylated metabolite lyso-GM2 (sialylgangliotriaosylsphingosine) in brain and plasma of patients and animal models of GM2 gangliosidosis – which has been presumed to have toxic properties when present in neural tissue [106, 107], and possibly resembling other pathological lysolipids, glucosylsphingosine in Gaucher's disease [108, 109] or galactosylsphingosine in Krabbe disease [110].

2) Anti-ganglioside autoantibodies - linked to the more severe late stages of the disease [111].

3) There is evidence that accumulation of sphingolipids in the lysosomal compartment perturbs endosomal transport and sorting, disrupting cellular homeostasis [112].

4) Impaired autophagy - the overwhelming accumulation of storage material restricts the normal fusion between autophagosomes and lysosomes. One manifestation of this inhibition is the formation of inclusion bodies containing aggregates of polyubiquitinated proteins [113-116] and protein p62/SQSTM1, which targets ubiquitinated proteins to the autophagosome for degradation [117, 118]. Of note is the work of Micsenyi et al. on ceroid lipofuscinosis type 2 (CLN2), suggestive of lysosomal membrane permeability as the principle pathogenic event - rather than a compromised degradative mechanism - where the release of lysosomal contents into the cytosol stimulates the formation of p62-containing protein aggregates [119]. Displayed in figure 2 are hitherto unpublished data showing ubiquitin and p62 co-staining of neurones in murine SD (Fig. **2**), which we take to signify the presence of dysfunctional autophagy and possibly increased lysosomal membrane permeability in experimental GM2 gangliosidosis.

5) Progressive accumulation of α-synuclein - a protein naturally expressed in neurones and glia [120], which binds proteins [121] and lipid vesicles [122], and implicated in lipid metabolism [123]. Suzuki et al. demonstrated the presence of α-synuclein accumulation in human lipidoses [124] and in the brains of SD mice [125]. We also detected extensive α-synuclein aggregation in many areas of the brain and spinal cord of these mice; the protein is present in young SD animals and increases with age and disease severity [116]. The significance of this alteration as a contributing factor to disease progression and severity is unknown. However, Suzuki and colleagues showed that ablation of α-synuclein reduced the extent of the pathology but offered only minor neurological benefit in SD mice [126]. Clearance of α-synuclein relies on the ubiquitin-proteasome system [127] and the autophagy-lysosomal pathway [118], and when these pathways are dysfunctional, as it is the case for many neurodegenerative disorders, α-synuclein accumulates. It has been recently shown that

truncation of α-synuclein by the inflammasome protein caspase-1 causes its aggregation with concomitant neuronal toxicity [128]. Taken together, these findings suggest that the inflammasome and the proteasomal/autophagosomal/lysosomal systems are inextricably linked and interdependent. The studies also go a long way in showing how the pathological accumulation of cellular macromolecules has far-reaching consequences, revealed by the plethora of pathology and disease manifestations, particularly in those LSDs with neuronopathic features.

In infantile cases of GM2 gangliosidosis most neurones are abnormal, but in later onset forms, particularly in adult patients, the visible abnormalities occur preferentially in particular anatomical sites. The result is a unique pattern and evolution of disease manifestations, which at first superficially may resemble other diseases and often lead to misdiagnosis [129-133].

**Demyelination**

Haberland and colleagues studied the white matter of infantile GM2 gangliosidosis and found histological evidence of severe and extensive demyelination, and biochemically a decrease of 50-85% in cerebrosides. These investigators postulated that arrested myelinogenesis played a major part in the clinicopathological evolution of GM2 gangliosidosis and that secondary myelin loss was a contributing factor. They attributed the failure in myelination to interference with cerebroside synthesis [134]. Alterations in myelin composition in animal models of GM2 gangliosidosis have also been described [135, 136]. In line with these observations, when we examined the expression of oligodendrocyte-specific genes in the SD mouse, we found them reduced in all regions of the brain at an early age, both at the level of transcription and translation. Moreover, mRNA expression of uridine diphosphate (UDP)-galactose:ceramide transferase, the key enzyme in galactocerebroside biosynthesis, was down-regulated at all ages examined (5-19 weeks), and well before the onset of obvious disease signs [116]. Myelin deficits identified in the early stages of other LSDs, Nieman-Pick type A [137] and fucosidosis [138] for example, suggests that demyelination is likely to be a common feature in these disorders and central to their pathogenesis.

**Neuroinflammation**

The innate inflammatory response evolved to protect and minimise the effects of injury to the affected tissue, but when chronically stimulated, as in neurodegenerative diseases, expression of cytokines and pro-apoptotic molecules results in an environment damaging to neurones, and a contributing factor to disease progression.

Myerowitz et al. concluded, from expression analysis of cerebral cortex from patients with Tay-Sachs and Sandhoff disease, that inflammation is a factor that leads to neuronal cell death [139]. Moreover, in a previous histological and gene expression study of the SD mouse, these authors found that microglia activation and expansion preceded “massive neuronal death” [140]. Studies by others have confirmed some of these results [141] (Fig. **2**). Inflammation is a consistent observation in all neurodegenerative disorders, regardless of the genetic defect, but it is difficult to prove unequivocally its contribution to disease progression and severity. The inflammatory response is a dynamic process, which involves numerous molecules, many of which have

overlapping functions. In an attempt to test experimentally the rôle of inflammation in GM2 gangliosidosis and anticipating an improved phenotype, several investigators have disrupted some of its elements, these include: ablation of cytokine expression - macrophage-inflammatory protein 1α (MIP-1α) [142], and tumour necrosis factor α (TNFα) [143] - or signalling molecules - sphingosine-1-phosphate, produced by sphingosine kinase 1 (Sphk1), and S1P3 receptor, a G protein-coupled receptor enriched in astrocytes [144]. Similarly, the effect of nonsteroidal anti-inflammatory drugs was examined [145]. Results from these manipulations confirmed a modest improvement in lifespan and disease features in the SD mouse; overall inflammation appears to be a net contributor to the adverse effects of the primary neurodegenerative process in GM2 gangliosidosis.

**Neuronal death and degeneration**

Investigations of neuronal death in GM2 gangliosidoses by Huang [146] and Wada et al. identified many TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) positive cells in human and murine brains, considered a signature of apoptotic demise [140]. However, Sargeant and colleagues after studying the SD mouse brain found no extensive TUNEL-positive neurones. Instead, only the ventroposterior medial and ventroposterior lateral (VPM/VPL) nuclei of the thalamus showed prominent staining, and as expected for a progressive neurodegenerative disease, neuronal numbers decreased with age at these sites, accompanied by increases in activated microglia (Fig. **2**). In addition, neuronal counts were taken from a number of other structures, and comparable loss of density was identified. Among those analysed, only the lateral vestibular nucleus of the brainstem showed abundant cell loss; small but statistically significant depletion of cells was detected in the ventral spinal cord [147]. Although neuronal loss might not be universal in the SD mouse at its humane end-point, neuronal dysfunction, as detected by silver staining, is widespread and demonstrable from a young age before disease signs are apparent [116] (Fig. **2**). Of note, in human GM2 gangliosidosis, the amount of neuronal loss at the time of death is reported to vary considerably between cases but is more evident and widespread than in the Sandhoff mouse [104, 134]. The implication from these studies is that neuronal dysfunction, and not necessarily extensive cell depletion, is sufficient to cause the protean disease manifestations that characterize GM2 gangliosidosis.

**Neuroimaging**

Magnetic resonance imaging (MRI) of infantile GM2 gangliosidosis typically shows extensive T2-weighted hyperintensities in cerebral white matter, basal ganglia, thalamus, pyramidal tract and cerebellum. Proton MR spectroscopy, which can be viewed as a non-invasive assay of brain metabolism, has shown that almost all brain regions have reduced MR signals due to N-acetylaspartate and N-acetylaspartylglutamate (tNAA), with signals suggesting increased abundance of myo-inositol in white and gray matter. The concentration of choline-containing compounds generally remains unchanged, while creatine and the energy-rich phosphocreatine appear to be present at increased abundance in cerebral white matter but markedly reduced in basal ganglia [148].

NAA localises almost exclusively to neurones and their processes [149], and its reduced abundance is associated with neuronal/axonal loss. Experimental and clinical studies suggest that diminished tissue

concentrations of NAA might indicate damage and neuronal dysfunction, rather than frank cell loss [150, 151]. Phosphorylcholine and glycerol phosphorylcholine appear to reflect membrane turnover. Phosphocreatine and creatine act as reserves for high-energy phosphates in the cytosol of neurons and glia [152]. Myo-inositol is a glial marker, predominantly found in astrocytes [153].

Wilken and co-workers identified a novel MR spectroscopic signal in the brain of an infant with Sandhoff dissease: this was presumed to be N-acetylhexosamine. While this signal was more prominent in white matter, it was also present in the thalamic nuclei [154]. Of interest, the newly identified MR signal was also detected in the SD mouse brain. High-performance liquid chromatography and mass spectrometry of mouse brain extracts identified N-acetylhexosamine-containing oligosaccharides as the most likely candidate for the new resonance signal - measurable in pre-symptomatic mice, and increasing in abundance over time as the disease progressed [155]. In summary, the pathology of infantile GM2 gangliosidosis as defined by neuroimaging is characterised by loss and/or dysfunction of neurones, demyelination and gliosis, and corresponds with the findings reported in serial studies using conventional histological staining methods for examining neuropathogical changes in this disease.

In contrast to the infantile forms of GM2, the only consistent abnormality noticed by MRI in late-onset patients is cerebellar atrophy and often hypodensity of the thalamus. MR spectroscopy has been reported in one patient by Jamrozik et al. who identified a lower ratio for N-acetyl aspartate/creatine in cerebellum, lenticular nucleus and thalamus, and an increased ratio for myo-inositol/creatine in the cerebellum. Fluorodeoxyglucose positron emission tomography carried out in the same patient revealed diminished glucose metabolism in cerebellum, and in temporal and occipital lobes [156]. Inglese and colleagues investigated nine patients by MR spectroscopy and found the cerebellum to be markedly abnormal; however, their study also concluded that neuronal injury is diffuse [157].

Refinements in the resolution and advancing technology of clinical neuroimaging with increased sensitivity and scope, offer opportunities for non-invasive monitoring of disease progression and therapeutic efficacy in several neuropathic lysosomal diseases and may ultimately prove to be sufficiently responsive to metabolic changes in a manner that will contribute usefully to the clinical assessment of potential therapies.

## THERAPEUTIC MODALITIES FOR GM2 GANGLIOSIDOSIS

### Enzyme replacement therapy and cell transplantation

With extraordinary prescience in 1964, de Duve foresaw the treatment of storage diseases by enzymatic supplementation [158]; the concept of inborn lysosomal storage diseases was proposed contemporaneously by HG Hers, who had identified the enzymatic deficiency of acid maltase in the glycogen storage disorder, Pompe disease [159]. Understanding of the underlying biology and methodology to implement enzyme supplementation came from studies in the laboratory of Elizabeth Neufeld. In their pioneering experiments, fibroblasts from MPS I (Hurler syndrome) and MPS II (Hunter syndrome) patients mutually complemented each other resulting in biochemical correction, leading them to conclude and later confirm, that functional complementation was due to the exchange of missing gene products or ‘corrective factors’ between the two fibroblast populations in culture [160-162]. The first and still the most successful treatment of any LSD by enzymatic supplementation is for type

I Gaucher disease; developed by Brady and co-workers over a period of almost 25 years and targeted to the diseased macrophage population via the avid mannose receptor[163]. Now known commercially as enzyme replacement therapy (ERT) this approach has also been developed for other LSDs with a prominent systemic phenotype, such as MPS I, Fabry disease, Pompe disease, MPS VI and MPS II.

Desnick and colleagues in the 1970s, injected purified HEX A intravenously in an infant with Sandhoff disease; there was no clinical benefit. HEX A was present in the liver, but not in the cerebrospinal fluid or brain parenchyma [164, 165]. Even when purified HEX A was injected directly into the cerebrospinal fluid, clinical outcomes were equally disappointing [166]. Recently, Tsuji et al. infused recombinant HEX A intraventricularly into the SD mouse brain. Their isozyme was produced in the methylotrophic yeast strain *Ogataea minuta*, resulting in a protein with a high content in mannose 6-phosphate (M6P)-type-N-glycan. Outcomes from this intervention showed a modest salutary effect [167]. Although there are ongoing clinical trials of enzyme therapy for some neuropathic LSDs - Metachromatic Leukodystrophy, IDEAMLD phase I/II clinical trial (NCT01510028) - it is unlikely that ERT will ever be a definitive treatment for GM2 gangliosidosis. This is because of limited enzyme spread to the neuraxis, and the need for repeated and frequent applications. However, recently enzyme augmentation has had one striking clinical success that also represents a potential proof of concept for rescue of an acute neurodegenerative lysosomal disease by enzymatic complementation. Recent outcomes from a clinical trial of repeated intraventricular infusions of recombinant human tripeptidyl peptidase 1 were shown to slow the loss of walking ability in symptomatic children aged 3 years and older who were affected by late-infantile neuronal ceroid lipofuscinosis type 2 (CLN2) which is caused by genetic deficiency of this lysosomal enzyme. This has led to the approval of the commercial enzyme developed by the Biomarin company (cerliponase alfa) as Brineura by the US Food and Drug Administration, in 2017. The protein is administered at doses of 300 mg over a period of 4-5 hours every two weeks and met the predefined criteria for safety and efficacy of this open-label study involving 22 patients with NCL2 compared with 42 untreated historical control patients [168].

Bone marrow transplantation is a cell-based method of delivering corrective enzyme to the brain. The aim is to persuade graft-derived monocytes to enter the host brain, secrete normal enzyme and cross-correct resident enzyme-deficient cells in a timely manner. Human patients have not benefited from this approach [169], but when the SD mouse was transplanted, survival was improved and disease manifestations progressed more slowly. Of note, however, no neurones were found to have detectable enzyme activity, nor was the amount of storage reduced. The authors attributed the beneficial effect to suppression of the innate inflammatory response by the graft [170]. Experimental evidence suggests that secretion of lysosomal enzymes by monocytes and other myeloid cells is protein- and species-specific. For example, unlike the well-secreted feline α-mannosidase or β-galactosidase, β-hexosaminidase is less abundant in the medium of cultured microglia [171, 172], and in line with these findings, bone marrow transplantation in human α-mannosidosis has been reported to be beneficial [173]. When delivered early to patients with MPS I, haematopoietic stem cell transplantation improves cognition and lifespan [174], but in the closely related disease MPS IIIA (mucopolysaccharidosis type IIIA or Sanfilippo type A), the same treatment extends life without neurological improvement [175]. Reports that the amount of enzyme produced by the graft impacts therapeutic outcomes, ‘more is better’, led Langford-Smith and colleagues to test this idea in the murine model of MPS IIIA. Enzyme augmentation resulted in a significant increase in lifespan and improved phenotype [176]

Should donor-derived myeloid cells be endowed will the ability to produce and secrete large quantities of HEX A by genetic manipulation, it is conceivable that cell transplantation might become a treatment option for GM2 gangliosidosis. Over recent years, better protocols for the management and prevention of graft-versus-host disease and improved survival from the myeloablative regimens used in the pre-engraftment conditioning, has encouraged clinical exploration of this stratagem in otherwise severe lysosomal diseases with neurological manifestations.

**Substrate reduction therapy**

Norman Radin was the first to propose reducing the burden of accumulated substrate in disorders such as storage diseases, by partially inhibiting the substrate-specific synthase. For this approach to succeed, significant residual or supplemented hydrolase activity is required. The rational is to balance the rate of substrate synthesis with that of diminished enzymatic degradative power [177]. Eliglustat tartrate (Genz-112638) is a synthetic analogue of glucosylceramide, a potent and specific inhibitor of glucosylceramide synthase (UDP-glucose: N-acylsphingosine transferase), that has proved to be effective and safe in patients with non-neuronopathic Gaucher disease [178-181]. Because glucosylceramide is the first committed step in the biosynthesis of glycosphingolidpids, including GM2 ganglioside, inhibitors of glucosylceramide could be used, in principle, to treat GM2 gangliosidosis, provided they cross the blood-brain barrier. Unfortunately, a characteristic not attributable to eliglustat, but its congener venglustat, which does traverse the blood-brain barrier and unlike eliglustat is not a substrate for the P-glycoprotein tranporter, is currently undergoing trials in type 3 (chronic neuropathic) Gaucher disease, Fabry disease and in patients with Parkinson disease who are heterozygous carriers of pathological mutation in the human *GBA1* gene.

Miglustat, the iminosugar N-butyldeoxynojirimycin, belongs to a different class of inhibitors of glucosylceramide synthase [182]. About 20% of the plasma concentrations of the drug have been found in human CSF after dosing, suggesting that it can to some extent cross the blood-brain barrier. The drug and is licenced as a second-line agent for the treatment of type 1 Gaucher disease [183], and as the only approved agent (in European patients) with the neurodegenerative disorder Niemann-Pick type C [184]. While miglustat increased lifespan and improved clinical features in the SD mouse [185], in a clinical trial of late-onset TSD patients, the drug did not meet its efficacy endpoint target [186, 187]. Despite the disappointments, the search for inhibitors to reduce accumulating substrates in LSDs, and indeed in other diseases, is an active field of scientific endeavour. As exemplified by eliglustat, currently approved as Cerdelga, and a first-line therapy in type 1 Gaucher disease, with efficacy comparable to enzyme therapy in non-neuronopathic Gaucher disease, substrate reduction therapy by means of orally active small molecules, has therapeutic advantages. In the further development of this field, substrate reduction therapy for some sphingolipid disorders may prove to be of particular therapeutic value if used in combination with therapies that complement the lysosomal degradative function that is compromised in a given disease.

**Enzyme enhancing therapy**

As pointed out above, most disease-causing missense mutations in GM2 gangliosidosis and in other LSDs, do not localise to the active site, but often cause instability of the native folded protein; targeted by the cellular quality control systems they are degraded before they can reach the lysosome. Strategies to reduce their degradation include the use of molecules that bind and stabilise the enzyme - chaperones - and in doing so protect it from degradation. Paradoxically, the most commonly used pharmacological chaperones are enzyme-specific competitive inhibitors [188]. An altogether different approach is to tamper with the ERAD system to either prevent the faulty protein being recognised in the first instance, or by enhancing the function of endogenous chaperones [82].

Pharmacological chaperones that have shown some in vitro findings which may offer promise for some late-onset forms of lysosomal diseases including Krabbe [189], GM1gangliosidosis [190], Fabry disease [191], Gaucher disease[192], and GM2 gangliosidosis [193]. A clinical trial with the Hex A inhibitor pyrimethamine has taken placed, and although therapeutic outcomes were not fully assessed because of the short evaluation period, leukocyte Hex A activity was increased up to a 4-fold in some patients [194]. However, no clinical benefit has yet been reported.

Kirkegaard and colleagues showed improved outcomes for animal models of several LSDs, including GM2 gangliosidosis, when the heat shock protein 70 (HSP70), an ER endogenous chaperone [195], was augmented *in vivo*. Intravenous or intraperitoneal injections of recombinant HSP70, or oral administration of its co-inducer, arimoclomol, both of which penetrate the brain, allowed for reduced cellular pathology and increased lifespan. In addition to its role as a chaperon of mutant proteins, HSP70 protects against permeabilization of lysosomes, which is another potential adverse pathological consequence of disturbed lysosomal physiology. Thus, HSP70 might constitute a supportive option with simultaneous effects on several mechanisms of disease [196]. Despite the scientific interest in enzyme enhancers, at the time of writing few if any of these enhancers have been rigorously tested in clinical trials, or indeed in authentic animal models of lysosomal diseases in which significant amounts of residual mutant enzyme are present.

**Gene therapy**

The once-in-a-life-time and definitive therapeutic intervention that gene therapy - the targeted manipulation of the genetic code - promises to deliver has long been awaited, and is particularly pressing for lysosomal diseases such as GM2 gangliosidosis, in which neurodegeneration is prominent.

Many neuropathic LSDs have characteristics ideally suited to this treatment modality. They are caused by defects in single genes whose products are naturally secreted – a property that can readily be exploited. Transduction of relatively few cells can supply sufficient corrective enzyme to complement the deficiency in a large volume of tissue, and abundant enzyme can apparently be synthesised and secreted without cytotoxic effects. Moreover, as known from the pseudo-deficiencies - healthy individuals with very low amounts of active protein – estimated to be about 10% of normal activity is all that is needed to avoid disease. Currently, the greatest challenge to the successful treatment of these conditions is in the safe delivery of the corrective gene to the sites where is needed and in a timely fashion. This is because often the entire nervous system, made up of many complex domains and connections, is implicated in the degenerative process, and because the lysosomal

disease, long established in the nervous system, is almost invariably diagnosed after the onset of symptoms. Notwithstanding the difficulties outlined above, the management of these disorders, with all their idiosyncrasies, has never been so promising. There is genuine interest, focused research and investment into rare diseases by industry and academia. Unprecedented progress has been made in the last few years towards fulfilling the ultimate goal, with impressive examples of disease rescue in authentic animal models and scientific discoveries likely ultimately to enable present technical problems to be circumvented.

Among viral vectors, recombinant Adeno-associated viruses (rAAV) have shown great potential for treating a variety of diseases, and have become the delivery vector of choice for the nervous system because most serotypes preferentially transduce neurons after intraparenchymal injection. Wild type AAVs are small, 20-25 nm in diameter, non-enveloped single-stranded DNA viruses of the Parvoviridae family. Unlike oncoretroviral and lentiviral vectors, their genome remains, to a large extent, episomal in the transduced cell, reducing the probability of insertional mutagenesis. For replication and gene expression, AAVs require a helper virus. Although they infect humans, integrating at a specific site in human chromosome 19, they are not associated with clinical disease [197], this feature is not preserved in recombinant AAVs. Many strains have been isolated from humans and primates, differing in cellular tropism and immunological properties [198, 199]. The 4.7 kb AAV genome is flanked by two inverted terminal repeats that contain the origin of replication, packaging and integration signals. Transgene-expressing rAAVs retain only the two inverted terminal repeats from their viral genome. Because rAAV could only be made in a small scale for many years, their use was restricted, but recent developments in their manufacture has permitted production in sufficiently large quantities for clinical applications [200]. rAAVs have been used in more than 100 clinical trials, a testament to their popularity, which has been attained as a result of their widespread tropism, capacity for inducing sustained gene expression in dividing and non-dividing cells and their safety record.

Guidotti and colleagues carried out one of the earliest experimental gene transfer procedures in an animal model of GM2 gangliosidosis. Adenoviral vectors, encoding human α- and β-subunits of β-hexosaminidase, were administered intravenously to TSD mice. This led to abundant expression of Hex A in serum and peripheral tissues, but was shown to be conditional upon both subunits being co-injected; α-subunit alone resulted in very low restoration of Hex A activity. As expected, neither vector nor β-hexosaminidase were able to cross the blood-brain barrier, but critically the study underscored the importance of co-expressing both hexosaminidase subunits, suggesting that if only one subunit is overexpressed the other becomes limiting [201]. Surprisingly, when Martino et al. infused non-replicating Herpes simplex vectors encoding human α-subunit alone directly into the internal capsule of TSD mice, they achieved distribution of Hex A and GM2 clearance throughout the brain, although not in the spinal cord [202]. Remarkable as these results are, the TSD mouse does not develop a clinical disease, and long-term efficacy was not reported, perhaps because the vector could not sustain extended transgene expression.

Encouraged by positive outcomes in animal models of other LSDs after gene transfer mediated by rAAV delivery, the authors of this review investigated the corrective power of this approach in the SD mouse. In marked contradistinction to the TSD model, murine SD - a knockout of the β-subunit of β-hexosaminidase - displays a progressive neurodegenerative disease. It closely recapitulates the human disease biochemically and pathologically and has neurological effects which in many ways mirror those observed in affected children. The

SD mouse appears indistinguishable from wild type or heterozygous littermates at birth, but by the age of 3 months motor abnormalities are noticeable, and soon after, the animal enters a phase of rapid decline with death occurring 1-2 months later. Stereotypic manifestations, which increase in severity as the disease progresses, include tremor, ataxia, rigidity, and spasticity [99]. Young adult SD mice, age 4-5 weeks, were injected with rAAV, serotypes 2/2 or 2/1 at intracranial sites. Monocistronic vectors expressing human α- (rAAV-α) or β-subunits (rAAV-β) were infused singly or in combination at one or multiple sites in the brain parenchyma (Fig. **3**). Rescue of the phenotype was achieved when the animals were given the combination treatment (rAAV-α + rAAV-β) or rAAV-β alone, but as expected, not when only rAAV-α was supplied. Mice were still alive and apparently healthy at the age of 2 years, the predefined end-point of the experiment. Concomitant with the phenotypic rescue, pathological features, namely, storage, inflammation and neurodegeneration, were reduced or undetectable in the brain and spinal cord. We have evidence indicating that the unprecedented outcome is the result of extensive spread of vector and protein through several routes: diffusion, axonal, perivascular and cerebrospinal fluid transport. We reported significant therapeutic benefit accrued after a single injection into one anatomical site. However, multiple injections improved outcomes, and are necessary for neuraxis-wide distribution of enzyme [203, 204] (Fig. **3**). We present here unpublished data, showing salutary effects in SD mice injected with the combination treatment (rAAV-α + rAAV-β) into the cerebrospinal fluid; animals aged 4-5 weeks were infused through one lateral ventricle. Abundant formation of HEX A at distant sites, such as the spinal cord was demonstrated, life was extended and classical pathological features reduced (Fig. **4**).

We explain the positive effects resulting from rAAV-β only interventions by the observed production of copious amounts of isozyme HEX B, and not from the abundant formation of interspecies hybrid HEX A. This notion is supported by separation of the different isozymes by ion-exchange chromatography and their identification using isozyme-selective artificial substrates, as well as cellulose-acetate electrophoresis of brain extracts [204]. In the presence of Hex B, the aforementioned biochemical bypass, mediated by an efficient apparently species-specific sialidase (Neu3) as occurs in the TSD mouse [101], would presumably operate also in the SD mouse transduced with rAAV-β alone. In humans, with no such endogenous sialidase, co-expression of both subunits by the simultaneous injection of rAAV-α and rAAV-β is an absolute requirement to maximise treatment efficacy. Because each of the recombinant viruses only drive expression of one of the subunits, simultaneous cellular infection by the two vectors must occur in order to form heterodimeric HEX A. This we confirmed by mRNA *in situ* hybridization of transduced brain sections; dual labelling of the two mRNA subunits was detected in individual cell nuclei, albeit at different ratios (Fig. **3**).

As with any treatment, early intervention is predicted to improve outcomes, particularly for neurodegenerative diseases, because of the limited regenerative capability of the nervous system. It is unknown how narrow the window of opportunity to treat GM2 gangiosidosis really is; whether we can prevent, halt or even reverse the disease or indeed when the point of no return has been reached. Using the SD mouse as a paradigm, we injected the animals with a combination of rAAV-α and rAAV-β at different ages and took them to their humane end points. These studies showed improved survival only when the animals were treated before disease signs were apparent or during their early manifestation, but not if treatment was initiated during the peri-symptomatic/symptomatic phase of the illness. A remarkable finding from these experiments was that despite the elapse of the therapeutic window so that the treatment had no beneficial effects on survival, abundant and widespread expression of β-hexosaminidase, which abrogated pathological features such as storage and

neuroinflammation, had still occurred. We concluded from these experiments that regardless of resolution of the cardinal pathological features mediated by gene transfer, a point is reached when functional deterioration and death are not preventable in GM2 gangliosidosis [116]).

Therapeutic outcomes in the SD mouse after intracranial injections with rAAV prompted us to seek reproducibility of the results in animal species with larger and more complex brains, as the most important next phase towards human clinical trials. Bearing in mind that the mouse, cat and sheep's brain is about 1/1000, 1/13 and 1/3 the size of a new-born human brain respectively, the cat represents an intermediate and appropriate candidate to test our gene transfer stratagem within a research environment. We approached Prof Henry J Baker (based at the Scott-Ritchey Research Centre, College of Veterinary Medicine, Auburn University) who with stubborn determination had maintained naturally occurring cat models of GM2 gangliosidosis for decades, understanding their importance to study mechanisms of disease and to test novel therapies, and a fruitful scientific collaboration was established.

The lifespan of normal cats is 12-14 years, while that of the SD cat is approximately 6 months. Onset of disease manifestations occurs at age 4-7 weeks, which include hypermetria, tremor, ataxia, paresis and seizures [205, 206]. Mutant cats were treated presymptomatically with a variety of rAAV serotypes. Monocistronic vectors expressing feline-specific α- and β-subunits were co-infused into either the thalamus and deep cerebellar nuclei [207, 208] or thalamus and lateral ventricle [209-211]. The different gene transfer modalities resulted in enzymatic activities above normal levels throughout the neuraxis, with concomitant reduction of storage compounds, amelioration of clinical signs and extension of life. Reproducibility of our mouse results in the larger and complex cat brain lends further support and justifies initiation of human clinical trials in the near future to treat these devastating and fatal diseases.

The need to co-express the two β-hexosaminidase subunits and the failure of single bicistronic rAAV vectors in co-synthesizing both peptides appropriately adds undesirable complexity and expense to the potential use of this treatment modality in the clinic; hitherto, only co-infusion of monocistronic vectors has been successful at generating therapeutic amounts of HEX A *in vivo*. However, a recent and exciting development might circumvent this difficulty. Tropak and colleagues reported the generation of a hybrid subunit they call μ that combines features of the two native forms. The μ-subunit contains the α-subunit active site, the stable β-subunit interphase, and a unique area in each of the subunits mandatory for interaction with the GM2 activator protein. This elegant "Frankenstein" approach to the problem results in homodimerization of μ-subunits and formation of the active isozyme named HEX M. Furthermore, when they intracranially injected 4-month old TSD mice with self-complementary AAV9 (scAAV9) expressing *HEXM*, there was a reduction in brain ganglioside and its clearance was comparable to outcomes observed in animals injected with scAAV9 expressing *HEXA*. The study was extended to SD mice injected intravenously during the neonatal period with scAAV9-HEXM. These animals showed decreased brain ganglioside despite the presence of low levels of HEX M, which the authors speculated was the result of poor transfer of vector from the circulation into brain structures [212]. In a different publication, the same group demonstrates that clearance of pathological storage is accompanied by an increased in lifespan and recovery of motor function in these animals [213]. These results are quite remarkable, but there remains a nagging concern about the functionality and safety of this "Frankenstein" creation for use in human patients; in particular the possibility that it may prove to be strongly

immunogenic. It is thus imperative that its long-term efficacy and safety record is comprehensively tested in larger animals.

The discovery that some AAV serotypes can cross the blood-brain barrier when injected intravenously, spreading widely throughout the neuraxis, constitutes a major leap forward for the treatment of neurodegenerative disorders. Among these, AAV9 [214], AAVrh8 and AAVrh10 [215, 216] have been extensively tested. Walia and co-workers intravenously injected rAAV9 expressing murine hexb into neonates and adult SD mice, extending life and reducing storage and inflammation [217]. The major concern raised by the study was the development of tumours in liver and lung in older mice. Others have reported similar accounts when rAAVs are injected systemically at a high dose. With the development of tumours only revealed in animals kept alive for prolonged periods, a cautious approach is warranted [218-220]. Undoubtedly, these initial aspect are disappointing, but a huge effort is being invested in generating viruses with improved specific targeting of cell types. Directed evolution of rAAV is an approach to select viruses that imitates natural evolution under selective pressure and experimental results with these viruses are encouraging [221-224].

## CONCLUDING REMARKS

The story of Tay-Sachs disease, the discovery of its complex genetics, biochemical components and their function is inextricably linked to some of the most important scientific discoveries, technical innovations and historical events of the 20$^{th}$ century. The next phase in the narrative of this rare but devastating disease must surely be the dawn of effective treatments. Of the stratagems outlined above, with its substantial therapeutic benefit convincingly reported in authentic living models, and because it aims definitively to correct the initiating cause of the disease, the most attractive hitherto appears to be gene transfer. However, for certain clinical forms of Tay-Sachs and its related diseases, combinatorial approaches may also be considered: for example, the use of substrate biosynthesis inhibitors and agents that simultaneously target distinct pathological mechanisms of disease may also offer much-needed clinical benefit.

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.


## ACKNOWLEDGEMENTS

MB C-G is principally funded by The National Institute of Health Research University of Cambridge Biomedical Research Centre (Metabolic theme), and E Z by the UK Medical Research Council. Cognate research in the host laboratory is currently funded by an MRC programme award to MB C-G and TMC (MR/K025570/1).


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LEGENDS TO FIGURES

**Fig. (1). Catabolism of GM2 ganglioside by the β-hexosaminidase system and transport to the lysosome.** GM2 ganglioside is hydrolysed *in vivo* in the lysosome by the concerted action of the isozyme Hex A and GM2 activator protein. Hex A is a heterodimer of the α- and β-subunits of β-hexosaminidase, and encoded by *HEXA* and *HEXB*, which localize to human chromosomes 15 and 5, respectively. Hex B and Hex S are homodimers of the β- and α-subunits. Only dimeric forms of Hex can hydrolyse specific natural and artificial substrates. Mutations in *HEXA*, *HEXB* and *GM2A* cause Tay-Sachs disease, Sandhoff disease and the GM2 activator protein deficiency respectively, also known as variants B, 0, and AB on the basis of the residual β-hexosaminidase isozyme activities in affected individuals. In a similar manner to other lysosomal hydrolases, Hex can travel to the lysosome directly (1, a) or indirectly (2, b). The latter route, known as the secretion/recapture mechanism, can be exploited for therapeutic applications; whereby the enzyme is secreted into the extracellular space and taken up by the same (d) or neighbouring cells, such as the axons of neurones

(c). The enzyme is transported in a retrograde manner to other parts of the cell thus correcting the enzymatic defect. Endoplasmic reticulum (ER); lysosome (L); mitochondria (M).

**Fig. (2). Pathologic features of GM2 gangliosidosis.** The most notable feature is the presence of grossly enlarged neurones throughout the nervous system, due to the presence of membranous cytoplasmic bodies (MCBs *), which are abnormal lysosomes, easily revealed by electron microscopy (EM). MCBs stain with Periodic acid-Schiff reagent (PAS) and antibodies against GM2 ganglioside. Activation and expansion of microglia is evident in many areas and revealed by staining for cell markers such as CD68, prominent here in the VPM/VPL nuclei of the thalamus. Neurodegeneration is detectable by silver staining, abundant in neuronal cell bodies and axons such as those of the internal capsule (IC). Impaired autophagy in neurones is shown by co-staining proteins p62 and ubiquitin. All sections are from Sandhoff mouse brain. Fields CA1 (CA1) and CA3 (CA3) of hippocampus (Hipp); fimbria (fi); granular layer of the dentate gyrus (GrDG); internal capsule (IC); nucleus (N); ventroposterior medial (VPM) and lateral (VPL or LP) thalamic nuclei; primary somatosensory cortex (S1).

**Fig. (3). Brain parenchymal gene transfer of rAAV monocistronic vectors rescues the Sandhoff mouse phenotype.** Infusion of rAAVs coding for human α- (hhex α) and β-subunits (hhex β) of β-hexosaminidase leads to distribution of Hex enzyme (red precipitate) throughout the entire neuraxis, seen here in a mutant animal aged 2 years. Dual mRNA *in situ* hybridization with probes specific for the α- and β-subunits detects the presence of both mRNAs in transduced neurones, a pre-requisite for the formation of abundant amounts of Hex A. Consequently, storage material is cleared or prevented as seen here in the spinal cord of a 2-year old mutant Sandhoff mouse. Central canal (cc); grey matter (gm); white matter (wm); CAG promoter is a composite promoter of the CMV enhancer, the chicken beta actin promoter, and the rabbit beta globin intron (CAGp); human immunodeficiency virus tat protein transduction domain (tat); woodchuck hepatitis virus posttranscriptional regulatory element (WPRE); inverted terminal repeat (ITR).

**Fig. (4). A single ventricular co-infusion of rAAVs into the Sandhoff brain rescues the mouse phenotype.** Four-week old Sandhoff mice were injected into one of the brain lateral ventricles with rAAV2/1 coding for α- and β-subunits of β-hexosaminidase. The intervention increased survival with supranormal formation of all three Hex isozymes, which are distributed throughout the entire central nervous system. Fractionation of the isozymes was carried out on the brain contralateral injection side by ion exchange chromatography. Hex activities were detected with the artificial substrate 4-MUG (4-methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside).