SUBSTRATE DEPRIVATION AS A NOVEL THERAPY FOR THE MUCOPOLYSACCHARIDOSES

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Children, Youth and Women’s Health Service

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Addenda

Chapter 1
p10, line 18: Insert words "and produce" after breed (add and produce)
p12, line 5: Change "relative permeable" to "relatively permeable"
p14, line 1: Remove comma after blood-brain barrier
p16, line 18: Insert after co-administration of antihistamine... "or by prolonging the infusion time or by a range of other drugs including those with anti-inflammatory and immunosuppressive properties such as corticosteroids (Schifffman and Brady 2002, Drugs; 62(5):733-742; Beck et al. 2004, Eur. J. Clin. Investig. 34:838-844).
p17, line 6: Add word "function" before reference.
p18, line 13: Replace words "Recent evidence has" with "It has been"
p19, line 14: remove the word "outcome"
p21, line 19: Insert underlined words "In another study of MPS mice were treated... and 12 month..."
p22, line 21: Insert "and cross-correction via mannose-6-phosphate mediated uptake following cell engraftment" at end of sentence.
p23, line 4: Replace word "production" with "localisation"
p24/25, Figure 1.7: Figure 1.7 A-D have been replaced. Legend line 9: replace "though" with "thought"
p26, line 1-3: Sentence should read "These patients showed a reduction of mean liver and spleen volumes and also an increased..."
p28, line 5 and line 8: Replace "Twitcher" with "twitcher" to indicate strain of mice
p28, last line: Replace sentence with "These compounds can be formulated to be administered orally, which is a major advantage"
p30, line 3: Add word "white" after "New Zealand"
p32, line 12: Add comma after "isoflavones"
p33, line 24: Replace "beta-glucuronidase" with "beta-glucosidase"
p34, lines 3, 4, 5, 8: Replace "beta-glucuronidase" with "beta-glucosidase"
p34, line 15: Insert word "the" after "stop codons in"
p34, line 20: After: "preclinical studies" insert "in MPS I"
Figure 1.9 legend, line 15 Replace "structure" with "structures"
p40, line 11: Replace "2-3" with "two to three"
p41, line 6: Replace "uncovered a role in TGF-β regulation in adult cells via protein-protein interaction" with "uncovered a role of syndecan-2 and its direct binding to TGF-β which regulates TGF-β signalling in adult cells"

Chapter 2
p71, lines 4-6: Replace sentence with "If a mouse failed to reach the escape platform within the allocated 60 seconds, the mouse was place manually onto the platform"

Chapter 3
p85, line 11 and Figure 3.5: Insert: "Female mice inbred in this study were not used for colony breeding and therefore were not pregnant or had litters." A power analysis to determine how many female mice would be needed to confirm the difference in bodyweight was conducted and to achieve a power analysis of 80% with a difference in weight of 15% a sample size of 4 mice would be needed and 2-5 mice were analysed for each group at 4 months of treatment.

Table 3.1: Insert "(N=3 per group)" after "normal treated mice" in table legend.
p86, line 22: Insert: "A reduction in total gag was seen in MPS IIIA treated mice, which on average was reduced to 74% of MPS IIIA untreated total gag levels in the liver (Figure 3.8A). This would incorporate total gag including a lysosomal component, and this decrease is likely to be due to reduced lysosomal gag. To confirm this, lysosomal gag was also analysed. Analysis of lysosomal
gag showed a greater reduction compared to total gag levels, with MPS IIIA treated mice on average reduced to 60% of MPS IIIA untreated lysosomal gag levels (Figure 3:80). This was expected since MPS IIIA is a lysosomal storage disorder and loss of the gag reduction was lysosomal gag.

p91, line 2: “si” should read “is”
p91, line 11: Add word “been” after “has not”

Chapter 4

p94, line 1: Change “Neurological pathology,” to “Neural pathology”
p98, bottom line: Change “showed an increased trend...” to “showed a trend to increased...” (reverse order)
p103, 2nd last line of 2nd last paragraph: Missing full stop at end of the sentence.
p114, insert before 3rd paragraph: “Qualitative changes in lysosomal storage in the brain or somatic tissues have not been observed in either study reported in chapters 3 and 4 using light microscopy analysis. However, a smaller decrease in brain lysosomal gag was seen when a quantitative assay was employed. The discrepancy between the biochemical measurement and visual assessment of storage may be due to the relative insensitivity of light microscopy to detect small changes in lysosomal storage. SDT acts to slow down the accumulation of gag substrate in the lysosomes, providing the opportunity for residual lysosomal enzyme activity to slowly turn over resident and newly acquired substrate. Thus conceptually SDT will not have the dramatic effect on gag storage observed with other types of treatment such as enzyme replacement therapy.”
p115, last line: Replace “4” with “four”

Chapter 5

p121, line 5: Insert sentences “These stains were chosen as good representative stains which are useful for detecting abnormalities in liver sections. H&E was used to detect general changes in morphology. Perl staining was used to stain iron and determine iron load, which is a marker of hepatic injury. Sirius Red staining was used to detect liver fibrosis and PAS staining is good stain for glycogen and mucins and is useful for outlining tissue structures such as basement membranes, capsules and blood vessels.”
p124, line 11: Replace “not” with “no”
p124, line 22: Replace “distal convoluted tubule” with “proximal convoluted tubule”
Figure 5.8 legend, line 4: Replace “distal convoluted tubule” with “proximal convoluted tubule”
Figure 5.9 legend, line 5: Replace “proximal convoluted tubule” with “distal convoluted tubule”
p127, insert before 3rd paragraph: “The colony was regularly checked every three months for a battery of pathogens including salmonella, enteric pathogens, respiratory pathogens including Mycoplasma spp., ectoparasitic examination, burrowing mites, helminths, protozoan mucosal scrapings and pinworm testing. No pathogens were observed in mice from the multi-generational study, therefore it is unlikely that pathogens would have been an influence of the visceral and neural pathology. All mice included in the multi-generational study were healthy and the only abnormalities were those associated with MPS IIIA disease progression in afflicted animals.”
p128, line 12: Remove “rad” after “Kapfer cells”
p129, line 24: Replace “natural level” with “minimal level”
p150, line 18: Remove “was” after “a toxicity study”

Chapter 6

p132, 2nd paragraph: Reword first sentence to clarify: “Even though EXT has been used to treat a large number of patients with non-neurological MPS pathology, there are still a number of problems associated with this type of therapy. Firstly, targeting...”
Thesis summary

Reduction of an enzyme required for the lysosomal degradation of glycosaminoglycan (gag) chains will result in a mucopolysaccharidosis (MPS) disorder. Substrate deprivation therapy (SDT), a new therapy option for MPS, aims to reduce the synthesis of gag chains, the natural substrate for the deficient enzyme. Reduced substrate levels would balance the reduced level of enzyme in patient cells resulting in normalised gag turnover. Rhodamine B, a non-specific inhibitor, reduced gag synthesis in a range of normal and MPS cells and also decreased lysosomal storage of gag in MPS VI (72%) and MPS IIIA (60%) cells. This positive response in vitro was extended to an in vivo therapy trial in the MPS IIIA mouse. Bodyweight gain of male MPS IIIA mice treated with 1 mg/kg rhodamine B was reduced compared to untreated MPS IIIA mice and was indistinguishable to that of normal mice. Liver size, total gag content and lysosomal gag was reduced in treated MPS IIIA animals as was urinary gag excretion. The alteration in MPS IIIA clinical pathology by rhodamine B combined with the observation that treatment had no effect on the health of normal animals demonstrates the potential for this type of therapy for MPS disorders. The water cross maze was found to be the only learning and memory test capable of detecting differences in learning behaviour in MPS IIIA and normal untreated mice. MPS IIIA mice treated with SDT rhodamine B showed an improved outcome with better learning capabilities than MPS IIIA untreated mice observed using this test. This means that rhodamine B is likely to cross the blood-brain barrier. These results are the first evidence of a positive response by the CNS to a systemic therapy for MPS IIIA. Rhodamine B administration over 4 generations did not produce any deleterious side effects in MPS IIIA. In utero therapy over four generations did not cause a reduction in litter size or bodyweight profile demonstrating that reduction of gag over a combined timeframe of two years was safe. A higher dose of 5 mg/kg rhodamine B did not produce any additional benefits on MPS IIIA pathology and no signs of hepatotoxicity were noted. Rhodamine B proved to be a
general inhibitor of gag synthesis and had a positive outcome on a number of clinical parameters in MPS IIIA mice. SDT in MPS IIIA mice improved learning capabilities as detected by the water cross maze which has not been previously reported. This provides evidence that small molecules such as rhodamine B, that are able to cross the blood-brain barrier, can have some effect on neurological pathology. This proof of principle study showed that SDT can be used to have a positive outcome on MPS pathology. Additional inhibitors of gag synthesis can also be investigated before this type of therapy can be translated into clinical use in MPS patients. Although it may be feasible to use rhodamine B as a SDT agent in vivo, other inhibitors may be more practical.
Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for photocopying and loan if accepted for the award of the degree

SIGNED:.......................................................... DATE:............................................
Acknowledgements

The list is long, but I have to thank all these people for helping me over the last 4 years.

I would like to thank my supervisors, Dr Sharon Byers, Dr Janice Fletcher and Dr Belinda Thomas, for which I greatly appreciate all the help, suggestions and advice they have offered me over the past four years.

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Well statistics is not my forte, so thank you to Janine Jones and particularly Kate Dowling at Biometrics SA for their help with statistical analysis for the bodyweight profiles, RAPC and cross maze data. Thanks for also answering my endless questions and narrowing the gap in my statistical understanding.
Thanks also to Dr. Sonja Klebe from the Department of Anatomical Pathology, Flinders Medical Centre for analysis of corneal clouding in mice with MPS IIIA both macroscopically and helping take pictures post-mortem.

The auditory evoked brainstem response analysis in the RAPC study would not have been possible without Dr Paul Weston from the Neurology department, CYWHS who helped set up the equipment and methodology and also to evaluate the hearing threshold in MPS IIIA and normal mice.

Thanks to my family, who have always been there and made sure that I had every opportunity to have the best start in life. They have always supported me through my studies and research and still don’t understand what MPS is after all these years.

Last but not least, to my fiancé Simon. Thanks for all your support, words of wisdom and encouragement over the years. This would not be possible without someone to share the anger when the experiments don’t work.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ABR</td>
<td>auditory evoked brainstem response</td>
</tr>
<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplantation</td>
</tr>
<tr>
<td>bp, kb</td>
<td>base pairs, kilobase pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulphate</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>dB</td>
<td>decibel(s)</td>
</tr>
<tr>
<td>DGJ</td>
<td>1-deoxy-galactonorjirimycin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide 5’triphosphate</td>
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<tr>
<td>DS</td>
<td>dermatan sulphate</td>
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<tr>
<td>dH2O</td>
<td>deionised water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
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<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERT</td>
<td>enzyme replacement therapy</td>
</tr>
<tr>
<td>EXTL</td>
<td>exostosin-like gene family</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>gag(s)</td>
<td>glycosaminoglycan(s)</td>
</tr>
<tr>
<td>Gen</td>
<td>generation</td>
</tr>
<tr>
<td>GET IT</td>
<td>gene-expression targeted isoflavone therapy</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary associated protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GSL</td>
<td>glycosphingolipid storage disorders</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan/hyaluronic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulphate</td>
</tr>
<tr>
<td>HSpag</td>
<td>heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>IMVS</td>
<td>Institute of Medical and Veterinary Science</td>
</tr>
<tr>
<td>kg, g, mg, μg, ng</td>
<td>kilogram, gram, milligram, microgram, nanogram</td>
</tr>
<tr>
<td>kDa, Da</td>
<td>kilodalton, dalton</td>
</tr>
<tr>
<td>KS</td>
<td>keratan sulphate</td>
</tr>
<tr>
<td>LSD</td>
<td>lysosomal storage disorder</td>
</tr>
<tr>
<td>LiCl</td>
<td>lithium chloride</td>
</tr>
<tr>
<td>L, ml, μl</td>
<td>litre, millilitre, microlitre</td>
</tr>
<tr>
<td>m, cm, mm, μm</td>
<td>metre, centimetre, millimetre, micrometre</td>
</tr>
<tr>
<td>mCi, μCi</td>
<td>milli Curie, micro Curie</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>M, mM, μM, pM</td>
<td>moles per litre, millimoles per litre, micromoles per litre, picomoles per litre</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>MGS</td>
<td>multi-generational study</td>
</tr>
<tr>
<td>MPS</td>
<td>mucopolysaccharidosis</td>
</tr>
<tr>
<td>MPS IIIA</td>
<td>mucopolysaccharidosis type IIIA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>Na³⁵SO₄</td>
<td>radioactive sodium sulphate</td>
</tr>
<tr>
<td>NB-DNJ</td>
<td>N-butyldeoxynojirimycin</td>
</tr>
<tr>
<td>NB-DGJ</td>
<td>N-butyldeoxygalactonojirimycin</td>
</tr>
<tr>
<td>NN-DNJ</td>
<td>N-(n-nonyl) deoxynojirimycin</td>
</tr>
<tr>
<td>NDST</td>
<td>N-acetylglucosamine N-deacetylase/N-sulphotransferase</td>
</tr>
<tr>
<td>NS</td>
<td>sulphaamidase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OGT 918</td>
<td>N-butyldeoxynojirimycin</td>
</tr>
<tr>
<td>PAPS</td>
<td>3’-phosphoadenosine 5’-phosphosulphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>abbreviation</td>
<td>definition</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
</tr>
<tr>
<td>pH</td>
<td>hydrogen ion concentration</td>
</tr>
<tr>
<td>% (w/v)</td>
<td>percent weight per volume</td>
</tr>
<tr>
<td>% (v/v)</td>
<td>percent volume per volume</td>
</tr>
<tr>
<td>RA</td>
<td>repeated acquisition</td>
</tr>
<tr>
<td>RAPC</td>
<td>repeated acquisition and performance chamber</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDT</td>
<td>substrate deprivation therapy</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) amino methane</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
</tbody>
</table>
1.1 Lysosomes and lysosomal storage disorders

Lysosomes are membrane-bound intracellular organelles, and the site of the catabolism of endogenous and exogenous macromolecules (Winchester et al. 2000). Lysosomes were first described by De Duve and colleagues as sac-like structures that contained a range of acid hydrolases and hence they provide an acidic microenvironment inside the cell (De Duve 1964, De Duve and Wattiaux 1966, De Duve 1983). Their cellular function is to degrade all naturally-occurring proteins, glycoproteins and glycolipids to their constituent monomeric components by using a number of proteases, glycosidases, sulphatases, phosphatases and lipases, which are contained within the lysosomal lumen. Once degraded these components are transported across the lysosomal membrane, where they are utilised by the cell (Sabatini and Adesnick 2001).

Hers described the first disease linked with lysosomal dysfunction in 1963, a deficiency in $\alpha$-glucosidase. This deficiency leads to the accumulation of glycogen in the lysosome and the clinical disease known as Pompe (Hers 1963). Over 43 lysosomal storage disorders have been identified (Gieselmann 1995, Futerman and van Meer 2004), with an overall Australian prevalence of approximately 1 in 7000-8000 live births (Meikle et al. 1999).

Most of the lysosomal enzymes are exohydrolases that allow the stepwise removal of terminal residues. They act in succession to degrade complex macromolecules to their monomeric components (Hopwood and Brooks 1997). A deficiency in one of these enzymes results in undegraded or partially degraded substrate, which is stored in the lysosome, since only monomeric components are transported out of the lysosome.

Lysosomal storage disorders (LSDs) are chronic and progressive clinical syndromes with a wide range of clinical severity. The clinical symptoms vary with each disorder, due to the
effects of the protein defect and the nature of the stored material (Winchester et al. 2000). LSDs can be broadly classified into nine subgroups based on the biochemical molecules stored (Hopwood and Brooks 1997): sphingolipidoses, mucopolysaccharidoses, glycogen storage disorders, oligosaccharidoses/glycoprotein disorders, multiple enzyme deficiencies, lipidoses, monomeric transport disorders, peptides disorders and S-acylated protein disorders (Table 1.1). Further discussion of the mucopolysaccharidoses and sphingolipidoses will follow in sections 1.2 and 1.3. Early detection of these disorders is essential for effective therapeutic intervention, as LSDs generally manifest early and can have a rapid clinical progression. Newborn screening programs are in development to provide the opportunity for earliest intervention, in the hope of normalising the outcome of those affected, before irreversible damage has occurred (Meikle et al. 2004, 2006, Fletcher 2006).

1.2 Mucopolysaccharidoses – General

The mucopolysaccharidoses (MPS) are a family of heritable lysosomal storage disorders caused by the deficiency of enzymes responsible for the degradation of complex carbohydrates (Neufeld and Muenzer 2001). They are all inherited in an autosomal recessive manner, with the exception of MPS II, which is X-linked. The complex carbohydrate substrates are composed of repeating disaccharide units [acidic sugar-amino sugar]ₙ and form five different glycosaminoglycan (gag) structures: chondroitin sulphate (CS), heparan sulphate (HS), dermatan sulphate (DS), keratan sulphate (KS) and hyaluronan (HA). The sequence of acidic and amino sugars, along with the sulphation and acetylation patterns on these sugars gives rise to these different carbohydrate structures (Hardingham and Fosang 1992). The functions of glycosaminoglycans are to form essential complexes of negatively charged heteropolysaccharide chains that are able to
Table 1.1: Lysosomal Storage Disorders

<table>
<thead>
<tr>
<th>Group</th>
<th>Stored substrates</th>
<th>Deficiency</th>
<th>Number of disorders</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sphingolipids</td>
<td>Hydrolases, activators</td>
<td>11</td>
<td>GM1 gangliosidosis, GM2 gangliosidosis (Tay-Sachs, Sandhoff), Fabry disease, Gaucher disease (types I, II and III), Krabbe disease, Niemann-Pick disease A and B</td>
</tr>
<tr>
<td>B</td>
<td>Mucopolysaccharides/ Glycosaminoglycans</td>
<td>Hydrolases, N-Acetyltransferase</td>
<td>11</td>
<td>See Table 1.2</td>
</tr>
<tr>
<td>C</td>
<td>Glycogen</td>
<td>Hydrolase</td>
<td>2</td>
<td>Pompe disease, Glycogen storage disease type IIB</td>
</tr>
<tr>
<td>D</td>
<td>Oligosaccharides/glycopeptides</td>
<td>Hydrolases</td>
<td>7</td>
<td>Mannosidosis, Fucoidosis, Schindler disease, Sialidosis, Aspartylglucosaminuria</td>
</tr>
<tr>
<td>E</td>
<td>Multiple</td>
<td>Multiple enzymes</td>
<td>3</td>
<td>Mucolipidosis II, III, I-cell disease, Pseudo-Hurler dystrophy, Multiple sulphatase deficiency, Galactosialidosis I, II</td>
</tr>
<tr>
<td>F</td>
<td>Lipid/cholesterol</td>
<td></td>
<td>2</td>
<td>Wolman disease, Niemann-Pick type C</td>
</tr>
<tr>
<td>G</td>
<td>Monosaccharide/amino acids/ monomers</td>
<td>Transporters</td>
<td>3</td>
<td>Salla disease, ISSD (severe), Cystinosis</td>
</tr>
<tr>
<td>H</td>
<td>Peptides</td>
<td>Proteases</td>
<td>1</td>
<td>Pycnodysostosis</td>
</tr>
<tr>
<td>I</td>
<td>S-acylated protein</td>
<td>Thioesterase</td>
<td>1</td>
<td>Batten disease</td>
</tr>
</tbody>
</table>
bind large amounts of water to form a gel-like matrix. Further discussion on the synthesis and functions of glycosaminoglycans will follow in section 1.6.

The outcome of a lysosomal enzyme deficiency is the incomplete breakdown of glycosaminoglycan chains, causing lysosomal accumulation of undegraded or partially degraded HS, DS, CS, HA and KS in the lysosome. This accumulation leads to cell, tissue and organ dysfunction. There are 11 known enzyme deficiencies giving rise to 7 distinct MPS disorders (Neufeld and Muenzer 2001) (Table 1.2).

The degradation of heparan sulphate-containing proteoglycans is the best characterised. Previous studies have shown that heparan sulphate proteoglycans (HSPGgs) are retrieved via endocytosis from the cell surface (Yanagishita and Hascall 1992). Glycosylphosphatidylinositol (GPI)-anchored HSPGs are endocytosed through a receptor-mediated mechanism where they are transported to the lysosome for degradation (Yanagishita and Hascall 1992). This occurs via heparan sulphate-specific endoglycosidases known as heparanases that can cleave the glycosidic bond of the heparan sulphate gag chain. Additionally, protein core degradation by proteases occurs in the endosomal compartment and gag fragments are sent to the lysosome. Lysosomal storage disorders, characterised by the type and amount of undegraded storage material, (Neufeld and Muenzer 2001) result from a deficiency in any one of the degradative enzymes. For heparan sulphate a complex combination of sulphatases and exohydrolases are needed for total degradation of the heparan sulphate gag chain. An example of specific heparan sulphate degradation and the LSDs that result from that deficient enzyme is shown in (Figure 1.1). As is apparent, the degradation of heparan sulphate chains within the lysosome is a highly regulated process and an enzyme deficiency in this pathway results in
Table 1.2: Classification of the Mucopolysaccharidoses
Data adapted from (Neufeld and Muenzer 2001)

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
<th>Data Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Hurler Syndrome</td>
<td>Neufeld and Muenzer 2001</td>
</tr>
<tr>
<td>Type II</td>
<td>Hunter Syndrome</td>
<td>Neufeld and Muenzer 2001</td>
</tr>
<tr>
<td>Type III</td>
<td>Sanfilippo Syndrome</td>
<td>Neufeld and Muenzer 2001</td>
</tr>
<tr>
<td>Type IV</td>
<td>Morquio Syndrome</td>
<td>Neufeld and Muenzer 2001</td>
</tr>
</tbody>
</table>

NOTE: This table is included in the print copy of the thesis held in the University of Adelaide Library.
Figure 1.1: Degradation pathway of heparan sulphate.

A diagrammatic representation of the stepwise degradation of a hypothetical HS oligosaccharide in the lysosome by nine exoenzymes. The disorder that results from a particular enzyme deficiency is labelled adjacent to the structure. Adapted from Neufeld and Muenzer, 2001.
Figure 1.1: Degradation pathway of heparan sulphate.

NOTE: This figure is included in the print copy of the thesis held in the University of Adelaide Library.
an inability to break down these substrates, leading to mucopolysaccharide storage and an associated clinical disorder.

A similar concert of enzymes is required for degradation of other gags such as CS, DS and KS and deficiencies in these enzymes also result in lysosomal storage disorders, including the MPS disorders (Table 1.3).

**Clinical presentation:**

For each MPS disorder a wide range of clinical severity is observed ranging from mild to severe. In general, severity depends on the amount of gag accumulated (Neufeld and Muenzer 2001) and thus correlates to residual enzyme activity. Severely affected patients generally have no residual enzyme activity and a large amount of gag accumulated. Mild clinical disease appears to be associated with the presence of some residual enzyme activity, allowing partial turnover of gag and later presentation. Studies in MPS I patients have shown a genotype/phenotype correlation in pathology (Bunge et al. 1998, Fuller et al. 2005) with severely affected patients having a small amount of enzyme with rapidly progressing pathology and patients with more enzyme activity having an attenuated clinical disease progression.

The MPS disorders share some common clinical features, including enlargement of the liver and spleen, bone and joint abnormalities, coarse facial appearance as well as impairments in hearing, vision (corneal clouding), respiration, sleep, heart disease and central nervous system deterioration (Neufeld and Muenzer 2001). In general, patients with a deficiency in an enzyme required for dermatan or keratan sulphate degradation are likely to suffer from bone, joint and other somatic symptoms, whereas patients with a deficiency in an enzyme required for heparan sulphate degradation present with primarily central
<table>
<thead>
<tr>
<th>Gag affected</th>
<th>Enzyme name</th>
<th>Type of enzyme</th>
<th>Resulting disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatan sulphate</td>
<td>iduronate-2-sulphatase</td>
<td>sulphatase</td>
<td>MPS II</td>
</tr>
<tr>
<td></td>
<td>α-L-iduronidase</td>
<td>glycosidase</td>
<td>MPS I</td>
</tr>
<tr>
<td></td>
<td>N-acetylglactosamine-4-</td>
<td>sulphatase</td>
<td>MPS VI</td>
</tr>
<tr>
<td></td>
<td>sulphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-hexosaminidase A, B, S</td>
<td>glycosidase</td>
<td>Sandhoff</td>
</tr>
<tr>
<td></td>
<td>β-glucuronidase</td>
<td>glycosidase</td>
<td>MPS VII</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>galactose-6-sulphatase</td>
<td>sulphatase</td>
<td>MPS IV</td>
</tr>
<tr>
<td></td>
<td>β-galactosidase</td>
<td>glycosidase</td>
<td>MPS IVB</td>
</tr>
<tr>
<td></td>
<td>N-acetylglucosamine-6-</td>
<td>sulphatase</td>
<td>MPS IIIID</td>
</tr>
<tr>
<td></td>
<td>sulphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-hexosaminidase A, B</td>
<td>glycosidase</td>
<td>Sandhoff</td>
</tr>
<tr>
<td></td>
<td>β-hexosaminidase A</td>
<td>glycosidase</td>
<td>Sandhoff</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tay-Sachs</td>
</tr>
<tr>
<td>Chondroitin sulphate</td>
<td>β-glucuronidase</td>
<td>glycosidase</td>
<td>MPS VII</td>
</tr>
<tr>
<td></td>
<td>N-acetylglactosamine-6-</td>
<td>sulphatase</td>
<td>MPS IV</td>
</tr>
<tr>
<td></td>
<td>sulphatase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
nervous system deterioration. Deficiency in enzymes that degrade both types of gags lead to a phenotype with both sets of symptoms.

1.2.1 Mucopolysaccharidosis type III

Mucopolysaccharidosis type III (MPS III or Sanfilippo syndrome) is the most common mucopolysaccharidosis disorder, with an Australian incidence of 1 in 66,000 live births (Meikle et al. 1999). MPS III is an autosomal recessive disorder caused by impaired heparan sulphate metabolism. Patients with MPS III make up a biochemically diverse, but phenotypically similar group, which is classified into four subtypes based on individual enzyme deficiencies (Neufeld and Muenzer 2001). Heparan N-sulphatase (sulphamidase) is the enzyme deficient in type A, α-N-Acetyl-glucosaminidase in type B, acetyl-CoA: α-glucosaminide acetyltransferase in type C and N-Acetylglucosamine 6-sulphatase in type D. All of these enzymes are essential for the complete degradation of heparan sulphate. Incomplete degradation of heparan sulphate leads to accumulation of undegraded or partially degraded heparan sulphate in the lysosome and abnormal excretion of heparan sulphate in the urine (Neufeld and Muenzer 2001).

Clinically, MPS III patients are characterised by severe central nervous system degeneration, but only mild somatic disease. The onset of clinical symptoms usually occurs between 2 and 6 years. Children often appear hyperactive and aggressive; have delayed development, coarse hair, and hirsutism, sleep disorders and mild enlargement of the liver and spleen. Neurologic degeneration is normally evident between 6 and 10 years of age, and loss of skills such as toileting and speech may be a presenting sign with social and adaptive skills affected. Unlike other MPS disorders, somatic involvement is minimal, with normal stature but disproportionate neurological involvement.
Specifically, MPS IIIA patients have 77 characterised mutations in the sulphamidase gene. These comprise 1 splice site, 9 small deletions, 7 small insertions and 60 different missense/nonsense mutations (Figure 1.2) (Yogalingam and Hopwood 2001). This is important since most missense/nonsense mutations result in various levels of residual enzyme activity, which is necessary to stimulate the turnover of gags in MPS disorders. Residual enzyme activity is needed for substrate deprivation therapy to be a viable therapeutic option.

In patients with MPS III and other MPS in which HS accumulates (MPS I, MPS II and MPS VII), secondary glycolipid storage is observed in addition to the accumulation of primary glycosaminoglycans (Figure 1.3A) (Constantopoulos et al. 1976, 1978 Constantopoulos and Dekaban 1978, Jones et al. 1998). This glycolipid is termed ganglioside and types $G_{M2}$ and $G_{M3}$ are accumulated in the brain and stored as “zebra bodies” (Figure 1.3B). It is proposed that the primary storage causes a secondary inhibition of glycolipid biosynthetic enzymes such as neuraminidases (Baumkotter and Cantz 1983), which are thought to correlate to brain pathology (Constantopoulos and Dekaban 1978, Constantopoulos et al. 1978).

### 1.3 Glycosphingolipid storage disorders (GSLs)

Like MPS, the GSLs are another group of lysosomal storage disorders. However, they differ in that the primary stored product is of glycolipid origin. Most GSLs have a common core structure composed of glucose or galactose sugars sequentially added to a hydrophobic lipid ceramide moiety, which is synthesised in the Golgi (van Echten and Sandhoff 1993, Ichikawa and Hirabayashi 1998). The GSLs with a glucose sugar, known as glucosylceramides, are widely distributed throughout the body, whereas those composed of galactose sugars, galactosylceramides, or the sulphated form, sulphatide, are localised to
Figure 1.2: Characterised mutations in the sulphamidase gene

The human sulphamidase gene has been characterised as 503 amino acids in length with 77 known mutation sites causing MPS IIIA. Yellow dots represent missense mutations and green dots represent nonsense mutations which make up 60 known mutations. Blue triangles represent small insertions (7) and red triangles represent small deletions (9). Data taken from http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SGSH and (Yogalingam and Hopwood 2001).
Figure 1.3: Electron microscopy of lysosomal inclusions in the MPS IIIA mouse CNS.

A perineuronal glial cell showing characteristic vesiculated cytoplasm (A, arrows) (4680x). Electron micrograph showing typical storage in a cortical neuron. Lysosomal inclusions characteristic of secondary ganglioside storage seen as stacks of membranes known as ‘zebra bodies’ (B, arrows) (7920x). Figure taken from (Bhaumik et al 1999). N indicates nucleus of the cell.
the central nervous system, where their role is in the formation and stability of myelin (Coetzee et al. 1998). A subset of the GSLs are gangliosides, which contain one or more sialic acid residues, and are found ubiquitously throughout the body, with a particular concentration around the surfaces of central nervous system cells. These gangliosides are thought to be secondarily stored in a number of mucopolysaccharide disorders where they contribute to the disease manifestations (Baumkotter and Cantz 1983, Lloyd and Furukawa 1998, McGlynn et al. 2004). Like glycosaminoglycans, glycolipids are part of a tightly controlled metabolic pathway where they are recycled in the Golgi (Schapiro et al. 1998) and later trafficked to the lysosome for glycosylhydrolase degradation. As expected, deficiencies in degradative enzymes lead to disorders known as GSL lysosomal storage disorders.

Because of the GSLs widespread expression, particularly of gangliosides in the central nervous system, the majority of these disorders result in neurological deficits. However, each individual disease is characterised by the type and severity of storage in the lysosomes as for the other LSDs. Figure 1.4 describes some of the GSL storage disorders, all of which are inherited in an autosomal recessive manner, with the exception of Fabry, which is X-linked. These disorders have a combined frequency of 1:18 000 live births (Meikle et al. 1999). All of the disorders mentioned in this figure result in the storage of glucosylceramide, whereas Krabbe and metachromatic leukodystrophy (MLD) are galactosylceramide storage disorders (Krivit et al. 1995).

Glycolipid disorders are also under investigation for therapies such as enzyme replacement, gene therapy, cell therapy and substrate deprivation therapy. Since all of the substrate deprivation therapy studies have been conducted in models of glycolipid storage disorders these will be discussed in more detail in section 1.5.5.
Figure 1.4: Glycolipid metabolism and resulting storage disorders.

The enzyme deficiencies are denoted by the rectangular boxes and the glycosphingolipid storage disorders are indicated in rounded boxes. Figure adapted from (Jeyakumar et al. 2002).
1.4 Animal models of Mucopolysaccharidosis

Animal models have provided invaluable information on the natural progression of disease pathology at a biochemical level and for the evaluation of new therapies for the MPS disorders. To date, all of the MPS disorders apart from MPS IIIC and MPS IX have corresponding animal models (Haskins et al. 2002). A large number of these result from naturally occurring mutations, including the MPS I dog and cat, MPS II dog and cat, MPS IIIA dogs and mouse, MPS IIIB dog and emu, MPS IIID goat, MPS VI dog, cat and rat and MPS VII mouse, cat and dog. (Table 1.4) The application of recombinant techniques has allowed the development of knockout mice for MPS I, MPS II, MPS IIIB, MPS VI and MPS VII (Table 1.4). The biochemistry and pathology of animal models are very close to their human counterparts, however in some disorders the clinical manifestations may develop at a slower pace (Neufeld and Muenzer 2001). The advantages of using rodent models in particular, is that sufficient numbers of animals can generally be obtained with ease, enabling the development of well controlled experiments using normal, carrier and affected animals (Suzuki and Mansson 1998). The development of larger animal models such as the MPS VI cat, MPS IIID goat, MPS I, II, and IIIA dogs are particularly useful in the study of slower developing pathology such as skeletal abnormalities. Generally, when a small animal model is present, a number of different therapeutic strategies are initiated to investigate the progression of disease and effect of the therapy, both physically, mentally and behaviourally before a larger animal model study is initiated, and finally progressing to human clinical trials.

1.4.1 MPS IIIA mouse model

MPS IIIA is caused by a deficiency of the lysosomal enzyme heparan N-sulphatase (sulphamidase) resulting in the accumulation of the glycosaminoglycan heparan sulphate in lysosomes. The MPS IIIA mouse model is a naturally occurring homologue of the human
Table 1.4: Animal models of Mucopolysaccharidosis.
Data adapted from (Neufeld and Muenzer 2001)

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Mucopolysaccharidosis Type</th>
</tr>
</thead>
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NOTE: This table is included in the print copy of the thesis held in the University of Adelaide Library.
disease which displays the same biochemical and clinical changes observed in human MPS IIIA patients (Bhaumik et al. 1999). MPS IIIA mice are outwardly characterised by their scruffy, unkempt looking fur, hunched posture, and abdominal distension caused by distension of the bladder in end stage disease (Bhaumik et al. 1999, Roberts et al. 2006) (Figure 1.5B). Upon death they display an enlarged liver and spleen and contain 1-1.5 mls turbid urine in their bladders depending on the age of the mice. Initial reports stated that the life expectancy of these mice is 7-10 months (Bhaumik et al. 1999). Under microscopic examination, MPS IIIA affected mice show extensive vacuolation in a variety of cell types in all tissues. Neurons within the cerebral and cerebellum cortices and other brain areas exhibited cytoplasmic distension. In addition to storage in the brain, the liver and kidneys respectively show storage in Kupffer cells of the hepatic sinusoid and the cortical regions of the kidney, in particular the distal convoluted tubules (Bhaumik et al. 1999).

On a biochemical level, the mutation in murine MPS IIIA has been characterised as a point mutation (G to A) at nucleotide position 91. This mutation results in an amino acid change from aspartic acid to asparagine at amino acid position 31 (D31N). A PCR based diagnostic test can be used to identify wildtype, heterozygote and affected littermates by the lack of the MspAII restriction site (Bhattacharyya et al. 2001). Homozygotes are fertile and able to breed 100% affected offspring. This mouse model is a good homologue of the human disease and provides a vital mechanism to investigate potential therapies for MPS.

1.5 Therapies for LSDs

LSDs are good targets for therapy and they should be easy to treat, since they are monogenic disorders and only require a small amount of enzyme to be produced in order to correct the defect. Essentially there are two approaches for therapies in the MPS disorders: one relies on increasing the amount of enzyme and the other focuses on reducing the
Figure 1.5: MPS IIIA mouse phenotype

Comparison of a normal mouse (top) and a MPS IIIA untreated littermate (bottom) at 9 months of age.
amount of stored substrate. Examples of the former are enzyme replacement therapy, gene/cell therapy and bone marrow transplantation and an example of the latter is substrate deprivation therapy. A number of different enzyme replacement therapies have been trialled and are in clinical practice for some LSDs (Kakkis et al. 2001, Muenzer et al. 2002, Harmatz et al. 2004) (Figure 1.6), however others remain in clinical trial. This figure also shows the relative proportion of each LSD and their current therapeutic status, such as BMT in the clinic. Even though therapy is available for some LSDs, a number of diseases are yet to have a suitable therapy (Figure 1.6).

1.5.1 Enzyme Replacement Therapy

Enzyme replacement therapy was first suggested three decades ago, after the realisation that storage in lysosomes is caused by a deficiency of lysosomal degradative enzymes (Hers 1963, De Duve 1964). However, early attempts at enzyme replacement failed due to a poor understanding of mannose-6-phosphate receptor mediated endocytosis and the inability to produce highly purified enzymes (Neufeld and Muenzer 2001). Currently, recombinant enzymes for the majority of MPS disorders, are expressed in cell culture systems (Oshima et al. 1987, Anson et al. 1992, Bielicki et al. 1993, 1995, 1996, Gehrmann et al. 1994, Kakkis et al. 1994, Unger et al. 1994, Weber et al. 1996, Litjens et al. 1997). These enzymes are secreted into the culture medium where they can be purified and used for therapeutic applications.

The first controlled studies of enzyme replacement therapy occurred in animal models. MPS VII mice have been treated with recombinant β-glucuronidase (Vogler et al. 1993, Sands et al. 1994), MPS I dogs with α-L-iduronidase (Shull et al. 1994, Kakkis et al. 1996), MPS II mice with iduronate-2-sulphatase (Muenzer et al. 2002), MPS VI cats with arylsulphatase B (N-acetyl-galactosamine-4-sulphatase) (Crawley et al. 1996, 1997, Byers
Figure 1.6: Incidence for LSD and their current treatment options.

Pie slices represent the relative prevalence of each disorder. Red shading represents disorders for which enzyme replacement or drug therapies currently exist for non-neuropathic patients. Green shading represents disorders for which ERT is undergoing or planning clinical trials for non-neuropathic patients. Purple shading represents disorders for which bone marrow transplantation is an option. All other disorders are represented by yellow shading. Figure adapted from Meikle and Hopwood, 2003.
et al. 1997) and MPS IIA mice with sulphamidase (Gliddon and Hopwood 2004). In all of these animal studies, there was a rapid clearance of the enzyme from circulation and histological analysis showed reduction of lysosomal storage vacuoles in most tissues, except cartilage, cornea and brain. Studies in mice have shown that administration of recombinant enzyme in the neonatal period, when the blood-brain barrier is relative permeable, has a positive outcome on brain pathology (O'Connor et al. 1998, Gliddon and Hopwood 2004); however the cartilage and cornea are not affected by neonatal administration (Crawley et al. 1997). In the human brain, ERT given in the neonatal period is unlikely to have the same outcome due to the more complete blood-brain barrier at birth (Urayama et al. 2004). A recent study showed that the the blood-brain barrier can be overcome using high dose recombinant enzyme which was able to penetrate into the brain and clear lysosomal storage (Vogler et al. 2005).

In humans, the first LSD to be successfully treated using enzyme replacement therapy was Gaucher disease, a disorder of sphingolipid degradation (Barton et al. 1991). β-glucocerebrosidase was initially purified from placental tissue and later recombinant enzyme was produced from Chinese hamster ovary (CHO) cells (Grabowski et al. 1995). Efficacy was improved by modification of the native enzyme to expose mannose residues, essential for targeting the enzyme to the lysosome in macrophages (Neufeld and Muenzer 2001). Since the early 1990s, over 3500 Gaucher disease patients have been treated using this strategy and it is considered a clinical success (Barranger and O'Rourke 2001). Another example of enzyme replacement is in the treatment of Fabry disease. Similar to Gaucher disease, this is also a disorder of sphingolipid degradation, resulting from the deficiency of α-galactosidase A. Three clinical trials using recombinant human α-galactosidase A have been initiated and showed a decrease in neuropathic pain,
stabilisation of renal function and improved quality of life (Brady et al. 2001, Desnick et al. 2002, Pastores and Thadhani 2002) which led to ERT being implemented for clinical use in Fabry disease (Eng et al. 2001a, 2001b, Pastores and Thadhani 2001, Schiffmann et al. 2001). ERT for another LSD Pompe disease, with a deficiency in α-glucosidase, is also under investigation (Van den Hout et al. 2001) and was recently approved for clinical use\(^1\).

MPS I (ERT approved for clinical use for patients in USA and Europe in 2003\(^2\)) (Kakkis et al. 2001), MPS II (ERT approved by the FDA in July 2006) (Muenzer et al. 2002) and MPS VI (ERT approved for clinical use in 2005) (Harmatz et al. 2004) are the only MPS disorders currently treated by ERT using recombinant α-L-iduronidase, iduronate-2-sulphatase and arylsulphatase B (N-acetyl-galactosamine-4-sulphatase) respectively (Figure 1.6). ERT clinical trials for MPS II (neurological forms) and MPS IVA\(^3\) are planned in the future (Figure 1.6). Since these disorders display primarily somatic pathology, the enzyme is able to access the target sites via the circulation and improve the clinical symptoms.

**Disadvantages of ERT**

Since intravenously administered enzyme relies on the circulation to reach its target sites for the therapeutic effect, it is expected that most tissues and organs with adequate blood supply are likely to benefit from enzyme replacement therapy, however symptoms such as macrocephaly, hydrocephalus, coarse facial features, mental retardation, bone deformities, and corneal clouding are not ameliorated (Brady 2006). For the cartilage and cornea it is the significant lack of vascularisation around these sites that limits the effects of even high dose enzyme replacement regimes (Crawley et al. 1997, Byers et al. 2000). Furthermore,

\(^1\) http://www.myozyme.com  
\(^2\) http://www.biomarinpharm.com/products-aldurazyme.shtml  
\(^3\) http://www.slu.edu/readstory/more/7115
the brain is protected by the endothelial blood-brain barrier, (reviewed in Bradbury 1993). This acts like a molecular sieve in order to prevent the brain from being exposed to large molecules and proteins that may cause irreversible damage. Since most recombinant enzymes are of high molecular weight and there are no specific receptors for lysosomal enzyme uptake on the blood-brain barrier, its presence prevents effective ERT on brain pathology.

Some studies have shown that administration of enzyme in mice from birth leads to a degree of correction of pathology, since the blood-brain barrier remains open for the first two weeks of post-natal life (O’Connor et al. 1998, Gliddon and Hopwood 2004). However, in humans the blood-brain barrier is permanently closed at birth, i.e. it has matured, and does not possess a transport system for phosphorylated lysosomal enzymes through the mannose-6-phosphate/IGF2 receptor seen in the immature form in neonates (Urayama et al. 2004). This is the major obstacle in the treatment of LSDs with CNS pathology. There are a number of research efforts which aim to address crossing the BBB in adult mice by means of cationic proteins and fusion proteins targeted to the brain in the hope of being able to administer enzyme to the brain (Triguero et al. 1989, Poduslo and Curran 1996a, 1996b, Wengenack et al. 1997). Other methods such as mannitol treatment have been used to gain temporary access to the brain (Ghodsi et al. 1999, Fu et al. 2003), however the application of this method is unlikely to be acceptable if translated to human therapy. A recent study showed that the the blood-brain barrier can be overcome using high dose recombinant enzyme which was able to penetrate into the brain and clear lysosomal storage (Vogler et al. 2005).
1.5.1.1 Localised therapy for “hard-to-reach” tissues

To circumvent the problem of targeting “hard to reach” tissues such as the brain and cartilage, local injections of recombinant enzyme have been used. This next section describes intra-articular joint ERT in a cat model of MPS VI. Direct injection into the cerebrospinal fluid (intra-thecal ERT) has also been shown to have therapeutic benefit in MPS I dogs.

1.5.1.1.1 Intra-articular ERT

Although MPS VI enzyme replacement therapy clinical trials have shown improvement in a number of symptoms, joints remain refractory to systemic treatment. Intra-articular ERT studies in MPS VI cats have shown an improvement in cat joint mobility and also a reduction in lysosomal storage in chondrocytes (Auclair et al. 2006). Therefore, this mechanism for delivering enzyme directly to the articular cartilage is a potential therapy for joint pathology in MPS VI.

1.5.1.1.2 Intra-thecal ERT

Intra-thecal therapy has been proposed as a way to expose the brain to replacement enzyme. This type of therapy, although invasive, is currently approved for some cancer patients who have their chemotherapy drugs administered via this route (Grabow et al. 2001, Rainov and Kramm 2001). Intra-thecal ERT relies on injecting recombinant enzyme into the cisterna magna of the patient, surrounding the brain with concentrated enzyme. Studies in MPS I dogs have shown that this type of therapy provides a good therapeutic outcome and clearance of lysosomal storage in the brain (Kakkis et al. 2004) due to penetration of the CNS with recombinant enzyme. Human studies are currently being undertaken to assess the potential of using this type of delivery system in MPS patients
with spinal cord compression and CNS storage\textsuperscript{4}. Recently administration of sulphamidase via intra-thecal injection in MPS IIIA mice led to a reduction in the level of heparan sulphate disaccharide in the brain, measured by mass spectrometry and microscopic examinations. This study also reported improved behavioural outcomes as measured by open field exploration (Hemsley \emph{et al.} 2006).

\textbf{1.5.1.2 Other problems with ERT}

Another drawback of ERT is the development of an immune response to the replacement protein in both human (Pastores \emph{et al.} 1993, Brady \emph{et al.} 1997, Ponce \emph{et al.} 1997, Brooks 1999, Rosenberg \emph{et al.} 1999, Kakkis \emph{et al.} 2001) and animal trials (Brooks \emph{et al.} 1997, 1998, 1999, Turner \emph{et al.} 1999, 2000). It should be noted that some of these studies were conducted using human enzyme in animal models; therefore an immune response to a protein of foreign origin would be expected. Any reaction could lead to the onset of a hypersensitivity reaction during or following enzyme administration which has been observed in some MPS I dogs with both human and canine enzyme (Kakkis \emph{et al.} 1996), MPS VI cats with human enzyme (Crawley \emph{et al.} 1996, 1997) and human Gaucher patients (Pastores \emph{et al.} 1993, Ponce \emph{et al.} 1997). If an immune response develops, it is generally controlled by co-administration of antihistamine. The significance of the immune response should also take into consideration the nature of the protein. LSD patients who have no residual enzyme activity, due to a deletion or truncation of the protein are likely to have a more severe response to the administered enzyme since there would have been no previous exposure to the enzyme and the immune system would detect it as a foreign protein (Brooks 1999). Further genotype/phenotype correlations and characterisation of the reactive areas of the protein, known as epitopes, are needed to predict which patients are likely to have an immune response. Action to prevent these immune responses may require

\textsuperscript{4}http://www.clinicaltrials.gov/ct/show/NCT00215527?order=1
the co-administration of immunosuppressants with ERT or treatment with high-dose enzyme at an early age before the immune system matures (Brooks 1999) to induce tolerance.

The second effect of an immune response is the interference of the circulating antibodies produced on the replacement protein (Kakavanos et al. 2003). During the long-term ERT clinical trial for type I Gaucher patients, two patients developed neutralising antibodies to glucocerebrosidase which developed at the same time as a plateau in therapeutic effect and disease progression (Brady et al. 1997, Ponce et al. 1997), however they continued with therapy. It should be noted that only two patients are recorded as developing neutralising antibodies out of thousands of Gaucher patients treated to date with ERT. This means there may be no serious therapeutic implications of antibody response in ERT patients as was first thought when this type of treatment regime commenced.

Another concern is the very high cost of enzyme replacement therapy for patients. Successful treatment relies on highly purified recombinant enzymes for administration to the patients. Cost estimates for ERT in Fabry patients is between AUD $200,000 and AUD$400,000 per patient per year\(^5\) (Sawkar et al. 2002) with similar expenditure expected to be associated per annum for ERT in other LSDs. This, along with the fact that enzyme has to be infused on a weekly basis, usually in a hospital setting, turns the focus to alternative treatment strategies such as gene/cell therapy and substrate deprivation therapy.

### 1.5.2 Bone Marrow Transplantation

Allogeneic bone marrow transplantation (BMT) increases the amount of enzyme by transplanting bone marrow cells from a closely matched donor to the affected patient.

Normal donor cells will secrete enzyme into the circulation. This approach to therapy has been limited by factors including the type of MPS, presence of CNS pathology, donor availability, age of the patient and severity of disease at the time of transplantation (Neufeld and Muenzer 2001).

Bone marrow transplantation studies in MPS I dogs have shown a reduction in intraneuronal storage with low levels of \( \alpha \)-L-iduronidase. Increased lifespan in this model is thought to be attributed to reduced musculoskeletal disease (Shull et al. 1982, 1987, 1988, Sabatini and Adesnick 2001). Similarly in the MPS VI cat improvements were noted in the liver, cardiovascular and skeletal system (Gasper et al. 1984, Wenger et al. 1986, Norrdin et al. 1995). Bone marrow transplantation in the MPS VII mouse led to increased longevity and correction of metabolic defects in most cells with the exception of the CNS and bone disease (Birkenmeier et al. 1991). Recent evidence has shown that neonatal transplants were more effective (Sands et al. 1993).

The major drawbacks of bone marrow transplantation are the high risk of morbidity and mortality associated with this procedure, the need for early treatment, and the incidence of graft-versus host disease (Neufeld and Muenzer 2001). Unfortunately, only MPS I and VI patients transplanted before the age of 2 have been shown to benefit from BMT. BMT is not a recommended therapy option for most other MPS disorders, particularly MPS II and MPS III (Resnick et al. 1992, Hoogerbrugge et al. 1995, Sivakumur and Wraith 1999, Vellodi et al. 1999) for reasons which are not yet understood.

### 1.5.3 Gene Therapy

Since LSDs are monogenic disorders, the use of gene therapy to supply enzyme to correct the lysosomal dysfunction is under trial. A number of different approaches are being
investigated. These include transduction of cells outside the body via *ex vivo* methods and *in vivo* methods using either adenoviral or retroviral-mediated correction. Cell therapy also has the potential to treat these disorders by supplying enzyme, leading to correction in the cell; however a lack of understanding of biological systems has prevented this therapy from being realised.

### 1.5.3.1 *Ex vivo* gene therapy

The *ex vivo* approach of gene and cell therapy has been successfully used in a number of animal studies. For example, in the MPS VII mouse, β-glucuronidase-deficient fibroblasts were transduced with the human β-glucuronidase gene (Moullier *et al.* 1993, Sly 1993) and implanted back into the mouse peritoneum. These ‘neo-organs’ showed enzyme expression and correction of storage in the liver and spleen (Moullier *et al.* 1993). This idea has more recently been utilised in the MPS II knockout mouse model (Friso *et al.* 2005) to improve the pathology outcome. Briefly, cells overexpressing iduronate-2-sulphatase were encapsulated to form particles, implanted in MPS II mice, which led to increased enzyme activity in liver, kidney and spleen and cleared lysosomal storage in these organs (Friso *et al.* 2005). No investigation of lysosomal correction in the central nervous system was reported, with a focus on somatic organs and their correction. Studies in MPS VII knockout mice involving transplantation of retrovirally-transduced cells secreting β-glucuronidase into the brain showed clearance of lysosomal distension from neurons and glial cells in the vicinity of the grafts and the ability to correct brain lesions (Taylor and Wolfe 1997).

In a larger animal model, gene therapy has been performed in the MPS VI cat (Yogalingam *et al.* 1999) where N-acetylgalactosamine-4-sulphatase (4S) was overexpressed in fibroblasts and implanted under the renal capsule of MPS VI kittens at 8-16 weeks of age.
Shortly after implantation, low level 4S expression was detected in peripheral blood leukocytes; however, this expression was not detected at longer time periods after implantation (Yogalingam et al. 1999). This demonstrates that this type of therapy is not likely to be effective in larger systems unless high concentrations of recombinant enzyme can be achieved and large numbers of transduced cells can be transplanted.

Early attempts using ex vivo gene therapy were conducted using retrovirally-transduced peripheral blood lymphocytes and were in clinical trial for Gaucher disease (Torchiana et al. 1998), MPS I (deficiency of α-L-iduronidase) and MPS II (deficiency of iduronate-2-sulphatase) (Braun et al. 1996). However, these trials failed because of inadequate targeted vector delivery mechanisms and only transient expression of the encoded gene (Eto and Ohashi 2002). This suggests that extensive investigation into a more efficient delivery system and mechanisms for long-term gene expression are needed for effective therapeutic outcomes.

1.5.3.2 In vivo gene therapy

In vivo gene therapy commonly relies on a virus to carry the cDNA for the enzyme of interest, to a number of different cell types. This results in integration of the gene of interest into the host genome and provides continual expression of the deficient enzyme. Two main virus types have been studied.

The first system is in retroviruses, and specifically lentiviruses (such as HIV-1), which are able to effectively transduce both dividing and nondividing cells and integrate into the host genome, thereby providing the possibility of long-term enzyme expression. Recombinant HIV-1 based lentiviral correction of lysosomal storage has been demonstrated in vitro in MPS I (Di Natale et al. 2002) MPS IIIA (Anson et al. 2007) and MPS IIIB (Villani et al. 2009).
2002) as well as in vivo for MPS I (Di Domenico et al. 2005, Kobayashi et al. 2005) and MPS VII (Bosch et al. 2000). In adult MPS VII mice, gene therapy for β-glucuronidase injected directly into the brain led to progressive accumulation of enzyme throughout the entire brain, and stable expression of the enzyme over a 16 week period (Bosch et al. 2000). Lysosomal inclusions in neurons, glia and perivascular cells, both proximal and distal from the injection site were reduced (Bosch et al. 2000). A drawback of lentiviral therapy is the potential for pathogenicity of the wild-type virus and the risk of insertional mutagenesis, however non-pathogenic and non-replicating vectors have been engineered to reduce this risk (Zufferey et al. 1998).

The second viral system is the adeno-associated virus (AAV), which can integrate into the host genome but may stay episomal until cell division (Anderson 1998, Miao et al. 2000) and can infect nondividing cells. Initial results proved promising with intramuscular injection of rAAV encoding the β-glucuronidase gene into MPS VII mouse neonates resulting in enzyme production and storage reduction in spleen and liver 16 weeks post-injection (Daly et al. 1999). Also, the administration of a single intravenous rAAV encoding the β-glucuronidase gene in newborns led to reduction of lysosomal inclusions and β-glucuronidase activity in many organs, including the brain, which persisted for longer than 4 months (Daly et al. 1999). In another study, MPS VII mice treated neonatally with systemic injections of rAAV, 12 months post-injection, treated mice showed increased survival, weight, bone length and improved retinal and auditory function compared to untreated MPS VII mice (Daly et al. 2001). However, another study showed that post-mortem examination after one year of treatment demonstrated the presence of hepatic tumours in treated MPS VII mice (Donsante et al. 2001). Since this is the only published long-term study of this type of therapy, this raises concerns about the safety of using virally-mediated systems as means of gene transfer. Recombinant AAVs have been
used for gene therapy in MPS I mice (Hartung et al. 2004), MPS IIIB mice (Cressant et al. 2004), MPS VI cats (Ho et al. 2002) and MPS VII mice (Sferra et al. 2004). Reports of long-term, high level expression of enzyme have been obtained using AAV expression systems. Recently, Sferra and colleagues showed that intrahepatic administration of an AAV vector for expression of β-glucuronidase in adult MPS VII mice led to stable hepatic expression for at least one year after injection. This expression of enzyme resulted in a reduction of lysosomal storage in both the somatic and central nervous systems (Sferra et al. 2004). However, each type of expression system is not without its drawbacks. AAV are small viruses, meaning they can only accommodate cDNA inserts of up to 4-5 kb and they also have the risk of insertional mutagenesis. Long-term studies using these sorts of systems need to demonstrate safety and efficacy before the success of gene therapy as a patient treatment can be realised.

Although gene therapy is expected to provide effective therapeutic benefits in the future, the need for improved viral delivery systems, regulation of gene expression (stable versus transient expression) and incidence of tumourigenesis from long-term expression must be addressed.

1.5.4 Cell therapy

Administration of stem cells should allow the correction of the enzyme deficiency through the production of a normal, fully functional enzyme. Although only a small amount of enzyme activity (1-10% of normal activity) is required to achieve a reduction in pathology (Cheng and Smith 2003), the ideal scenario would be to over-express the enzyme of interest using genetic modification strategies, enabling sufficient amounts of enzyme to be expressed by the donor cells (Zhou et al. 1995, Hahn et al. 1998).
Therapeutic examples of cell therapy in LSDs include the systemic infusion of mesenchymal stem cells into irradiated acid sphingomyelinase deficient mice which led to the production of donor-derived cells in non-haemopoietic tissues, including the brain (Pereira et al. 1998). Further studies in these mice have shown that these donor-derived cells are able to cross the blood-brain barrier and differentiate (Priller et al. 2001a, 2001b), and direct injection of these cells into the brain results in engraftment and migration of the donor-derived cells distant to the site of injection (Azizi et al. 1998, Kopen et al. 1999, Schwarz et al. 1999).

A recent study in newborn MPS VII mice showed that injection of foetal neural stem cells grown into neurospheres, allowed donor cells to migrate far beyond the site of injection within 24 hours, and some of them could reach the olfactory bulb (Fukuhara et al. 2006). Histological analysis showed decreased storage in hippocampal, cortical, and ependymal regions of the brains of treated mice and functional assessment tests showed improvement in behaviour patterns via an object recognition task (Fukuhara et al. 2006).

Similar results have been demonstrated using embryonic human neural progenitor cells which overexpress β-glucuronidase (Buchet et al. 2002), which when injected into the striatum of nude mice, showed long-term expression and differentiation into neurons and astrocytes. Specifically in MPS VII, transplanted neural progenitors expressing β-glucuronidase into the cerebral ventricles of affected mice led to engraftment and expression of β-glucuronidase throughout the brain, which resulted in widespread clearance of lysosomal storage (Snyder et al. 1995).
Cell therapy is a potential avenue for investigation in the MPS disorders; however, a greater understanding of stem cell biology and control of differentiation of these cells is needed before the potential of this type of therapy is recognised.

1.5.5 Substrate Deprivation Therapy (SDT)

Increasing the amount of deficient enzyme either by administering recombinant enzyme (ERT) or by increasing the amount of enzyme by gene and cell therapy or bone marrow transplantation has been discussed. Substrate deprivation therapy (SDT) aims to decrease the amount of stored material by administering small molecules that are capable of inhibiting the synthesis of the substrate, which can be glycolipid, glycoprotein or gag in origin (see Figure 1.7 for examples of inhibitors), resulting in the restoration of the balance between synthesis and degradation (Butters et al. 2003). This type of therapy relies on the presence of residual enzyme activity in order to effectively turn over the stored substrate. It was first suggested by Vunnam and Radin that analogues of ceramide inhibit glucocerebroside synthetase in the mouse brain (Vunnam and Radin 1980). Later, Gaucher disease was shown to be treated by enzyme inhibitors (Radin 1996). There is a correlation between residual enzyme and clinical pathology (Bunge et al. 1998), and by reducing the amount of substrate available, any remaining enzyme can metabolise the storage. In MPS disorders, this would rely on the reduction of glycosaminoglycan synthesis by inhibiting enzymes vital for the synthesis of these structures. The residual enzyme activity in the patient would therefore be more likely to turn over the stored substrate initially and hence reduce pathology. Substrate deprivation agents can be administered as oral drugs and therefore are likely to have better patient compliance, especially when treating children (Pawar and Kumar 2002).
Figure 1.7: Chemical structure of SDT molecules.

A = N-butyldexyronorjirimycin, B = N-butyldexyrgalactonorjirimycin, C = 4-deoxy-4-fluoro-2-acetamido-2-deoxy-D-glucose, D = 4-deoxy-4-fluoro-2-acetamido-2-deoxy-D-galactose, E = sodium chlorate, F = rhodamine B, G = genistein. Compounds A and B have been used as SDT agents in GSL disorders, whereas C and D have been shown to inhibit heparan sulphate synthesis in vitro. Compound E is an inhibitor of O-sulphation and E and F are general inhibitors of gag synthesis. Compound G is an isoflavone though to inhibit gag synthesis.
1.5.5.1 Substrate deprivation therapy for GSL disorders

1.5.5.1.1 N-butyldeoxynorjirimycin (NB-DNJ)

The effect of substrate deprivation was first demonstrated in an *in vitro* model of Gaucher disease. Administration of *N*-butyldeoxynorjirimycin (NB-DNJ, figure 1.7A), inhibited the primary conversion of ceramide to CMH glucosylceramide and therefore prevented lysosomal accumulation of glucosylceramide in cells (Figure 1.8) (van Echten and Sandhoff 1993, Platt *et al.* 1994a). Subsequent *in vivo* experiments showed a clear reduction in the amount of ganglioside storage in neurons of Tay-Sachs and Sandhoff mice (Platt *et al.* 1997a, 1997b, Jeyakumar *et al.* 1999). A combination of bone marrow transplantation and NB-DNJ treatment in Sandhoff mice showed that mice on the combination therapy survived significantly longer than those treated with either BMT (Norflus *et al.* 1998) or NB-DNJ alone (Jeyakumar *et al.* 2001). This may be important for patients with no residual enzyme activity, usually those with infantile-onset disease. Further studies using NB-DNJ in Fabry mice showed a partial prevention of endothelial dysfunction (Platt *et al.* 2003, Heare *et al.* 2006) seen in some Fabry patients (Moore *et al.* 2006).

NB-DNJ is currently used clinically in the glycolipid storage disorders where gangliosides are the primary storage product (Platt and Butters 1999). This small molecule has been registered since 2002 in Europe and since July 2003 in the USA for treatment of mild to moderate type I Gaucher patients who are unsuitable to receive enzyme replacement therapy (Aerts *et al.* 2006).

NB-DNJ, known also as Zavesca/miglustat/OGT 918, is used clinically for treatment of some non-neuropathic Gaucher disease (Cox *et al.* 2000, 2003, Butters *et al.* 2003). In the non-comparative phase I/II study, 28 type I Gaucher patients were enrolled, with 22
Figure 1.8: Glycosphingolipid (GSL) biosynthesis and SDT therapeutics.

Mechanism of action of substrate deprivation therapy agents and their interference in the synthesis pathway of glycosphingolipids. L-cycloserine inhibits the conversion of Palmitoyl-CoA to 3-ketosphinganine, whereas NB-DNJ and NB-DGJ inhibit the conversion of ceramide to CMH glucosylceramide. Agents and their site of inhibition are indicated in red. Figure adapted from Jeyakumar et al. 2002.
Figure 1.8: Glycosphingolipid (GSL) biosynthesis and SDT therapeutics.

NOTE: This figure is included in the print copy of the thesis held in the University of Adelaide Library.
patients completing the treatment regime for 12 months. These patients showed a mean reduction of liver and spleen volumes and mean increased haemoglobin concentration and platelet counts. Common side effects of the substrate deprivation therapy treatment included diarrhoea (89% of patients) and weight loss (50%) which subsided with time. These specific gastrointestinal problems led to the withdrawal of some patients from the trial, however these symptoms settled in patients who persisted with the therapy. Some patients developed peripheral neuropathy, but this was not assessed prior to commencement of the therapy. These promising results support the investigation of the substrate deprivation approach in other lysosomal storage disorders. Clinical trials are currently underway using Zavesca/miglustat for Niemann-Pick C, Tay-Sachs and Sandhoff patients with a clinical trial in infantile Tay-Sachs showing no effect on neurological deterioration (Bembi et al. 2006) and late-onset Tay Sachs showing no change in the progression of the disease after 24 months of treatment. Promising results have been shown in Niemann-Pick C patients after 12 months of miglustat treatment, which showed improvement in key features of the disease including saccadic eye movement, swallowing ability, auditory acuity and cognitive performance as measured by the Mini Mental Status Examination (Patterson et al. 2006). An ongoing trial in type III Gaucher disease (neurologic form) is in progress and miglustat levels in the brain are reported as 30-40% of those in the plasma, which represents significant CNS penetration (Patterson 2005). However the results at 12 months of treatment were inclusive.

6 http://ntsad.org/ S05/S05 research _init.htm
1.5.5.1.2 *N*-butyldeoxygalactonorjirimycin (NB-DGJ)

Like NB-DNJ, this compound is derived from the same family, but incorporates an *N*-alkylation around the galactose structure (Figure 1.7B). It was developed as a safer alternative to NB-DNJ, to avoid the inhibition of other glycosidases and glycosyl transferases seen with NB-DNJ (Winchester and Fleet 1992). The mechanism of action is the same as for NB-DNJ, being an inhibitor of the ceramide-specific glucosyltransferase (Figure 1.8) and thus inhibiting the glycosylation of many sphingolipid components (Butters *et al.* 2000). However, NB-DGJ has no effect on the maturation of *N*-linked oligosaccharides or on lysosomal glucocerebrosidase, meaning that it does not inhibit other lysosomal enzymes such as *α*-glucosidase I and II (Schweden *et al.* 1986, Tan *et al.* 1991, Jacob and Scudder 1994). *In vitro* testing in Gaucher disease showed that NB-DGJ is as effective as NB-DNJ at preventing glycolipid storage in the range of 5-50 μM (Platt *et al.* 1994b) and mice treated with either NB-DNJ or NB-DGJ showed equivalent distribution and degree of GSL depletion in the liver (Andersson *et al.* 2000). *In vivo* daily injections from post-natal day 2 to 5 in β-galactosidase-knockout mice treated with 600 mg/kg showed a reduction in total brain ganglioside content (Kasperzyk *et al.* 2004). When a high dose of 1200 mg/kg/day was administered a further reduction was seen in total brain ganglioside as well as a reduction of *Gm*₁ ganglioside in brain, with no side effects noted with either dose of treatment. This type of treatment may be effective in managing early-onset *Gm*₁ gangliosidosis and may also have an important role in other glycolipid and MPS disorders. However, the search for safer and more effective agents than those used in preliminary studies is ongoing.
1.5.5.1.3  **L-cycloserine**

L-cycloserine is an inhibitor of 3-ketohydrlosphingosine synthase, which catalyses an early step in the biosynthesis of psychosine and cerebrosides (Figure 1.8). When injected subcutaneously into normal mice, the levels of sphingolipids, including ceramide and sphingomyelin, in the brain were reduced (Sundaram and Lev 1984a, 1984b, 1989) and the galactosylceramide levels in myelin were reduced (Miller and Denisova 1998). In Twitcher mice, a model for Krabbe disease, L-cycloserine treatment prolonged the life-span and delayed the progression of clinical symptoms such as rapid weight loss and demyelination of axon fibres (LeVine *et al.* 2000). In mixed genetic background Twitcher mice, Biswas and colleagues showed that administration before the onset of pathology increased the lifespan and delayed the clinical disease progression but had no effect of pathology progression if administered after the disease onset (Biswas *et al.* 2003).

When L-cycloserine administration was combined with bone marrow transplantation at post-natal day 10 in Twitcher mice, this doubled the survival times for either bone marrow treatment or L-cycloserine treatment alone (Biswas and LeVine 2002), suggesting a synergistic effect. The combined-therapy mice also showed a decrease in lectin-positive globoid cells, and glial fibrillary associated protein (GFAP) staining; however treated mice still showed extensive demyelination. This supports findings from BMT treatment in pre-symptomatic Krabbe children which was effective, however patients still had problems related to demyelination (Escolar *et al.* 2005).

All studies to date using substrate deprivation therapy in glycolipid storage disorders have shown positive outcomes; albeit with some side-effects. Substrate deprivation therapy thus shows potential to treat a number of lysosomal storage disorders. The ability for oral administration is a major advantage over other therapies where more invasive methods are
needed. This is important especially when treating children, since orally administered 
drugs are likely to have better patient acceptance compared to regular infusions and 
transplants (Pawar and Kumar 2002). The application of this type of therapy to MPS 
disorders, although not reported to date, should be evaluated, particularly in those MPS 
disorders with secondary ganglioside storage.

1.5.5.2 Substrate Deprivation Therapy for MPS

All current studies for substrate deprivation therapy have been conducted in GSL models. 
Since positive results were seen for these disorders, glycosaminoglycan synthesis 
inhibitors are a logical next step. Inhibiting the formation of the primary storage product 
should delay the development of pathology in MPS disorders. In addition to this, the 
primary storage is thought to lead to development of secondary storage, in the form of 
glycolipid. Since the glycolipid is thought to cause the neurological deficits in some MPS 
disorders, the reduction in primary storage may also have a flow-on effect on glycolipid 
storage. This means that SDT is a potential strategy for reducing the neurological problems 
associated with MPS disorders. There are a number of inhibitors of glycosaminoglycan 
synthesis, which will be discussed in the following section.

1.5.5.2.1 Rhodamine B

Rhodamine B is a colouring agent also known as DMC Red 37 that was used in cosmetics 
such as lipsticks and soaps (Figure 1.7F). In vitro experiments in cultured human lip 
fibroblasts showed that rhodamine B at concentrations of 25 μg/ml and 50 μg/ml led to a 
reduction in glycosaminoglycan synthesis (Kaji et al. 1991a, 1991b). However, various 
reports suggest that this dye has the potential to cause growth retardation and liver damage 
(Bonser et al. 1956, Hansen et al. 1959), erythrocyte haemolysis (Gangolli et al. 1967),
mutagenicity (Nestmann et al. 1979, Lewis et al. 1981), immune suppression (Wess and Archer 1982) and carcinogenicity when given at high doses, corresponding to approximately 10 milligrams per week (Umeda 1952, 1955). Studies in the New Zealand rabbit with 1mg/kg did not produce deleterious side-effects (Mason and Edwards 1989). Carcinogenic effects were only seen when rats were administered with 50-200 mg/kg/wk, where the formation of sarcomas was seen (Umeda 1952, 1955). However, the administration of low concentrations of rhodamine B is thought to decrease the synthesis of gags without side-effects. Since gags are important in the formation of extracellular proteoglycans, only minimal suppression of these compounds is desirable since altering the biosynthetic pathways may lead to other changes in protein expression. Although other inhibitors of gag synthesis were investigated, this thesis reports the effect of rhodamine B on inhibition of gag synthesis in vitro and in vivo (Roberts et al. 2006).

1.5.5.2.2 Sodium chlorate

Sodium chlorate is a known competitive inhibitor in vitro of the enzyme sulphate adenylytransferase. This inhibition gives rise to a reduction in the synthesis of the sulphate donor PAPS (3’-phosphoadenosine 5’-phosphosulphate) (Greve et al. 1988, Humphries and Silbert 1988, Keller et al. 1989). Sodium chlorate therefore reduces protein and carbohydrate sulphation (Figure 1.7E). In 1997, van Kuppeveld and colleagues (van Kuppeveld et al. 1997) demonstrated a reduction in protein sulphation but not in protein synthesis using 3 mM sodium chlorate and also showed that increasing the concentration of sodium chlorate to 10 mM led to a reduction in protein synthesis, which is not desirable. Sodium chlorate has also been shown to inhibit sulphation of tyrosyl residues of proteins without inhibiting protein synthesis or causing cell toxicity (Baeuerle and Huttner 1986). Phamantu and colleagues (Phamantu et al. 1999) showed that chlorate treatment abolished
the sulphate incorporation into gags, conferring a decrease in the resulting cell-associated proteoglycans charge density. No investigation of the use of sodium chlorate as a substrate deprivation therapeutic has been reported to date, although it is a potential candidate for treatment of MPS disorders, since a number of these disorders result from the deficiency of lysosomal sulphatase enzymes. Altering the sulphation patterns of cellular glycosaminoglycans and proteoglycans may have some effect on ameliorating the clinical symptoms by reducing the highly sulphated glycosaminoglycans that are stored in these patients.

1.5.5.2.3 Analogues of glucose and galactose

Berkin and colleagues (Berkin et al. 2000a, 2000b) first described the synthesis of 4-deoxy-4-fluoro analogues of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose (Figure 1.7C and 1.7D, respectively). They showed that these synthetic heparan sulphate analogues, which are able to disrupt glycosaminoglycan biosynthesis, could be useful for a number of clinical disorders. These compounds have a fluorine group introduced at carbon-4 instead of the normal hydroxyl group. This is thought to interfere with glycosaminoglycan synthesis since incorporation of these analogues will essentially stop elongation of the gag chain (Berkin et al. 2000a), however, this is still under investigation. In vitro studies with the glucose analogue in cultured mouse hepatocytes demonstrated a decrease of glucosamine by 12% and sulphate by 18% measured by radiolabel incorporation compared to control cells (Berkin et al. 2000a). This correlates to a decrease in intracellular hepatocyte glycosaminoglycan synthesis at 1 mM. The galactose analogue demonstrated a greater reduction in glucosamine and sulphate radiolabel incorporation, 1% and 9% respectively compared to control cells (Berkin et al. 2000a). The
galactose analogue also decreased protein synthesis by 43% so may not be as useful in a therapeutic setting.

All of the inhibitors mentioned above are smaller than 500 Daltons and should therefore theoretically cross the blood-brain barrier, where they can affect the biosynthetic pathways of numerous cell types. This is of particular importance for those MPS disorders which display CNS pathology, and which cannot be treated successfully by current measures.

1.5.5.2.4 Genistein

The isoflavone, genistein (Figure 1.7G), which inhibits tyrosine kinase is thought to decrease gag synthesis (Nikitovic et al. 2003, Mitropoulou et al. 2002) or the expression of the parent proteoglycan molecule depending on cell type (Schonherr et al. 1997). Recent evidence has suggested that expression of some of the genes encoding enzymes involved in gag synthesis might be controlled by pathways dependent on oestrogen receptors and protein tyrosine kinase (Piotrowska et al. 2006). Genistein, a substance from the group of isoflavones has a structure resembling that of oestrogen and may act on the oestrogen-dependent pathway and have an effect on the signal transduction which may alter gene expression. Studies done by Piotrowska and colleagues (Piotrowska et al. 2006) showed that skin fibroblast cultures from control and MPS patients treated with genistein showed a decrease in radiolabelled sulphate incorporation, a measure of gag synthesis, and had a flow-on effect on gag storage. This study also showed that treatment with 10 μM genistein led to similar biochemical results in MPS I patient skin fibroblasts when compared to recombinant enzyme (α-L-iduronidase) treatment. Electron microscopic examination of MPS I cells treated with genistein showed a reduction in zebra bodies, flocculent inclusions and complex vacuoles to similar levels as shown with enzyme treatment (Piotrowska et al. 2006). Clinical trials in MPS IIIA and IIB patients using genistein as
gene-expression targeted isoflavone therapy (GET IT) were reported to have effects on clinical parameters such as reduced urinary heparan sulphate, improved hair structure, and parental observations such as better walking and sleeping (Wegrzyn 2006).

1.5.5.3 OTHER AGENTS – MISCELLANEOUS

1.5.5.3.1 Chemical Chaperones

Some lysosomal diseases have residual enzyme activity despite the mutant enzyme being misfolded or mistrafficked in the cell (Berg-Fussman et al. 1993). This opens up the possibility of using chemical chaperones within the secretory pathway as facilitators for proper folding or enzyme stability (Morello et al. 2000). 1-deoxy-galactonorjirimycin (DGJ) has been shown to chaperone α-galactosidase, the enzyme deficient in Fabry disease, by acting as a competitive inhibitor for the enzyme (Fan et al. 1999, Asano et al. 2000). These studies showed that incubation of Fabry patient lymphocytes with DGJ for 4 days led to a 7-fold increase in α-galactosidase activity, which stayed elevated after the DGJ was removed from culture media. In vivo, transgenic mice treated with the same inhibitor showed an increase in α-galactosidase activity in all major organs (Ishii et al. 2004). This was probably due to the inhibitor binding to the mutant enzyme and allowing stabilisation at a neutral pH. Since the enzyme complex was stable, correct trafficking from the endoplasmic reticulum to the lysosome occurred and stability was maintained due to the high concentration of substrate and the reduced pH in the lysosome.

Another example of enzyme stabilisation using chemical chaperones is using N-(n-nonyl) deoxynojirimycin (NN-DNJ). At a concentration of 10 µM this inhibitor, when added to fibroblast culture medium for 9 days, led to a 2-fold increase in N370S β-glucuronidase activity (Sawkar et al. 2002), the most common mutation in non-neuronopathic Gaucher
disease (Beutler and Kuhl 1986). However, even after the inhibitor was removed from the culture media, activity remained elevated for up to 6 days. NN-DNJ has also been shown to increase the wild-type $\beta$-glucuronidase activity, but not the mutant L444P (a less common Gaucher disease variant) $\beta$-glucuronidase activity (Sawkar et al. 2002). Co-incubation of the recombinant $\beta$-glucuronidase enzyme, Ceredase with the NN-DNJ led to increased resistance to heat denaturation, which was improved with increasing NN-DNJ concentrations. The mechanism of correction was suggested to be correct folding of $\beta$-glucuronidase at neutral pH, allowing transport from the ER to the Golgi and proper trafficking to the lysosome (Sawkar et al. 2002). This type of therapeutic strategy is important, since most LSDs are thought to only need a small amount of corrected enzyme in order to see an effect on clinical outcome (Cheng and Smith 2003).

1.5.5.3.2 Stop codon read-through

This type of therapy may provide an alternative or adjunct therapy for MPS disorders. Stop-codon read through therapy relies on the fidelity of mRNA stop codons in enzyme sequence. From molecular-genetic studies, a large number of patients with lysosomal storage diseases are reported to have a premature stop codon mutation in one or both alleles of enzymes needed for lysosomal degradation (Brooks et al. 2006). MPS I and neuronal ceroid lipofuscinosis have a high incidence of these premature stop codons. Preclinical studies have shown that gentamicin induced the read-through of premature stop codons resulting in enzyme activity which subsequently reduced lysosomal storage (Brooks et al. 2006). The effect on neurological pathology remains unknown, as the ability for these small drugs to pass through the blood-brain barrier is currently undetermined.
RNA interference (RNAi) is an in-built mechanism of eukaryotic cells to suppress the expression of genes that determine fundamental roles in cell differentiation and survival (van der Krol et al. 1990, Cogoni and Macino 1997). RNAi is a form of post-transcriptional gene regulation which occurs through the targeted degradation of mRNA sequences complementary to the interfering RNA which prevents translation of the mRNA into protein. This natural ability of cells to self-regulate gene expression has led to the possibility of using this technology as a biomedical therapy. The earliest studies, conducted in *Caenorhabditis elegans*, showed that long double stranded RNA ranging from 50 to 1000 nucleotides was able to silence homologous mRNA transcripts, resulting in a measurable decrease in gene-specific expression (Fire et al. 1998).

In MPS disorders, RNAi technology could be used to knockdown genes involved in specific gag synthesis. Specifically, the introduction of small interfering RNA directed against the enzymes responsible for gag chain elongation and/or sulphation should result in a decrease in the amount of sulphated glycosaminoglycan synthesised by the cell. This decrease in gag synthesis would cause a reduced amount of substrate to be delivered to the lysosome for subsequent degradation. In MPS patient cells this should result in a reduction of lysosomal gag storage, as the residual enzyme activity possessed by the patient’s cells would be able to more effectively turnover the reduced level of gag within the lysosome. Since lysosomal gag storage has been shown to correlate with clinical pathology (Bunge et al. 1998), this is likely to have a positive therapeutic outcome for the patient. No reports of this type of therapy for MPS have been published to date.
1.6 Glycosaminoglycans and Proteoglycans

1.6.1 Structure and function

Glycosaminoglycans (gags) are long, unbranched, carbohydrate chains composed of repeating acidic sugar and amino sugar residues. Their function is to stabilise and support the cellular and fibrous components of tissues and also to help maintain water and salt homeostasis in the body (Hardingham and Fosang 1992). In addition, they also have an important role in the regulation of tissue growth via interactions with a number of growth factors (Prydz and Dalen 2000). The amino sugar can be either D-glucosamine or D-galactosamine, which can be sulphated on C4 or C6 and/or N-acetylated. The acidic sugar is either L-iduronic acid or its C5 epimer, D-glucuronic acid. The exception to this is keratan sulphate, which contains galactose instead of an acidic sugar residue. There are five major types of glycosaminoglycans; chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate/heparin and hyaluronic acid (Table 1.5) (Hook et al. 1984).

Proteoglycans compose the biologically active form of the gag and are classified according to their protein core structure. The diversity and heterogeneity of proteoglycans is achieved by both the core protein sequence and the type and specific structure of the attached gags to the protein core, and their function is related to distinct domain structures on the gag chains. Some proteoglycans are incorporated into the plasma membrane, where they are able to interact and bind with signalling molecules, such as growth factors and γ-interferon (Prydz and Dalen 2000). Cell surface gags can be internalised and have been detected in numerous organelles from the secretory endocytosis pathway (Fedarko and Conrad 1986, Ishihara et al. 1986, Tumova et al. 1999). Other localisation mechanisms can occur via the trans-Golgi network where proteoglycans can be released along with other vesicles and storage material (Prydz and Dalen 2000).
Table 1.5: Classification and structure of glycosaminglycans

<table>
<thead>
<tr>
<th>GAG</th>
<th>Hexuronic or IDuronic Acid</th>
<th>Galactose</th>
<th>Hexosamine Residue</th>
<th>Disaccharide Composition</th>
<th>Cellular Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparan sulphate (HS)/Heparin</td>
<td>D-glucuronic acid (GlcA) L-iduronic Acid (IdoA)</td>
<td>D-glucosamine (GlcNAc)</td>
<td><img src="image1" alt="HS structure" /></td>
<td>HS: basement membranes Heparin: Mast cells</td>
<td></td>
</tr>
<tr>
<td>Keratan sulphate (KS)</td>
<td>Galactose (Gal)</td>
<td>D-glucosamine (GlcNAc)</td>
<td><img src="image2" alt="KS structure" /></td>
<td>Cartilage proteoglycans aggregates with CS. Also cornea.</td>
<td></td>
</tr>
<tr>
<td>Chondroitin sulphate (CS)</td>
<td>D-glucuronic acid (GlcA)</td>
<td>D-galactosamine (GalNAc)</td>
<td><img src="image3" alt="CS structure" /></td>
<td>Major component of cartilage, tendons, ligaments and aorta</td>
<td></td>
</tr>
<tr>
<td>Dermatan sulphate (DS)</td>
<td>D-glucuronic acid (GlcA) L-iduronic acid (IdoA)</td>
<td>D-galactosamine (GalNAc)</td>
<td><img src="image4" alt="DS structure" /></td>
<td>Major component of skin, blood vessels and heart valves</td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid (HA)</td>
<td>D-glucuronic acid (GlcA)</td>
<td>D-glucosamine (GlcNAc)</td>
<td><img src="image5" alt="HA structure" /></td>
<td>In synovial fluid of joint, vitreous humour of eye, umbilical cord and loose connective tissue</td>
<td></td>
</tr>
</tbody>
</table>

Table adapted from (Prydz and Dalen 2000) and cellular localisation adapted from (Champe and Harvey 1994)
1.6.2 Synthesis of glycosaminoglycans and proteoglycans

For correct gag synthesis, a different enzyme is required for the addition of each monosaccharide residue (Sugahara and Kitagawa 2000). All gags, except hyaluronic acid, are synthesised bound to a protein core and are known as proteoglycans. Hyaluronic acid is synthesised as a free gag on the plasma membrane (Laurent and Fraser 1992).

All gag synthesis, with the exception of keratan sulphate and hyaluronic acid is initiated by the gag-protein linkage region tetrasaccharide sequence (composed of xylose (Xyl), galactose (Gal), galactose (Gal) and glucuronic acid (GlcUA)) which is attached to serine residues on the protein core (Figure 1.9A). Gag-attachment sites have the consensus peptide sequence Ser-Gly/Ala-X-Gly (where X is any amino acid), which allows for gag chain initiation (Esko and Zhang 1996). A number of different transferase enzymes are required for the addition of each alternating N-acetylhexosamine and glucuronic acid residue (Sugahara and Kitagawa 2002). The addition of either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) to the linkage region commits gag synthesis to either heparan sulphate or dermatan/chondroitin sulphate. In the case of heparan sulphate, gag chain initiation is conducted by EXTL2 or EXTL3 (enzymes with GlcNAcT-I activity) and chain elongation occurs using enzymes with GlcNAcT-II activity, EXTL3 and EXTL1 (reviewed in Sugahara and Kitagawa 2002).

Following successful chain elongation, the glucuronic acid residue can be C5-epimerised to iduronic acid and the glucuronic acid residue can be 2-O- or 3-O- sulphated to give heterogeneity in the chain structure (Sugahara and Kitagawa 2002) (Figure 1.9B). The glucosamine residues can be N-deacetylated and then N-sulphated which is catalysed by the N-acetylglucosamine N-deacetylase/N-sulphotransferase (NDST) enzymes. The residues can also be O-sulphated by glucosaminyl 6-OST and 3-O (3OST) and 2-O
Figure 1.9: Biosynthesis and modification of sulphated gags.

(A) Biosynthesis of sulphated gags.

The linkage region of heparan sulphate is synthesised on specific serine residues (Ser) on the core protein. The linkage region is composed of a xylose residue (xyl), two galactose residues (Gal), and one glucuronic acid residue (GlcA), and is common for heparan, chondroitin and dermatan sulphate. The addition of either N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) to the linkage region commits gag synthesis to either heparan sulphate or dermatan/chondroitin sulphate. Gag chain initiation (1) is conducted by EXTL2 or EXTL3 (enzymes with GlcNAcT-I activity) and chain elongation (2) occurs using enzymes with GlcNAcT-II activity, EXTL3 and EXTL1. Figures adapted from Sugahara and Kitagawa 2002.

(B) Post polymerisation modification of heparan sulphate occurs through the action of various epimerases, N-sulphotransferases and O-sulphotransferases to generate a myriad of heparan sulphate structure with specific function. Figure adapted from Esko and Lindahl 2001.
Figure 1.9: Biosynthesis and modification of sulphated gags.

NOTE: This figure is included in the print copy of the thesis held in the University of Adelaide Library.
sulphotransferases (2OST) (Sugahara and Kitagawa 2002). These modifications allow the discrimination between heparin and heparan sulphate and gives rise to a variety of heparan sulphate structures that have different biological functions.

1.6.5 Abnormalities in heparan sulphate biosynthesis

The synthesis of heparan sulphate and its subsequent O-sulphation, N-sulphation and epimerisation is tightly regulated, and disruptions in this pathway have various outcomes. Targeted deletion of Ext1 in mice results in early embryonic lethality and Ext1-null embryos die around the time of gastrulation (Lin et al. 2000). The EXT1 mice embryos produce no HS and possess an abnormal distribution of the morphogen Indian hedgehog (Ihh) that does not associate with the cell surface and causes increased chondrocyte proliferation and delayed hypertrophic differentiation (Hilton et al. 2005). In addition to this, mice with a targeted knockout of Ext2 develop normally until embryonic day 6.0, when they become growth arrested and fail to gastrulate, suggesting that heparan sulphate plays an essential role in embryogenesis (Stickens et al. 2005). The Ext1 enzyme has also been conditionally disrupted in embryonic mice to define the developmental role of HS in brain (Inatani et al. 2003). This study showed that HS is pivotal for mammalian brain development since the mutant mice displayed patterning defects caused by numerous HS-binding morphogens.

Mutations in the EXT genes in humans lead to hereditary multiple exostoses (HME). The main consequence of these mutations is development of bony outgrowths near the ends of long bones, due to inappropriate chondrocyte proliferation and bone growth (Duncan et al. 2001).
Studies in *Drosophila* have shown that ablation of the HS polymerising enzymes *ext1* homologue, *tout velu (ttv)* and *ext2* homologue, *sister of tout velu (sottv)* is important for establishing morphogen gradients in the developing embryonic tissue. Any disruption in HS synthesis causes aberrations in the three major signalling pathways of *hedgehog (Hh)*, wingless (*Wg*) and *decapentaplegic (Dpp)* (Bellaiche *et al.* 1998, Karsenty and Wagner 2002, Han *et al.* 2004, Takei *et al.* 2004).

Mutant mice that have targeted disruption of N-deacetylase/N-sulphotransferase 1 (NDST1) result in some embryonic lethality, and those that survive to term die soon after from pulmonary insufficiency and hypoxia due to malformed lungs (Ringvall *et al.* 2000). Skull and eye abnormalities were also observed in these mutant mice with a range of different phenotypes (Ringvall *et al.* 2000, Grobe *et al.* 2002). NDST-2 knockouts only displayed abnormal mast cells, which were reduced greatly in number and had depleted sulphated heparin chains (Forsberg *et al.* 1999, Humphries *et al.* 1999). Knockout mice for NDST-3 are viable and fertile and do not show any overt phenotype (Grobe *et al.* 2002). NDST-4 knockouts are currently being generated (Grobe *et al.* 2002).

### 1.6.6 Heparan sulphate proteoglycans (HSpgs)

Proteoglycans can be grouped into families depending on the composition of their gag side chains. For heparan sulphate side chains, these form heparan sulphate proteoglycans (HSpgs) which comprise of the glypican, syndecan and perlecan families (reviewed in Hacker *et al.* 2005 and Whitelock and Iozzo 2005). The first two are either transmembrane or GPI-anchored membrane bound proteoglycans that contain predominantly HS side chains whereas the latter is localised to cell surfaces (Hacker *et al.* 2005). These are conserved throughout different species and specifically regulated and expression levels change in a stage- and tissue-specific manner. The presence of HS side chains allows
interaction with numerous ligands including growth factors and hormones and regulation of a myriad of cell processes (reviewed in Hacker et al. 2005 and Whitelock and Iozzo 2005).

1.6.6.1 Glypicans

Glypicans are HSpgs that are linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Fransson 2003). There are six different glypicans present in mammals (Films et al. 1988, David et al. 1990, Stipp et al. 1994, Watanabe et al. 1995, Saunders et al. 1997, Veugelers et al. 1997, 1999, Paine-Saunders et al. 1999), two in Drosophila melanogaster (Nakato et al. 1995, Baeg et al. 2001) and at least one in zebrafish (Topczewski et al. 2001) and C.elegans (Hacker et al. 2005). They all have the common region of 14 conserved cysteine residues and 2-3 gag attachment sites near the C-terminal close to the membrane; however they share a small amount of homology along the rest of the protein. In general, glypicans are expressed predominantly during development. Expression levels change in a stage- and tissue-specific manner, suggesting that glypicans are involved in the regulation of morphogenesis (Litwack et al. 1994, 1998, Li et al. 1997, Saunders et al. 1997, Pellegrini et al. 1998)

1.6.6.2 Syndecans

These are type I transmembrane proteins which can have up to five gag attachment sites that can carry HS, CS or DS side chains, but are mainly HS. There are four syndecan genes, syndecan-1 to -4 present in mammals, but only one in invertebrates (reviewed in Tkachenko et al. 2005). Syndecan-3 is specifically expressed on the growing axonal surface during brain development (Watanabe et al. 1996, Carey et al. 1997, Hsueh et al. 1999). Other roles of syndecan-3 include regulation of skeletal muscle differentiation and development and regulation of hedgehog signalling, which is important in embryogenesis
and differentiation. Syndecan-3 also has important roles in cell adhesion and migration, neurite outgrowth and long-term potentiation (Chernousov et al. 1996, Kinnunen et al. 1998, Lauri et al. 1999, Erdman et al. 2002). Syndecan-1 is an important regulator of cell-cell and cell-extracellular matrix interactions. Mice engineered to overexpress syndecan-1 have delayed dermal wound healing (Elenius et al. 2004). Syndecan-2 is the predominant syndecan expressed during embryogenesis. Recent studies have uncovered a role in TGF-β regulation in adult cells via protein-protein interaction (Tkachenko et al. 2005). Syndecan-4 is the best characterised member of the syndecan family with roles in FGF signalling, regulation of cell migration and control of adhesion via cytoskeletal modifications (Tkachenko et al. 2005).

HSpgs have also been implicated in the control of feeding behaviour (Reizes et al. 2001). The over-expression of syndecan-1 results in abnormal expression of this HSpg in the hypothalamic nuclei that controls energy balance and transgenic syndecan-1 mice develop mature-onset obesity. In contrast, mice engineered to over-express heparanase displayed reduced food consumption and decreased bodyweight (Zcharia et al. 2004). Heparanase is an endo-glycosidase that catalyses the first step in HS gag degradation (Hulett et al. 1999, Kussie et al. 1999, Toyoshima and Nakajima 1999, Vlodavsky et al. 1999) and transgenic animals possessed lower tissue levels of HS. Thus HS levels in the hypothalamus are intimately involved in the signals that regulate feeding behaviour and maintain energy balance. In addition to this, syndecan-3 knockout mice have reduced feeding behaviour in response to food deprivation (Reizes et al. 2003) as well as impaired performance in tasks using hippocampal function, suggesting abnormalities in learning and memory (Kaksonen et al. 2002).
1.6.6.3 Perlecans

Perlecan is the major HSpg which makes up basement membranes and other connective tissues (Whitelock et al. 1999). While found predominantly as a HSpg, some forms of perlecan lack gag chains and some have CS and DS chains attached to the proteoglycan (Isemura et al. 1987, Iozzo and Hassell 1989, Couchman et al. 1996). Perlecan is expressed early in mouse embryonic development in tissues associated with the major blood vessels of the cardiovascular system (Handler et al. 1997) and also in cartilage development and endochondral ossification (Handler et al. 1997, Arikawa-Hirasawa et al. 1999, 2001a, 2001b, 2002, Costell et al. 1999, French et al. 1999). An important function of perlecan is binding and presentation of growth factors, particularly in events of matrix remodelling such as early migration and proliferation in endothelial wound healing (Sephel et al. 1996), angiogenesis and tumour invasion. Domain I of perlecan is important in binding to basic fibroblast growth factor (FGF-2) which may modulate the effects of growth factor activity by releasing enzymes such as plasmin, thrombin, heparanase and collagenase (Whitelock et al. 1996).

A reduction in the level of gag synthesis has important consequences on proteoglycan function. The presence of gag side chains allows interactions with growth factors and hormones and disruption of these processes can have dire consequences. Interference with gag synthesis using substrate deprivation therapy needs to be controlled so that normal functioning of gags is not affected (Templeton 1992).
1.7  Aims and hypotheses

The overall objective of this project was to evaluate the efficacy of SDT with rhodamine B in the murine model of MPS IIIA. A number of specific objectives were proposed to achieve this.

1) To characterise a number of different SDT agents in cultured normal and MPS patient cells to determine their effect on reducing gag synthesis and gag storage.

2) To administer 1 mg/kg rhodamine B over 6 months in the murine model of MPS IIIA and examine the effects on clinical disease progression.

3) To evaluate a range of learning and memory tests: A) repeated acquisition and performance chamber B) Morris water maze C) water cross maze and D) open field test as measures of behavioural dysfunction in MPS IIIA and normal mice and where applicable MPS IIIA treated mice

4) To administer 1mg/kg rhodamine B over 4 generations of MPS IIIA mice with pregnant dams being treated during pregnancy to evaluate the effect of rhodamine B treatment over an extended timecourse and any toxicity associated with this.

5) To administer 5mg/kg high dose rhodamine B over 6 months in MPS IIIA mice and determine effect of higher dose on clinical disease progression and toxicity.
CHAPTER 2
MATERIALS AND METHODS
2.1 Materials

2.1.1 Animal experimentation

- 0.5 ml insulin syringe (27G) BD (North Ryde, NSW, Australia)
- Baby shampoo Johnson and Johnson (New Jersey, USA)
- Glutaraldehyde Sigma Chemical Co. (St Louis, USA)
- Ketamine (100 mg/ml) Parnell Laboratories Pty. Ltd. (NSW, Australia)
- Isofluorane (fluorothane) Abbott Australasia Pty Ltd. (NSW, Australia)
- Lacri-Lube® Allergan (Buckinghamshire, England, UK)
- Needle electrodes Radiometer Pacific (Victoria, Australia)
- Neonatal ear tips Radiometer Pacific (Victoria, Australia)
- Noise event meter Type 2208 Brüel and Kjör (Copenhagen, Denmark)
- Rhodamine B Sigma Chemical Co. (St Louis, USA)
- Sodium saccharin ICN Biochemicals Inc. (Aurora, Ohio, USA)
- Saline (0.9% NaCl) BDH Laboratory Supplies (Poole, England)
- Full cream milk powder Diploma, Unigate Australia (Victoria, Australia)
- Ilium Xylaxil-20 (20 mg/ml) (Xylazine) Troy Laboratories Pty. Ltd. (NSW, Australia)

2.1.2 Histochemical Reagents

- APES (3’-aminopropylethoxysilane) Sigma Chemical Co. (St Louis, USA)
- Eosin Y C.I. 45380 BDH Laboratory Supplies (Poole, England)
- Haematoxylin C.I. 75290 Surgipath Medical Industries, Inc.
  (Richmond, Illinois, USA)
2.1.3 Histochemical Solutions

The solutions routinely used were kindly donated by the Department of Histopathology at the Children, Youth and Women’s Health Service (South Australia, Australia). They were made using distilled water and were as follows:

- Lithium carbonate: 10 mls of saturated stock solution and 40 mls of distilled water
- Mayer’s haematoxylin: 0.2% haematoxylin C.I. 75290, 5% ammonium aluminium sulphate, 0.02% sodium iodate, 5 mM citric acid, 5% chloral hydrate
- Stock Eosin Y: 1% eosin Y C.I. 45380, 0.5% potassium dichromate, 10% saturated aqueous picric acid, 10% absolute ethanol
- Working eosin solution: 50 mls of stock eosin and 50 mls of water
- Toluidine blue solution: 1% toluidine blue C.I., 1% borax, filtered
2.1.4 Cell culture reagents

Cell scrapers Nalge Nunc International (Rochester, NY, USA)

Dulbecco’s Modification of Eagles’s Medium (DMEM) ICN Biochemicals Inc. (Aurora, Ohio, USA)

Dimethyl sulphoxide (DMSO) Sigma Chemical Co. (St Louis, USA)

Ethanol (70% v/v) Pharmacia Pty Ltd. (Bentley, Australia)

Foetal Calf Serum (FCS) JRH Biosciences (Lenexa, USA)

Ham’s F12 Medium ICN Biochemicals Inc (Aurora, Ohio, USA)

Penicillin/Streptomycin solution CSL Biosciences (Melbourne, Vic, Australia)

RPMI 1640 Medium ICN Biochemicals Inc (Aurora, Ohio, USA)

Tissue culture flasks Greiner Bio-one (Frickenhausen, Germany)

Tissue culture plates and disposables Corning (Lindfield, NSW, Australia)

Trypan blue (0.5%) Cytosystems Pty. Ltd. (Castle Hill, NSW, Australia)

Trypsin (0.12% (w/v))/ Na2EDTA (0.02% (w/v)) JRH Biosciences (Lenexa, USA)

2.1.5 Radiochemicals

\(^3\)H-glucosamine Amersham Biosciences Pty Ltd. (NSW, Australia)

Na\(^{35}\)SO\(_4\) Amersham Biosciences Pty Ltd. (NSW, Australia)
2.1.6 Molecular reagents

Agarose, DNA grade
Progen Industries Ltd. (Darra, Qld, Australia)

Agarose, High resolution
Progen Industries Ltd. (Darra, Qld, Australia)

Bromophenol blue
BDH Chemicals Ltd. (Poole, Dorset, England)

dNTP mix (10 mM)
Roche (Castle Hill, NSW, Australia)

Ethanol (99.7-100%)
Merck (Kilsyth, Victoria, Australia)

Ethidium bromide
Ameresco (Ohio, USA)

Glycerol
Merck (Kilsyth, Victoria, Australia)

Hot Start Taq polymerase
Qiagen (Doncaster, Victoria, Australia)

Isopropanol
Asia Pacific Specialty Chemicals Ltd. (Seven Hills, NSW, Australia)

Lithium chloride (LiCl)
BDH (Merck) Ltd. (Kilsyth, Victoria, Australia)

Magnesium chloride (25 mM)
Qiagen (Doncaster, Victoria, Australia)

PCR buffer (10X)
Qiagen (Doncaster, Victoria, Australia)

Phenol
Sigma Chemical Co. (St Louis, USA)

Primers
Life Technologies, Invitrogen (Victoria, Australia)

Proteinase K
Boehringer Mannheim, (Indianapolis, USA)

Restriction endonuclease (AcI)
New England Biolabs (Beverly, Mass., USA)

SPP-1 EcoRI, pUC19 HpaII size markers
Geneworks (Adelaide, SA, Australia)

Syringe filters (0.2μm)
Sartorius AG (Goettingen, Germany)
Chapter Two: Materials and Methods

*Taq* polymerase Roche (Castle Hill, NSW, Australia)

2.1.7 **Protein reagents**

3-phenylphenol Sigma Chemical Co (St Louis, USA)

4-methylumbelliferyl-2-acetamido-2-deoxy-β-glucopyranoside Sigma Chemical Co. (St Louis, USA)

4-methylumbelliferyl-phosphate Sigma Chemical Co. (St Louis, USA)

4-methylumbelliferyl-sulphate Sigma Chemical Co. (St Louis, USA)

Bio-Rad Dye Reagent Concentrate Bio-Rad Life Science Group (Hercules, California, USA)

Bovine serum albumin (BSA) Sigma Chemical Co (St Louis, USA)

DEAE-Sephacel resin Sigma Chemical Co (St Louis, USA)

D-glucuronolactone Paton Scientific (Stepney, SA, Australia)

Optiphase Hisafe scintillation fluid Perkin Elmer Life Sciences, (Turku, Finland)

Percoll Amersham Biosciences (Buckinghamshire, England)

Triton X-100 Sigma Chemical Co. (St Louis, USA)

2.1.8 **Buffers and solutions**

*Unless otherwise stated, all buffers were made in MilliQ filtered water. The buffers and solutions routinely used were as follows:*

Citrate buffer 0.054 M Na₃citrate.2dH₂O, pH 4.8

Cpc/citrate 0.1% (w/v) cpc in citrate buffer
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sephacel column wash buffer</td>
<td>0.1 M NaAc, pH 5.0</td>
</tr>
<tr>
<td>DEAE-Sephacel column elution buffer</td>
<td>0.1 M NaAc, 2M NaCl, pH 5.0</td>
</tr>
<tr>
<td>10 x DNA loading buffer</td>
<td>50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue</td>
</tr>
<tr>
<td>EM fixative</td>
<td>2% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M Na cacodylate buffer pH 7.4</td>
</tr>
<tr>
<td>10% buffered formalin</td>
<td>40% (v/v) formaldehyde solution was diluted 1/10 in PBS pH 7.2</td>
</tr>
<tr>
<td>Glycine buffer</td>
<td>0.2 M glycine, 0.125 M Na₂CO₃, 0.146 M NaOH</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris, pH 8.0, 2 mM NaCl, 1 mM EDTA, 0.5% Tween-20</td>
</tr>
<tr>
<td>Meta-hydroxydiphenol solution</td>
<td>15 mg 3-phenylphenol in 10 ml 0.5% NaOH stored in dark for one month.</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS)</td>
<td>137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.2</td>
</tr>
</tbody>
</table>
## Chapter Two: Materials and Methods

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteoglycan extraction buffer</td>
<td>6 M urea, 50 mM NaAc, 100 mM aminocaproic acid, 10 mM Na&lt;sub&gt;2&lt;/sub&gt;EDTA, pH 5.0</td>
</tr>
<tr>
<td>Sulphuric acid/tetraborate solution</td>
<td>0.0125 M sodium tetraborate in concentrated sulphuric acid (18.3 M)</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris-acetate, 2 mM EDTA, pH 8.5</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5</td>
</tr>
<tr>
<td>Tissue extraction buffer</td>
<td>6 M urea, 0.05 M Na acetate pH 5.0, 0.1% Triton X-100 containing protease inhibitors (0.1 M aminocaproic acid, 0.1 M Na&lt;sub&gt;2&lt;/sub&gt;EDTA, 5 mM benzamidine, 0.5 mM phenyl methyl sulphonyl fluoride and 10 mM n-ethylmaleimide)</td>
</tr>
</tbody>
</table>
2.2 Cell culture methods

2.2.1 Cell culture maintenance

Normal skin fibroblasts and MPS patient fibroblasts were purchased from the Coriell Cell Repository (Camden, New Jersey, USA), or were obtained from the Department of Genetic Medicine, CYWHS after obtaining informed consent. Be(2)-C cells were obtained from ATCC (Manassas, VA, USA). CHO-K1 cell lines were maintained in Ham’s F12 supplemented with 10% (v/v) FBS, 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate. Skin fibroblasts were maintained in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% (v/v) FBS, 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate. Be(2)-C cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate. Cells were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO₂ in either a NAPCO incubator (Model 5415) or a Forma Scientific water-jacketed incubator (Series 3250, Thermo Electron Corporation, Waltman, MA, USA). Gas exchange was ensured by loosening the culture flask caps. All cell culture was performed under sterile conditions in either a laminar flow or biohazard hood in a designated tissue culture laboratory area within a PC2 facility.

Cell cultures were maintained by feeding three times weekly unless otherwise specified. Old media was aspirated under sterile conditions and replaced with fresh, pre-warmed medium. T₇₅, T₂₅ and 6-well plates were given 12 ml, 6 ml or 2 ml of fresh media respectively per flask or well.
2.2.2 Subculturing cells

Culture media was removed and the cells were washed three times with an excess of phosphate buffered saline (PBS). Cells were harvested in 0.012% (w/v) trypsin, 0.002% (w/v) Na₂EDTA in PBS at 37°C for 5-10 minutes. Cells were then detached from the flask surface via pipetting, pooled and collected by centrifugation in a Heraeus Megafuge 1.0R for 5 minutes at 750 x g at room temperature. The supernatant was discarded and cells were resuspended in an appropriate volume of media (i.e. 3 ml for a 1:3 subculture) from which partial volumes (i.e. 1 ml) were transferred as desired to new T75 flasks containing fresh, pre-warmed media to a total volume of 12 ml, or an equivalent for other tissue culture vessels. Skin fibroblasts were subcultured 1:3 and Be-2(C) and CHO-K1 cells were subcultured 1:10.

2.2.3 Cell counts

Cells were harvested as per section 2.2.2 and the cell pellet was resuspended in 1 ml of Ham’s F12 media per T75 flask. A 1:2, 1:5 or 1:10 dilution of cell suspension was then made in 0.05% trypan blue in a 12 cm x 75 mm glass culture tube. The mix was then pipetted onto a Neubauer Brightline haemocytometer enclosed by a cover slip and allowed to fill the haemocytometer via capillary action. Cells were then counted at a 10 x magnification on an Olympus (model: CK2) inverted light microscope and the number of cells excluding trypan blue per millilitre of suspension determined. Cells were then plated at the required density and fresh pre-warmed medium was added to make up the required volume.
2.2.4 Cryopreservation

Cells were harvested as per section 2.2.2 and resuspended in 1 ml media supplemented with 10% (v/v) FBS, 50 U/ml penicillin G, 50 μg/ml streptomycin sulphate and 15% (v/v) dimethyl sulphoxide. The cell suspension was then transferred to a 1.8 ml screw-cap cryotube and incubated overnight at -70°C (Forma Scientific Bio-Freezer) in a Nalgene™ Cryo 1°C freezing container before being transferred to liquid nitrogen storage (Locator 4 Cryo Biological Storage System).

2.3 In vitro methods

2.3.1 SDT

2.3.1.1 Effect of SDT on gag synthesis

Skin fibroblasts or Be(2)-C cells were seeded into 6-well plates and grown to confluency as described in sections 2.2.1 and 2.2.2. Cells were pre-incubated with between 0 and 100 μg/ml rhodamine B or between 0 and 50 mM sodium chlorate for 1 hour at 37°C. Cells were then incubated in the same concentration of rhodamine B or sodium chlorate plus 5 μCi/ml Na35SO4 for 6 hrs at 37°C. The medium was removed and the cells extracted into 4 M guanidine HCl, 50 mM sodium acetate pH 5.8 buffer containing protease inhibitors (0.1 M amino hexanoic acid, 0.05 M Na2EDTA) and 0.1% Triton X-100.

Excess radiolabel was removed using Sephadex G-25 size exclusion columns (Amersham Biosciences). Columns were equilibrated in PD-10 buffer (4 M GuHCl, 0.1 M Na2SO4, 0.05 M Tris base, 0.5% Triton X-100, pH 7.5), then media (500 μl) or cell layer extract (250 μl) was applied to the column. Ten 0.5 ml fractions were collected into 6 ml scintillation vials and 4 ml of Optisafe scintillation fluid was added and mixed thoroughly.
Na$^{35}$SO$_4$ and $^3$H incorporation was determined by scintillation counting using a Wallac System 1409 Liquid Scintillation counter (Wallac, Turku, Finland).

2.3.1.2 Effect of SDT on protein synthesis

Be(2)-C cells and MPS patient skin fibroblasts were grown to confluency in T$_{75}$ flasks as described in sections 2.2.1 and 2.2.2. Once confluent, cells were split into 6-well plates and grown until confluent. To measure protein synthesis cells were pre-incubated in 0 to 100 μg/ml of rhodamine B or 0 to 50 mM sodium chlorate as above for 1 hr at 37°C. This media was removed and replaced with the same concentration of rhodamine B or sodium chlorate plus 5 μCi/ml $^3$H-serine. Cells were incubated for 6 hrs at 37°C. Medium was removed and the cell layer scraped into 20 mM Tris, 0.5 M NaCl, pH 7.0 and stored at -20°C.

Radiolabelled proteins from both media and cell layer were precipitated with 5% (w/v) trichloroacetic acid containing 20 mM L-serine. This was followed by centrifugation at 10,000 x g in a microfuge for 5 minutes and the supernatant was discarded. The precipitated protein pellet was resuspended in 0.5 ml of 0.5 M NaOH and then transferred to a 6 ml scintillation vial (Perkin Elmer), followed by the addition of 4 ml of Optisafe scintillation fluid and the incorporated radioactivity determined by scintillation counting on a Wallac System 1409 Liquid Scintillation counter (Wallac, Turku, Finland).

2.3.1.3 Effect of SDT with rhodamine B on gag storage

MPS II, IIIA, VI patient and control skin fibroblasts were grown to confluency as described in sections 2.2.1 and 2.2.2 for 14-days. On day 14, 3 μCi/ml Na$^{35}$SO$_4$ was added to each T$_{75}$ flask and incubated overnight at 37°C. Cells were harvested as per section 2.2.2.
in 1:10 trypsin/EDTA and centrifuged at 750 x g for 5 mins. Each pellet was resuspended in 2 ml of percoll buffer A (0.025 M sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.4) and cells were drawn into a 10 ml syringe via a 23G needle and disrupted by drawing back and releasing the syringe barrel six times. After centrifugation at 750 x g for 10 minutes at 4°C the supernatant was removed and stored on ice. The disruption step was repeated a second time, the supernatants pooled and made up to a total volume of 20 ml with percoll buffer A and centrifuged at 3000 x g for 15 minutes at 4°C in a Beckman JA-20 centrifuge. The pellet was resuspended in 3 ml percoll buffer A and layered over 17 ml of 30% (v/v) percoll solution (Amersham Pharmacia) in percoll buffer A. The cell extract was centrifuged at 7700 x g for 60 minutes at 4°C in a Beckman JA-20 centrifuge. Each gradient was fractionated into 1.0 ml aliquots and stored at -20°C. A 100 μl aliquot of each fraction was added to 4 ml of Optisafe scintillation fluid (Perkin Elmer) and mixed thoroughly. Na\textsuperscript{35}SO\textsubscript{4} was determined by scintillation counting using a Wallac System 1409 Liquid Scintillation counter (Wallac, Turku, Finland).

2.4 Animal experimentation methods

2.4.1 Mouse information

Mice were bred from an in-house colony, originally established from MPS IIIA heterozygotes and consist of a background of predominantly 129SvJ, C57Bl/6 with a smaller contribution from SJL and CD1 mouse strains (Bhaumik et al. 1999). All normal control and MPS IIIA mice were derived from this background strain.

2.4.2 Mouse maintenance

Mice were housed under standard conditions with 12 hour light/dark cycles. They had access to ad libitum water and joint stock ration (Ridley AgriProducts, SA, Australia) as
required. All procedures involving live animals were approved by the Children, Youth and Women’s Health Service (formerly Women’s and Children’s Hospital), University of Adelaide and Institute of Medical and Veterinary Sciences Institutional Animal Ethics Committees where appropriate.

### 2.4.3 Colony breeding management

Mice were bred as needed. As MPS IIIA mice are fertile, homozygous crosses were performed on 10-15 week old mice. Age-matched normal control mice were also paired during these times. Generally, one male was paired with one female mouse and the male was allowed to remain in the cage for 7-10 days, after which he would be removed and caged separately. Females were then checked regularly for the two weeks post-pairing and daily in the third week after pairing for any signs of pups. The birth date of mouse pups was taken as being the date on which pups were first observed with the dam. Pups were toe-tagged on day seven and were weaned from the dam at three weeks of age. Male and female mice were housed separately after weaning. Bodyweights were recorded every week and urine collected on a fortnightly basis using metabolic cages.

### 2.4.4 PCR analysis of genotype

MPS IIIA mice were identified by mutation analysis in the week after birth following toe-tagging. A sample of toe tissue corresponding to mouse identification number was cut from pups seven days after birth. Sterile scissors were used to cut the toes which were cleaned with 70% ethanol in between pups. Pups were removed from the dam and placed under soft towel under a heating lamp. The toe tissue was placed in an Eppendorf and stored on ice for transportation to the laboratory. Pups were monitored closely over the next week for
any signs of rejection from the dam. No infections or death have resulted from this procedure in the colony.

Genomic DNA from the toes was obtained by incubating toes in 50 µl lysis buffer + proteinase K solution (1 ml of 50 mM Tris-HCl, pH 8.0, 2 mM NaCl, 1 mM EDTA, 0.5% (v/v) Tween-20, 0.5% (v/v) Triton X-100 and freshly added 0.4 mg/ml proteinase K) overnight at 37°C. Following overnight incubation, the proteinase K was inactivated by heating to 100°C for 5 minutes and the material was pelleted by centrifugation in a microfuge at 10 000 x g for 1 minute. Toe lysates were stored at -20°C until used in PCR analysis.

2.4.5 Genotyping PCR

Under standard PCR conditions, a 105 bp fragment was amplified from murine genomic DNA (extracted as per section 2.4.4). Briefly, 2 µl of genomic DNA from mouse toe tissue was added to 5 µl 10 X PCR buffer (Qiagen) and mixed with 100 ng of oligonucleotides 5’ MS12 (5’ NNT CTG TCT TCC TCA GCG 3’) and 3’ MS4 (5’ GAT AAG GCT GTG GCG GGA CAG GG 3’), 5 mM dNTP mix and 2.5 units Taq polymerase (Roche). Distilled water was added to final volume of 50 µl. The mutation region was amplified following 2 min at 94°C, 35 cycles of 45 sec at 94°C, 45 sec at 60°C, 40 sec at 72°C and 4 min final extension at 72°C. The 105 bp PCR fragment generated was digested with 5 units AcI at 37°C overnight. This was then electrophoresed in 1 x TAE buffer using a 5% high resolution agarose gel. The point mutation G91A present in MPS IIIA results in the loss of the AcI restriction site (Bhattacharyya et al. 2001). MPS IIIA genotype show a digested band size 94 bp and mice of normal genotype show a band size of 78 bp with heterozygote mice displaying both the MPS IIIA and normal band.
2.4.6 Sex determination PCR

To determine the sex of pre-weaned litters a sex determination PCR was employed (Lambert et al. 2000). This amplifies two genes, an autosomal interleukin-3 (IL-3) and a Y chromosome Sry gene product. Briefly 2 μl of genomic DNA from mouse toe tissue was added to 5 μl of 10 X PCR buffer (Qiagen) and mixed with 10 pmol of Sry oligonucleotides 5’ Sry 8276-8295 (5’ TGG GAC TGG TGA CAA TTG TC 3’) and 3’ Sry 8677-8658 (5’ GAG TAC AGG TGT GCA GCT CT 3’), 10 pmol of IL-3 oligonucleotides 5’ IL-3 792-801 (5’ GGG ACT CCA AGC TTC AAT CA 3’) and 3’ IL-3 1335-1316 (5’ TGG AGG AGG AAG AAA AGC AA 3’) 5 mM dNTP mix and 2.5 units Taq polymerase (Roche). DNA fragments of 402 bp for Sry PCR products present only in male samples and 544 bp for the IL-3 PCR products present in both male and female samples were analysed on a 3% (w/v) agarose gel electrophoresed in 1 x TAE buffer.

2.4.7 In vivo administration of rhodamine B

MPS IIIA and normal littermates were injected weekly starting at four weeks of age with 1 mg/kg rhodamine B (made up in sterile saline weekly and stored at 4°C) via the tail vein. Briefly, mice were weighed to determine the volume of rhodamine B to be administered and were placed in a holder attached to a retort stand. The mouse’s tail was heated using a heat lamp for 10 seconds or until tail veins became dilated. The tail was held firmly by the observer and mice were infused over 10 seconds with 1 mg/kg rhodamine B (0.2 mg/ml solution) using a 27G insulin needle. Gauze padding was immediately placed at the site of injection to stop bleeding. Upon completion, mice were removed from the holder and returned to their cage.
2.4.8 Urine collection

Urine was collected from mice on a regular basis by two methods. Firstly, metabolic cages were used to collect urine overnight. Secondly, at the time of injection or weighing the mouse was placed over a collection tube and the bladder gently manipulated to cause urination. Urine was stored at -20°C until analysis of creatinine or gag levels as described in sections 2.7.4 and 2.7.3 respectively.

2.4.9 Euthanasia

All mice were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation except for those used for liver function testing. Post-mortem procedure involved weighing the mouse to determine total bodyweight and dissecting organs for histological examination. Specimens analysed were brain (cerebral hemisphere and cerebellum), heart, liver, kidney, leg muscle, spleen and lung. These were weighed and placed in Eppendorf tubes or 10 ml collection tubes for transportation to the laboratory where all samples were stored at -20°C. Histological specimens were taken for light and electron microscopy as described in sections 2.8.1 and 2.8.2 respectively.

2.4.10 Liver function tests

Mice were anaesthetised by inhalation of isofluorane (Abbott Australasia Pty Ltd.) and blood was collected using the cardiac puncture method using a 27G needle and syringe. After sufficient blood collection, mice were euthanised by overdose of inhaled fluorothane. Half a millilitre of mixed venous/arterial blood was transferred to plasma separator tubes with lithium heparin (Becton Dickinson, New Jersey, USA) and stored on ice. Plasma was isolated using microcentrifugation and used in liver function tests as routinely conducted by the core laboratory at the Children, Youth and Women’s Health Service. Briefly
samples were measured for total protein, albumin, globulin, and total bilirubin. The enzymes ALT, GGT and ALP were also measured. All tests were conducted using a Synchro CX analyser (Beckman, California, USA) using appropriate reagent kits (Beckman, California, USA).

2.5 Mouse biochemistry methods

2.5.1 Isolation of urinary gags

Urinary gags were precipitated from mouse urine by adding 2 volumes of 0.1% cetylpyrinium chloride and incubating at 37°C for 30 minutes followed by centrifugation at 1500 x g for 10 minutes. The pellet was resuspended in 150 µl 2 M LiCl and 0.8 ml absolute ethanol added to precipitate gags at -20°C for 1 hour. The precipitate was recovered by centrifugation at 1500 x g for 10 minutes and the pellet resuspended in water. Gag was measured as described in section 2.7.3.

2.5.2 Tissue homogenisation

Brain and liver samples were homogenised in 5 volumes of tissue extraction buffer (6 M urea, 0.05 M Na acetate pH 5.0, 0.1% Triton X-100 containing protease inhibitors (0.1 M aminocaproic acid, 0.1 M Na₂EDTA, 5 mM benzamide, 0.5 mM phenyl methyl sulphonyl fluoride and 10 mM n-ethylmaleimide), transferred to 10 ml tubes and rotated at 4°C for 48 hours. The tissue homogenate was centrifuged at 7700 x g for 25 mins in a Beckman JA-20 centrifuge at 4°C and the supernatant applied to 0.5 ml DEAE-Sephacel resin equilibrated in 0.1 M Na acetate buffer, pH 5.0. The columns were washed in 10 column volumes of the same buffer and glycosaminoglycans eluted in 5 column volumes of the same buffer containing 2 M NaCl. The uronic acid content was determined and the total gag content normalised to tissue weight.
2.5.3 *Tissue percoll fractionation*

Brain and tissue samples were homogenised briefly in 5 volumes 0.25 M sucrose, 1 mM EDTA, pH 7.5 buffer, centrifuged at 650 x g for 10 mins at 4°C and the supernatant transferred to a new tube. Following centrifugation of the supernatant at 750 x g for 10 mins at 4°C the supernatant was removed and the pellet was resuspended in 1 ml of 0.25 M sucrose, 1 mM EDTA, pH 7.5 buffer. This was transferred to a 2 ml Potter-Elalijhem test tube and pestle apparatus and 5 strokes of the pestle was applied and then aspirated through a 23G needle using a 1 ml syringe and centrifuged at 1250 x g for 10 mins at 4°C. The supernatant was layered over 20 mls of 18% percoll gradient in 0.25 M sucrose, 1 mM EDTA, pH 7.5 buffer and centrifuged in a Beckman centrifuge with JA-20 rotor at 20 000 rpm for 60 mins at 4°C. The gradient was fractionated into 20 x 1 ml fractions and assayed for β-hexosaminidase and acid phosphatase activity as described in sections 2.7.5 and 2.7.6 respectively.

2.6 *Mouse behaviour methods*

2.6.1 *Repeated Acquisition and Performance Chamber (RAPC)*

2.6.1.1 *RAPC apparatus*

The RAPC apparatus is a rectangular Plexiglas chamber with a start box and a goal box situated at opposite ends. The chamber interior is divided into five compartments of equal size separated by four transparent panels, each equipped with three one-way doors that can be latched individually, permitting access to successive compartments (Figure 2.1). Passage to the goal box and the associated saccharin reward (a 20 μl drop of 0.2% saccharin solution) was dependent upon entry into the fifth compartment. Access to the first compartment was controlled by a guillotine style divider. The chamber was connected via a chamber controller and used the computer software programs RAPC experiment™.
**Figure 2.1: Setup of the RAPC apparatus.**

A diagrammatic view of the RAPC setup and location of the start box, doors and saccharin reward (A). An actual view of the setup and the location of the saccharin reward (B). Trials were conducted in a dimly lit room with the observer sitting near the start box. For the performance phase of all trials all doors were locked with the exception of A3, B3, C3 and D3. For the repeated acquisition phase the sequence of open doors was randomly assigned on each day with the remaining doors locked.
Repeated Acquisition and Performance Chamber (RAPC)

A

Start Box

One Way Doors

B

C

D

Goal Chamber

Saccharin Reward

B

Saccharin reward
and RAPC edit™ to edit and run the testing protocols for each mouse. For the performance phase of all trials all doors were locked with the exception of A3, B3, C3 and D3. For the repeated acquisition phase the sequence of open doors was randomly assigned on each day with the remaining doors locked (Appendix I). All testing was conducted in a dimly lit room to promote motivation and done between the hours of 8am and 1pm.

2.6.1.2 Saccharin habituation

Before introducing the mice into the Repeated and Performance Chamber (RAPC), normal control mice and MPS IIIA age-matched mice were habituated to a 0.2% (w/v) sodium saccharin solution. Mice were water deprived for 18-20 h overnight and the following morning a 0.2% (w/v) solution of saccharin was administered for 2 x 30 minute sessions instead of normal drinking water. Mice were observed during this period to ensure that they consumed the saccharin solution. After the two sessions were completed, normal water was restored to each mouse and mice were observed for any signs of dehydration in the 24 h period after water deprivation. Mice were weighed to ensure that they returned to normal bodyweight after the water was restored.

2.6.1.3 Habituation A

Prior to testing in the Repeated Acquisition and Performance Chamber (RAPC), normal control mice and MPS IIIA age-matched mice (approx 11 weeks of age) were habituated to the chamber via the following protocol. The day before testing, mice were deprived of water overnight (18-20 hours). Habituation A consisted of the mice being placed in the enclosed start box, and then entering the chamber through the guillotine door. The 12 doors located inside the chamber were taped open with electrical tape, to allow the mice to explore freely. In front and behind all doors (total 24 positions) a 20 µl drop of 0.2% (w/v)
saccharin solution was placed. The mouse remained in the chamber for 20 minutes after
which it was removed and returned to its cage. The number of drops consumed by the
mouse was recorded.

2.6.1.4 Habituation B

Habituation B was performed under the same conditions as Habituation A, however
instead of all the doors being taped open with electrical tape, the doors remained closed,
but unlocked. Once the mouse reached the final goal box, it was removed and placed back
into the enclosed start box and the procedure was repeated until the 20 minute timeframe
was completed. The sequence of door entry was recorded as well as the number of
saccharin drops consumed by each mouse.

2.6.1.5 Habituation C

Habituation C was performed under the same conditions as Habituation B, however,
instead of putting saccharin drops in front and behind all the door entry and exit points, the
saccharin drops were only placed at the exit points of the chamber C and chamber D doors.
In addition to these six drops, one drop of saccharin solution was placed in the middle of
the goal box (along the furthest edge) giving a total of 7 saccharin drops.

2.6.1.6 Habituation D

Habituation D was performed under the same conditions as Habituation C, however,
instead of putting saccharin drops at the exit points of the chamber C and D doors, they
were only placed at the exit points to the chamber D doors as well as the goal box (total 4
drops).
2.6.1.7 Performance habituation

The performance phase of the test was distinguished by a static audio signal which was activated on the computer when each performance trial commenced. A performance habituation was necessary to teach the mice that only one correct door (with the rest being locked) in each set of doors could be entered successfully. This was because during the other habituation protocols all the doors remained closed but unlocked, allowing the mice to ‘choose’ which door to enter. Under the performance habituation conditions, this was no longer the case, and required the mouse to remember which door sequence contained the unlocked doors. Performance habituation started by the observer locking all doors except A3, B3, C3 and D3 and placing a drop of saccharin solution in the goal box area as shown in Figure 2.1. The mouse was removed from cage, weighed and placed in the enclosed start box. The mouse entered the first chamber and proceeded to explore this chamber. Errors and the time the doors were attempted to be opened were recorded by the computer program. Once the mouse reached the goal box and consumed the reward solution the time was stopped. After completion of the trial the mouse was removed from the goal box and transferred to the enclosed start box. The chamber was then wiped over quickly with a dilute solution of baby shampoo and dried with a towel. The reward solution was replaced in the goal chamber and the second test commenced. This was repeated until three trials were completed. After the third trial the mouse was returned to its cage and water was restored.

2.6.1.8 Performance protocol

The performance (P) protocol was performed as for the performance habituation, however, due to a lack of motivation of the mice used, only two trials were completed. This was interchanged with two repeated acquisition trials and then repeated 2 x performance trials, 2 x repeated acquisition trials, 2 x performance trials, 2 x repeated acquisition trials as
Tables 2.1 and 2.2: Setup of the RAPC trials.

Table 2.1 shows the setup of the sequences used for the performance and repeated acquisition phases in the 12 week study. The performance sequence (sequence 1) remains constant throughout the 3 testing sessions, whereas the repeated acquisition sequence (sequences 2-4) changes on a random basis with each session. Table 2.2 illustrates the testing protocol during one testing session which comprises of 12 trials, 3 x 2 blocks of testing for each phase with a 2 minute inter-trial interval for each mouse.
Table 2.1: Sequences used for the different phases of RAPC

<table>
<thead>
<tr>
<th>Testing Session</th>
<th>Performance</th>
<th>Repeated Acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sequence 1</td>
<td>Sequence 2</td>
</tr>
<tr>
<td>2</td>
<td>Sequence 1</td>
<td>Sequence 3</td>
</tr>
<tr>
<td>3</td>
<td>Sequence 1</td>
<td>Sequence 4</td>
</tr>
</tbody>
</table>

Table 2.2: Overview of a testing session setup

<table>
<thead>
<tr>
<th>Session</th>
<th># Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performance</td>
<td>2 (A1, A2)</td>
</tr>
<tr>
<td>Learning</td>
<td>2 (A1, A2)</td>
</tr>
<tr>
<td>Performance</td>
<td>2 (B1, B2)</td>
</tr>
<tr>
<td>Learning</td>
<td>2 (B1, B2)</td>
</tr>
<tr>
<td>Performance</td>
<td>2 (C1, C2)</td>
</tr>
<tr>
<td>Learning</td>
<td>2 (C1, C2)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>12</td>
</tr>
</tbody>
</table>
shown in Table 2.2 to give a total of 12 trials. Latency was measured as the time required for the mouse to leave the start box, navigate through the four compartments, and consume the saccharin solution in the goal box. Mice were placed manually in the goal box if they failed to reach it within 6 min on any trial and a latency score of 360 seconds was recorded along with any errors they made up until 360 seconds. Errors were defined as attempts to go through a locked door. Mice were tested at 12 weeks (session #1-4), 20 weeks (sessions #5-8) and 31 weeks (session #9-12) of age to determine if there were learning deficits associated with MPS IIIA pathology.

2.6.1.9 Repeated acquisition protocol

The repeated acquisition (RA) analysis was carried out in the same manner as the performance testing, however this phase of the test did not have an auditory stimulus from the internal speakers. On each day a random door sequence was chosen (see Table 2.1 and Appendix 1 for sequences) and this consisted of 2 trials per presentation with a total of 3 presentations (A, B, C) (i.e. total of 6 RA trials). RA trials were performed after a presentation of performance trials as shown in Table 2.2. Latency was measured as the time required for the mouse to leave the start box, navigate through the four compartments, and consume the saccharin solution in the goal box. Mice were placed manually in the goal box if they failed to reach it within 6 min on any trial and a latency score of 360 seconds was recorded along with any errors they made up until 360 seconds. Errors were defined as attempts to go through a locked door. Mice were tested at 12 weeks (sessions #1-4), 20 weeks (sessions #5-8) and 31 weeks (session #9-12) of age to determine if there were learning deficits associated with MPS IIIA pathology.
2.6.1.10 Auditory Evoked Brainstem Response

The auditory evoked brainstem response was conducted according to the MPS VII mouse testing protocol (Sands et al. 1995) and has also been used in a variety of mouse strains (Erway et al. 1993). Briefly, mice were anaesthetised by intraperitoneal injection of 5 mg/kg (w/v) xylazine and 75 mg/kg (w/v) ketamine and LacriLube was applied to eyes to prevent dehydration. Needle electrodes were placed subdermally on the top of the head (active electrode), below the right or left ear (reference electrode) and below the opposite ear to reference (ground electrode), as shown in Figure 2.2. The electrodes were used to receive the brainstem response output which was detected after the stimulus was applied and were collected with a Medelec MS92 Averager (OUTPUT). Ear tips for human neonates (Radiometer Pacific) were used to deliver broadband clicks generated from a Medelec ST10 Sensor Pre-amplifier (INPUT). The auditory brainstem response threshold was determined in descending intensities (10dB) and finally at 5dB increments (Erway et al. 1993). A range of -10 to 40 dB was usually tested. Each ear was tested and recorded separately. Animals were allowed to recover from anaesthesia in a heated environment and were given pre-warmed 0.18% NaCl/ 4% dextrose subcutaneously to prevent dehydration.

2.6.2 Morris water maze analysis

Morris water maze (MWM) analysis was carried out according to Gliddon and Hopwood (2004) at 35 weeks of age as a test of learning and memory in mice.

2.6.2.1 MWM apparatus

A plastic tub 130 cm in diameter and 40 cm in height was arbitrarily divided into four quadrants, northwest (NW), northeast (NE), southwest (SW) and southeast (SE), as shown in Figure 2.3. The observer stood near the NW quadrant. The pool was filled with water.
Mice were anaesthetised with intraperitoneal injections of 5 mg/kg xylazine and 75 mg/kg ketamine. The mouse represented by the diagram is setup for testing in the right ear. Needle electrodes were inserted subdermally as indicated (top of head (red - active), non-test ear (green - ground) and test ear (black – reference)) and connected via appropriate adaptors to a sensor receiver box. Neonatal ear tips were used to deliver broadband clicks generated from a Medelec Pre-amplifier (INPUT) ranging from -10dB to 40dB and collected using a Medelec Averager (OUTPUT) from the sensor receiver box which the needles were attached to. Brainstem responses were printed out and the threshold was determined. Each ear was tested and recorded separately. Diagram not drawn to scale.
Medelec Sensor Pre-amplifier
INPUT SIGNAL

Sensor receiver box

Medelec Averager
OUTPUT SIGNAL
Figure 2.3: Setup of the Morris water maze apparatus.

A plastic tub of 130 cm in diameter and 40 cm in height was arbitrarily divided into four quadrants, northwest (NW), northeast (NE), southwest (SW) and southeast (SE). The observer stood near the NW quadrant and the escape platform (10 cm in diameter and 1 cm below the surface of the water) was located in the SW quadrant as indicated. The pool was filled to a depth of 25 cm with water (21-25°C) containing approximately 500 g skim milk powder to make the water opaque. Mice were subjected to 4 trials, one from each of the cardinal points of pool (i.e. N, S, E, and W) randomly determined for each day and the time taken to reach the hidden platform was recorded. Posters were placed on the walls, as indicated in light blue, to provide visual cues in order for mice to spatially orient themselves in the pool. All other major fixtures in the testing room are labelled on the figure. Diagram not drawn to scale.
(21-25°C) to a depth of 25 cm, and contained approximately 500 g skim milk powder to make the water opaque. Posters were placed on the walls, to provide visual cues in order for mice to spatially orient themselves in the pool.

2.6.2.2 Habituation

All mice were acclimatised to the pool and surroundings on day 1 without the platform in the pool. Each mouse was released from the north quadrant marker and was allowed to swim for a total of 30 seconds. On day 2, habituation was repeated as per day 1 but a 10 cm platform was placed 1 cm below the surface and 30 cm from the edge of pool in the SW quadrant of the pool, providing an escape route for the mice. Each mouse was released from the north quadrant marker with its head facing the side of the pool and allowed 90 seconds to find the platform. If the mouse did not find the platform within the required time the researcher guided it to the platform allowed it to remain there for 10 seconds.

2.6.2.3 Acquisition phase

Testing officially began on day 3, whereby mice were subjected to 4 trials, one from each of the cardinal points of the pool (i.e. N, S, E, and W) in a random order. Each mouse was released with its head facing the side of pool and allowed 90 seconds to locate the platform. If the mouse did not locate the platform in time, a fail was recorded (90 seconds) and the mouse was guided to it and allowed to remain on the platform for 10 seconds. The acquisition phase was performed on days 3-7. An inter-trial time of approximately 4 minutes was standard for all acquisition trials, where mice were towel dried after their swim and allowed to rest under a heat lamp until the next trial.
2.6.2.4 Probe phase

On day 8, mice were subjected to a probe phase. This involved removing the platform from the pool and timing the amount of time spent in each quadrant for a total time of 60 seconds. A video recorder was used to record the visual path of each mouse and then each quadrant was scored on a per second interval as shown on the video recording.

2.6.2.5 Relearning phase

The relearning phase was conducted on days 9-12, which involved returning the platform in the SW quadrant and was carried as per the acquisition phase method in section 2.6.2.3.

2.6.2.6 Reversal phase

The reversal phase was conducted on days 13 and 14 whereby the platform location was switched to the opposite quadrant (NE). Even though the quadrant location was different for these trials the same method was used as for the acquisition phase method in section 2.6.2.3.

2.6.3 Cross maze analysis

Cross maze analysis was carried out according to methods published in previous studies (Dumas and Rabe 1994, Summers et al. 2006) at 30 weeks of age in MPS IIIA and age-matched normal littermates.

2.6.3.1 Cross maze apparatus

The apparatus consisted of a clear perspex plastic plus cross maze which was placed in a circular pool of water (100 cm in diameter) (23.5°C-24.5°C). The pool was completely
surrounded by a 90 cm high wall covered in black plastic. The water was made opaque by
the addition of full cream milk powder (Diploma) to conceal an escape platform (EP),
which was submerged 0.5 cm below the surface of the water. The EP was located in the
distal half of the east arm. Constant visual (spatial) cues were placed around the maze and
were provided by various objects around the room and by the observer who always stood
at the west arm (Figure 2.4).

2.6.3.2 Habituation (Day 1)
All mice were acclimatised to the pool and surroundings on day 1 without the platform in
the pool. Each mouse was released from the west arm with its head facing the side of the
pool and allowed to swim for a total of 60 seconds. Mice were towel dried and returned to
their cages.

2.6.3.3 Learning phase (Days 2-6)
Testing officially began on day 2, whereby mice were subjected to 6 trials, in 2 blocks of 3
trials, separated by a 30 minute rest. The order of the release arms without the escape
platform was randomly determined. Each mouse was released with its head facing the side
of pool and allowed 60 seconds to locate the platform. If the mouse did not locate the
platform within this time, a fail was recorded and the mouse was guided to the platform
and allowed to remain there for 20 seconds. The learning phase was performed on days 2-
6. An inter-trial time of 10 seconds was the time for all acquisition trials, where mice were
towel dried after their swim and allowed to rest under a heat lamp until the 30 minute
interval had elapsed. The latency (seconds) to find the escape platform was recorded and
entry into an arm without the escape platform was recorded as an incorrect entry. As well
as this, entry into the arm with the escape platform (east), without climbing onto the escape
Figure 2.4: Setup of the Cross maze apparatus.

A plastic tub of 100 cm in diameter and 40 cm in height was filled with water (23.5-24.5°C) containing approximately 500 g milk powder to make the water opaque. A clear perspex cross maze was placed into the opaque water and oriented so that each arm of the cross was opposite a visual cue (as indicated). A clear platform was placed 5 cm in from the wall of the east arm lengthwise and submerged 0.5 cm below the surface of the water (EP_. The whole apparatus was elevated on a metal apparatus, which was surrounded by a 90 cm wall of black plastic. The observer stood behind the metal surrounds in the west arm. Mice were subjected to 1 trial from each arm (determined randomly, trials 1-3) separated by a 30 min interval and repeated with an additional 1 trial from each arm (determined randomly, trials 4-6, with a different start position to trial 1). No other fixtures besides doors are indicated, due to the presence of the black plastic surroundings which restrict the view of mice within the pool. Diagram not drawn to scale.
platform (i.e. swimming around the platform) was also recorded as an incorrect entry. A correct entry was recorded only if the mouse went from the release arm directly to the east arm containing the escape platform and this was binomial (1/0), whereby 1 was a correct entry and 0 was an incorrect entry. If a mouse failed to reach the escape platform within the 60 seconds, allocated the mouse was placed onto the platform and left for 20 seconds before allowed on the 10 second inter-trial interval. This learning phase was conducted on days 2-6.

2.6.3.4 Memory phase (Day 16)

Mice were given a 10 day rest from the end of their learning phase. The memory phase was conducted on day 16 and consisted of 6 trials, 2 blocks of 3 separated by a 30 minute interval. Testing was conducted as for the learning phase and randomly assigned release points were used for the testing protocol.

2.6.4 Open field analysis

Open field analysis was conducted according to published methods (Hemsley and Hopwood 2005). Briefly, the apparatus consisted of a plastic box which contained 15 (5 x 3) squares measuring 9 cm x 10 cm. Each mouse was introduced in the bottom left hand corner with the head pointing towards the corner of the box and recorded for 3 minutes. During this time the number of line crosses and rears (counted as both front paws off the floor) were recorded. This was performed at 3 weeks and 10 weeks of age. The box was cleaned with ethanol in between trials. After the trial was complete mice were returned to the home cages.
Chapter Two: Materials and Methods

2.7 General protein methods

2.7.1 Protein assay: Bradford

Protein concentration was determined using the method of Bradford (Bradford 1976). Briefly, a standard curve of bovine serum albumin was constructed from 0-20 µg protein. After the addition of 200 µl Bradford reagent (BioRad) the samples were incubated at room temperature for 20 minutes. The absorbance was determined at A 595 on an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences).

2.7.2 Protein assay: Lowry

Protein concentrations were also measured using the method of Lowry (Lowry et al. 1951). Briefly, 2 µl samples of 1:10 diluted tissue extracts were assayed in a total volume of 100 µl and their protein concentration calculated from a standard curve of 0, 4, 8, 16 and 32 µg DADE Human Protein standard. One hundred microlitres of 1.7% (w/v) Na₃C₆H₅O₇.2H₂O was mixed with 100 µl 1% (w/v) CuSO₄ in dH₂O and 10 mls 2% Na₂CO₃ in 0.1 M NaOH and 1 ml was added to each sample, vortexed and left at room temperature for 1 minute. One hundred microlitres of diluted Folin and Ciocoulteau’s Reagent was added to each sample and vortexed immediately. This was incubated at room temperature for 30 minutes. The absorbance was read on a spectrophotometer at wavelength 750 nm.

2.7.3 Uronic acid assay

The concentration of uronic acid in samples was determined by the method of Blumenkrantz and Asboe-Hansen (Blumenkrantz and Asboe-Hansen 1973). Briefly, a standard curve of D-glucuronic acid lactone was constructed using concentrations of 0-10 µg uronic acid. Samples were appropriately diluted in a final volume of 200 µl and 1.2 ml sulphuric acid/tetraborate solution was added and vortexed. After hydrolysing in a 100°C
water bath, samples were cooled on ice and 20µl of meta-hydroxyphenol solution was added. The samples were vortexed and left at room temperature for 5 minutes. A 200 µl aliquot of each sample was transferred to a flat bottomed 96-well plate (Nalge Nunc, Rochester, NY, USA) and read at an absorbance of 520 nm. Unknown samples were calculated from the standard curve.

2.7.4 Determination of urine creatinine levels

Urinary creatinine concentration of 1:2 diluted mouse urine samples was routinely determined by the Core Laboratory at the CYWHS. The method used was the creatinine Jaffe Method for urine using a Creatinine Reagent kit (Beckman) and this was measured on a Beckman Synchron CX5 CE autoanalyser (Synchron CX Systems).

2.7.5 β-Hexosaminidase enzyme activity assay

β-Hexosaminidase enzyme activity was measured according to Leaback and Walker (Leaback and Walker 1961). Briefly, 30 µl aliquots of each percoll fraction along with 20 µl saline and 50 µl 4-methylumbelliferone substrate (4MU-2-acetamido-2-deoxy-β-D-glucopyranoside, 0.142 mmol/l) were incubated at 37°C for 60 minutes. The reaction was stopped by increasing the pH through the addition of 0.2 M glycine buffer, pH 10.7. The relative fluorescence of the tube was read on a Perkin-Elmer LS-50B spectrofluorimeter using a micro-flowcell and auto-sampling system and referenced to 2.84 nmol of 4-methylumbelliferone standard.

2.7.6 Acid phosphatase enzyme activity assay

Acid phosphatase enzyme activity was measured according to Kolodny and Mumford (Kolodny and Mumford 1976). Briefly, 20 µl aliquots of each percoll fraction along with
30 µl saline and 50 µl 4-methylumbelliferone substrate (4MU-phosphate, 10 mmol/l) were incubated at 37°C for 30 minutes. The reaction was stopped by increasing the pH through the addition of 0.2 M glycine buffer, pH 10.7. The relative fluorescence of the tube was read on a Perkin-Elmer LS-50B spectrofluorimeter using a micro-flowcell and auto-sampling system and referenced to 2.84 nmol of 4-methylumbelliferone standard.

2.8 Histopathology methods

2.8.1 Processing of tissue for paraffin embedding

Tissues for paraffin embedding were fixed in 10% buffered formalin for 24 h at room temperature after which they were transferred to 70% ethanol. Fixed tissues were routinely processed by the Department of Histopathology, CYWHS (South Australia, Australia) using a Tissue Tek VIP processor overnight under pressure and vacuum and embedded in paraffin wax.

2.8.2 Processing of tissue for Spurr’s resin embedding

Tissues for embedding in Spurr’s epoxy resin were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 24 h at 4°C and then transferred to 0.1 M sodium cacodylate buffer pH 7.4 for storage. Tissues were routinely processed by the Department of Histopathology at the Children, Youth and Women’s Health Service (South Australia, Australia) and also Adelaide Microscopy (Adelaide, South Australia, Australia).
2.8.3 Preparation of APES coated slides

Clean microscope slides were loaded into racks and immersed in ethanol for 5 minutes at room temperature. The slides were transferred to ethanol containing 2% (v/v) APES solution for 10 seconds, rinsed in ethanol and washed in distilled water. Slides were dried in an oven at 60°C overnight.

2.8.4 Sectioning of paraffin embedded tissues

Paraffin sections (4 μm) were cut on a Leitz 1512 rotary microtome. The sections were floated on a 50°C waterbath and transferred to APES coated slides. The slides were dried on a 50°C hot plate for 10 minutes and stored until stained.

2.8.5 Sectioning of Spurr's epoxy resin embedded tissues

Resin embedded tissues (1 μm) were cut using a glass knife on a Reichert Ultracut S ultramicrotome (Leica Microsystems, Wetzlar, Germany). The sections were floated in a boat of distilled water formed by securing aluminium tape around the diagonal face of a glass knife. Sections were removed from the boat with a paintbrush and transferred to glass slides and left to dry. Sections were stained with toluidine blue as described in section 2.8.6. To determine eye pathology, 1 μm sections were cut and stained with toluidine blue at Adelaide Microscopy using routine methods.

2.8.6 Toluidine blue staining

Slides with 1 μm thick resin embedded sections were prepared as per section 2.8.5. Slides were placed on a 70°C heating block to melt the plastic resin. All sections were covered in filtered 1% toluidine blue in 1% borax solution and left on the heating block until the edges
of the stain turned metallic black-green. Slides were washed thoroughly under running distilled water and mounted using DPX mounting media and coverslipped.

### 2.8.7 Light microscopy

Toluidine blue stained sections were analysed using an Olympus BX40 compound light microscope with attached Olympus DP50 camera. Images were collected using Viewfinder Lite version 1.0 and Studio Lite version 1.0 software. Sections from the special stains analysis were analysed using an Olympus BX51 light microscope with an Olympus U-D03 camera attachment.

### 2.8.8 Electron microscopy

Tissue samples were prepared for electron microscopy by staff at Adelaide Microscopy. Samples embedded in Spurr’s resin were cut at a thickness of 70-80 nm using a Reichart ultracut S microtome. The sections were stained for 10 minutes with uranyl acetate and 10 minutes in lead citrate. Electron microscopic analysis was conducted at Adelaide Microscopy using a Philips CM100 transmission electron microscope. Images acquisition was done using analySIS ® image analysis software (Olympus Soft Imaging Solutions GmbH, Japan).

### 2.9 Statistical analyses

#### 2.9.1 Bodyweight analyses

The relationship between bodyweight and time for normal and MPS IIIA mice with treatment was estimated using Restricted or Residual Maximum Likelihood (REML). Statistical analysis was conducted by Kate Dowling and Janine Jones at Biometrics SA.
2.9.2 Tissue weight and gag analyses

All other statistical analyses were conducted using one-way ANOVA with a post-hoc Tukey’s HSD test was used to measure the statistical significance between designated variables (SPSS, version 11.0).

2.9.3 RAPC analyses

Statistical analysis for the RAPC was conducted by Kate Dowling at Biometrics SA.

Latency:

The latency data from the P Phase and RA phase trials were analysed separately. Wald tests were used to determine the effects of the three-way interaction of genotype, session and trial, the two-way interactions of genotype and session and session and trial and the genotype, session and trials main effects. If a factor or interaction was found to be statistically significant then least significant difference (LSD) tests were conducted. A value of less than 0.05 was determined to be statistically significant.

Errors

The errors response is count data and so was analysed using a generalised linear model assuming the Poisson distribution and a log link (a log-linear model). Deviance tests were used to determine the significance of the genotype, session and trial main effects and their two- and three-way interactions. LSD tests were conducted if a factor or interaction was found to be statistically significant. A value of less than 0.05 was determined to be statistically significant.
2.9.4 Cross maze analyses

Statistical analysis for the cross maze was conducted by Kate Dowling from Biometrics SA.

The relationship between escape latency, genotype, treatment and sex was analysed using Restricted or Residual Maximum Likelihood (REML (Patterson and Thompson 1971)) for both learning and memory tests. Wald tests were used to determine the effects of day, treatment, sex and their two and three-way interactions. If a factor or interaction was found to be statistically significant then least squares difference (LSD) tests were conducted. Binary data from the correct trials for both the learning and memory trials was analysed using logistic regression. Data from the incorrect entries/re-entries data was analysed using a log-linear model. Both parameters used deviance tests to determine the significance of the day (learning only), sex and treatment main effects and their interactions.
CHAPTER 3
INHIBITION OF GAG SYNTHESIS *IN VITRO*
AND IN MURINE MPS IIIA
3.1 Introduction

Intravenous enzyme replacement therapy is the first effective multi-tissue treatment for children with MPS. It is available clinically for MPS I (approved for use in Japan, EU and USA⁹), MPS II (approved for use in USA by FDA in July 2006 and awaiting approval in early 2007 in the EU¹⁰) and MPS VI (approved for use in EU and USA in 2006¹¹). However, not all symptoms of MPS are treated by intravenous ERT, and pathology in the brain, cartilage and cornea remain unaffected (Crawley et al. 1997, O’Connor et al. 1998, Byers et al. 2000, Gliddon and Hopwood 2004, Brady 2006). The development of additional and/or adjunct therapies to address these sites of pathology is the subject of intensive research.

Substrate deprivation therapy (SDT) aims to reduce the amount of substrate produced by the cell, rather than increasing the amount of deficient enzyme, in order to correct pathology. SDT has proven effective in a number of non-MPS lysosomal storage disorders (reviewed in Butters et al. 2003, Platt et al. 2003). An inhibitor of ceramide synthesis, NB-DNJ, has been used in an in vitro Gaucher disease model and subsequently in animal models of Fabry, Sandhoff and Tay-Sachs disease (Platt et al. 1994a, 1997a, 2003, Jeyakumar et al. 1999). Treated Tay-Sachs mice displayed a reduction in glycolipid storage and a reduction in neuropathology (Platt et al. 1997a). Sandhoff mice had delayed onset of pathology, reduced storage in the brain and peripheral tissues, and their life expectancy was increased by 40% (Jeyakumar et al. 1999). Treatment also reduced endothelial dysfunction in Fabry mice (Platt et al. 2003, Heare et al. 2006). Currently, NB-DNJ is approved for use in patients with type I Gaucher disease (Cox et al. 2000, 2003).

Improvements in clinical parameters such as reduction in liver and spleen volumes were

⁹ http://phx.corporate-ir.net/phoenix.zhtml?c=106657&p=irol-newsArticle&ID=924259&highlight=
¹⁰ http://www.shire.com/shire/NewsAndMedia/PressReleases/showShirePress.jsp?ref=661&tn=3&m1=8&m2=
observed. Clinical trials are currently underway to evaluate NB-DNJ in type III Gaucher disease, juvenile Tay-Sachs and Sandhoff, and Niemann-Pick type C.

Transfer of this concept to the MPS disorders requires the inhibition of gag chain synthesis. Conceptually, this could occur at the level of chain initiation, elongation or sulphation (reviewed in Funderburgh 2002, Silbert and Sugumaran 2002, Sugahara and Kitagawa 2002). Rhodamine B is thought to inhibit chain elongation but is not specific to one particular gag type (Kaji et al. 1991a, 1991b). The more specific inhibitors of individual gag synthesis, such as 4-deoxy-4-fluoro-2-acetamido-2-deoxy-D-glucose (F-GlcNAc) and 4-deoxy-4-fluoro-2-acetamido-2-deoxy-D-galactose (F-GalNAc), which inhibit heparan sulphate gag synthesis, are also thought to affect chain elongation (Berkin et al. 2000a). Alternatively, inhibitors that alter the gag sulphation patterns (such as sodium chlorate) are applicable to five of the MPS types (Greve et al. 1988, van Kuppeveld et al. 1997). The isoflavone, genistein, which inhibits tyrosine kinase, is thought to decrease gag synthesis (Nikitovic et al. 2003, Mitropoulou et al. 2002) or the expression of the parent proteoglycan molecule depending on cell type (Schonherr et al. 1997). A recent in vitro study has shown that genistein is able to reduce the amount of gag synthesised in human skin fibroblasts and have a flow-on effect on gag storage as measured using biochemical and electron microscopic analysis (Piotrowska et al. 2006).

Murine MPS IIIA is a naturally occurring mouse model of MPS IIIA. It results from a missense mutation (G91A) in the sulphamidase gene, causing a reduction in sulphamidase activity to 3-4% of normal levels (Bhaumik et al. 1999, Bhattacharyya et al. 2001). Mice display increased bodyweight compared to age-matched normal mice, have a hunched posture, facial dysmorphism and scruffy appearance, as well as widespread lysosomal storage (Bhaumik et al. 1999). This mouse model provides a good homologue of the
human disease since it displays similar biochemical and clinical changes to those observed in human MPS IIIA patients and is a good model for evaluation of the effects of lysosomal storage on both somatic and CNS pathology (Bhaumik et al. 1999, Bhattacharyya et al. 2001, Gliddon and Hopwood 2004).

No in vivo studies have been reported for any inhibitor of gag synthesis in MPS disorders. In this chapter the effect of rhodamine B and sodium chlorate on gag synthesis was first examined in cultured cells. The rhodamine B study was then extended to determine its effect on gag storage in MPS cells and, in vivo on the progression of pathology in the MPS IIIA mouse over a 6 month period. Evaluation included biochemical, histological and physical parameters to determine the effect of rhodamine B treatment on somatic disease.
Chapter Three: Inhibition of gag synthesis in vitro and in murine MPS IIIA

3.2  In vitro results

3.2.1  Inhibition of gag synthesis by rhodamine B in normal and MPS cells

The addition of increasing concentrations of rhodamine B to the culture medium of MPS IIIA skin fibroblasts or Be(2)-C neuroblastoma cells (Section 2.3.1.1) resulted in a dose-dependent decrease in Na\(^{35}\)SO\(_4\) incorporation into gags (Figures 3.1A and B). All concentrations of rhodamine B tested resulted in a decrease in gag synthesis in Be(2)-C cells but was only apparent in skin fibroblasts at rhodamine B concentrations of 50 \(\mu\)g/ml or greater. Protein synthesis (Section 2.3.1.2), as measured by the incorporation of \(^3\)H-serine, was unaffected by the addition of rhodamine B up to 75 \(\mu\)g/ml (Figure 3.1A), however at concentrations of greater than 75 \(\mu\)g/ml, protein synthesis was reduced.

To determine whether the inhibition of gag synthesis results in a reduction in lysosomal gag storage, MPS IIIA, MPS VI patient and control skin fibroblasts were incubated with 25 \(\mu\)g/ml rhodamine B for 14 days (Section 2.3.1.3). On day 14, cells were labelled with Na\(^{35}\)SO\(_4\) and then separated on a percoll density gradient to isolate lysosomal compartments (Section 2.3.1.3) (Byers et al. 1998), as determined by the presence of the lysosomal enzyme \(\beta\)-hexosaminidase (Leaback and Walker 1961) (Section 2.7.5). Rhodamine B treatment reduced stored gag in the lysosomes of both MPS IIIA and MPS VI skin fibroblasts by 60% and 72.5% respectively (Figure 3.2). This confirms that rhodamine B is a non-specific inhibitor of gag synthesis, since MPS IIIA and MPS VI store different gags (heparan sulphate and dermatan sulphate, respectively). These results suggest that inhibition of gag synthesis does have a flow-on effect to reduce gag storage in vitro and the mechanism is not specific for one type of gag.
Figure 3.1: *In vitro* dose response with rhodamine B.

MPS IIIA skin fibroblasts (A) or Be(2)-C neuroblastoma cells (B) were incubated with varying concentrations of rhodamine B for 6 hrs at 37°C. Na$^{35}$SO$_4$ (5 μCi/ml) was added to the culture medium to determine gag synthesis or 5 μCi/mL $^3$H-serine to determine protein synthesis. Results are the mean ± standard deviation of n=3 replicates.
Figure 3.2: Lysosomal gag content with rhodamine B.

Skin fibroblasts were incubated with 25 μg/mL rhodamine B for 2 weeks prior to isolation of the lysosomal fraction by percoll gradient centrifugation. Lysosomal gag was determined by uronic acid assay. Gag storage in untreated fibroblasts was denoted as 100% and gag storage in rhodamine B treated fibroblasts expressed as a percentage of that in untreated cells. Results are the mean ± standard deviation of n=2 replicates.
3.2.2 Inhibition of gag sulphation by sodium chlorate in normal cells

The addition of increasing concentrations of sodium chlorate to the culture medium of Be(2)-C neuroblastoma cells (Section 2.3.1.1) resulted in a dose-dependent decrease in Na$^{35}$SO$_4$ incorporation into gags (Figure 3.3). To achieve a reduction of greater than 50% incorporation of Na$^{35}$SO$_4$, a concentration of greater than 10 mM sodium chlorate was required. The addition of sodium chlorate up to 50 mM had minimal effect on protein synthesis (Section 2.3.1.2). No further investigation using sodium chlorate was carried out in this study.

3.3 Effect of rhodamine B treatment in an in vivo MPS IIIA mouse model

An in vivo SDT trial was set up in the MPS IIIA mouse (Section 2.4.1). Normal and MPS IIIA mice were administered 1 mg/kg rhodamine B by weekly intravenous tail vein injection for 6 months (Section 2.4.7). The effect of rhodamine B on disease pathology was evaluated using physical parameters (Section 2.4) biochemical analysis (Sections 2.5 and 2.7) and histological analysis (Section 2.8).

3.3.1 Physical appearance

MPS IIIA mice have scruffy fur, a hunched posture, abdominal distension and a flattened face (Figure 3.4 right-hand side) compared to normal mice (Figure 3.4, bottom left-hand side). Both normal and MPS IIIA animals received rhodamine B weekly over a six-month treatment period from four weeks of age. Treated MPS IIIA mice had a sleeker, less scruffy and hunched appearance (Figure 3.4, top left-hand side) compared to their untreated counterparts (Figure 3.4, right-hand side). No difference was observed between normal treated and untreated mice with respect to overall physical appearance (image not shown).
Figure 3.3: *In vitro* dose response with sodium chlorate.

Be(2)-C neuroblastoma cells were incubated with varying concentrations of sodium chlorate for 6 hrs at 37°C. Na<sup>35</sup>SO₄ (5 μCi/ml) was added to the culture medium to determine gag synthesis (□-□) or 5 μCi/mL <sup>3</sup>H-serine to determine protein synthesis (♦-♦). Results are the mean ± standard deviation of n=3 replicates.
Figure 3.4: Rhodamine B treatment effect on physical appearance

Physical appearance of an MPS IIIA mouse treated with rhodamine B (top left), an untreated MPS IIIA mouse (right) and untreated normal (bottom left) at 7 months of age. Scale bar indicates 1 cm.
3.3.2 Bodyweight profile

All untreated MPS IIIA mice gained weight more rapidly than normal mice, such that by 16-weeks MPS IIIA males were 129% of normal mice bodyweight (p< 0.001, REML) and MPS IIIA females were 117% of normal bodyweight (p=0.0025, REML, Figures 3.5A and B respectively). A decrease in bodyweight gain was observed in treated, male MPS IIIA animals compared to their untreated littermates (p=0.011, REML) which became evident after 100-days of age (Figure 3.5A), such that at 16-weeks of age, the bodyweight of treated MPS IIIA animals was 81% of the untreated MPS IIIA bodyweight and 105% of normal weight. A reduction in bodyweight gain by female MPS IIIA mice was also observed upon treatment with rhodamine B (to 87% of the untreated bodyweight at 16-weeks, Figure 3.5B), however, the difference was not significant (p>0.05, REML). No difference between normal untreated and treated mice was observed in bodyweight profiles for either males or females (Figures 3.5A and B respectively; p>0.05, REML).

3.3.3 Urinary gag output

Total urinary gag levels (μg/nmol creatinine) in untreated MPS IIIA mice varied between individual animals but generally were elevated by up to 2-fold compared to normal mouse urinary gag (Figure 3.6). Treatment of MPS IIIA mice with 1 mg/kg rhodamine B reduced the urinary excretion of gag towards normal, an effect that was particularly evident at earlier times, less than 117 days of age. Treatment of normal animals with 1 mg/kg rhodamine B had no significant effect on urinary gag levels (p>0.05, one-way ANOVA).

3.3.4 Organ weights

One of the clinical symptoms in MPS IIIA patients (Neufeld and Muenzer 2001), which is also a feature of the MPS IIIA mouse (Bhaumik et al. 1999), is an increase in abdominal
Figure 3.5: Effect of rhodamine B treatment on bodyweight gain

Bodyweight of male (a) and female (b) normal untreated (□), normal treated (■) MPS IIIA untreated (■) and MPS IIIA treated mice (♦). Arrow denotes when treatment was started. p< 0.001, REML, untreated MPS IIIA versus normal males. p=0.0025, REML, untreated MPS IIIA versus normal females. p=0.011, REML, untreated versus treated MPS IIIA males. p>0.05, REML, untreated versus treated MPS IIIA females. p>0.05, REML, treated versus untreated normal females.
Figure 3.6: Effect of rhodamine B treatment on urinary gag excretion

Urinary glycosaminoglycan excretion expressed as µg of uronic acid normalised to urine creatinine. MPS IIIA untreated (○) and MPS IIIA treated mice (●). The green solid line and the dotted lines represent the mean uronic acid concentration in normal urine ± 1 standard deviation respectively. Number of replicates varies from at least n=3 from each group.
organ size. It was found that MPS IIIA mice displayed up to 2-fold increase in liver (Figure 4.7A, p<0.05, one-way ANOVA) and up to a 3-fold increase in spleen size (Figure 4.7B, p>0.05, one-way ANOVA) compared to normal mice, confirming the report of Bhaumik et al. (Bhaumik et al. 1999). Specifically, liver and spleen size of normal mice were not altered following treatment with rhodamine B (p>0.05, one-way ANOVA). A reduction in liver weight was observed in the MPS IIIA animals treated with 1 mg/kg rhodamine B compared to untreated mice at 4 months of treatment (p<0.05, one-way ANOVA), but this difference was not statistically significant at 6 months of treatment (p>0.05, one-way ANOVA) (Figure 3.7A). Rhodamine B treatment had no effect on spleen size, which was still increased in the MPS IIIA treated mice (Figure 3.7B) and was not statistically different to MPS IIIA untreated counterparts (p>0.05, one-way ANOVA). Weights of all other tissues were in the same range as the normal mice and treatment of normal mice with rhodamine B did not alter any of the post-mortem organ weights (Table 3.1, p>0.05, one-way ANOVA).

3.3.5 Gag content

Total liver gag content was elevated in MPS IIIA untreated compared to normal untreated gag content (Figure 3.8A, p<0.05, one-way ANOVA). Treatment of MPS IIIA reduced the gag content towards normal, however this was not statistically significant compared to MPS IIIA untreated mice (Figure 3.8A, p>0.05, one-way ANOVA). No difference was noted in total gag content of normal untreated or treated livers (Figure 3.8A p>0.05, one-way ANOVA). After 6 months of treatment, lysosomal gag content, as determined by percoll fractionation in MPS IIIA liver, was also reduced towards normal levels (Figure 3.8B). Lysosomal brain gag was also reduced in MPS IIIA treated mice (Figure 3.9) compared to MPS IIIA untreated mice.
Figure 3.7: Effect of rhodamine B treatment on liver and spleen weight

Liver (A) and spleen (B) weight at post-mortem. Normal untreated (◆), normal treated ( userType), MPS IIIA untreated (■), MPS IIIA mice treated with 1mg/kg rhodamine B (■) at 2, 4 and 6 months of treatment with Rhodamine B. *Significant difference (p<0.05) (Tukey’s HSD), MPS IIIA versus normal. †Significant difference (p<0.05) (Tukey’s HSD), MPS IIIA treated versus MPS IIIA untreated. Normal treated versus untreated p>0.05.
Table 3.1: Normal untreated versus treated post-mortem tissue weights

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age (days)</th>
<th>Normal Untreated</th>
<th>Normal Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>60</td>
<td>0.110 ± 0.042</td>
<td>0.147 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.150 ± 0.040</td>
<td>0.187 ± 0.035</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.150 ± 0.026</td>
<td>0.177 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.210 ± 0.000</td>
<td>0.180 ± 0.000</td>
</tr>
<tr>
<td>Kidney</td>
<td>60</td>
<td>0.335 ± 0.021</td>
<td>0.373 ± 0.047</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.400 ± 0.030</td>
<td>0.427 ± 0.104</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.450 ± 0.087</td>
<td>0.480 ± 0.078</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.450 ± 0.000</td>
<td>0.520 ± 0.000</td>
</tr>
<tr>
<td>Lung</td>
<td>60</td>
<td>0.140 ± 0.000</td>
<td>0.157 ± 0.021</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.157 ± 0.057</td>
<td>0.207 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.180 ± 0.010</td>
<td>0.187 ± 0.042</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.220 ± 0.000</td>
<td>0.240 ± 0.000</td>
</tr>
<tr>
<td>Brain</td>
<td>60</td>
<td>0.425 ± 0.007</td>
<td>0.387 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.357 ± 0.068</td>
<td>0.410 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.443 ± 0.021</td>
<td>0.420 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>0.400 ± 0.000</td>
<td>0.460 ± 0.000</td>
</tr>
</tbody>
</table>

Post-mortem tissues weights are reported for normal untreated and normal treated mice at 60, 90, 150 and 210 days of treatment (i.e. 1, 2, 4, and 6 months of treatment) Results are expressed as the average ± standard deviation of each tissue for each group. No statistical significance was reached for any tissue (p>0.05, one-way ANOVA)
Figure 3.8: Effect of rhodamine B treatment on tissue gag

Total liver gag (A) content in normal untreated (◆), normal treated (◆) MPS IIIA untreated (■) and MPS IIIA treated mice (■). Normal treated versus untreated p>0.05. Lysosomal gag content (B) in liver of normal (green bars), MPS IIIA treated (purple bars) and MPS IIIA untreated (blue bars) (of 7 month old mice). Data is expressed as the mean ± standard deviation of n=5 for liver size and n=3 for liver gag. * indicates p<0.05, Tukey’s HSD, MPS IIIA versus normal and ** indicates p<0.05, Tukey’s HSD, untreated versus treated MPS IIIA.
**A**

Lysosomal gag content (ȝg UA)

µg UA/g liver tissue

Age (days)

90 150 210

**B**

Lysosomal gag content (µg UA)

Normal UnTx  MPS IIIA Tx  MPS IIIA UnTx

0 2 4 6 8 10 12 14 16
Figure 3.9: Effect of rhodamine B treatment on brain gag

Lysosomal gag content in brain of normal (green bars), MPS IIIA treated (purple bars) and MPS IIIA untreated (blue bars) mice. Data is expressed as the mean ± standard deviation of n=3 at 7 months of age.
3.3.6 Tissue Histology

Analysis of liver sections from normal treated mice showed no aberration of normal cell architecture (Figure 3.10D) compared to normal untreated mice (Figure 3.10B). Liver sections from both normal untreated and treated mice showed hepatic lobules typical of liver structure. In contrast, extensive lysosomal vacuolation was observed in the liver sections of MPS IIIA untreated mice. This vacuolation was observed in hepatocytes (Figure 3.10A) and was not altered by treatment with 1 mg/kg intravenous weekly injections of rhodamine B (Figure 3.10C). Storage was also evident in the endothelial cells (Figure 3.10A and 3.10C).

Treatment with rhodamine B in normal mice (Figure 3.11D) did not alter the morphology of kidney sections from normal mice (Figure 3.11B). The proximal convoluted tubules (PCT) with their prominent brush border of tall microvilli which almost completely filled the lumen were clearly evident. The distal convoluted tubules (DCT) were identified by the absence of their brush border, a larger more clearly defined lumen and more nuclei evident per cross-section. In untreated MPS IIIA mice, the proximal convoluted tubule in the kidney showed minimal vacuolation, however the distal convoluted tubule showed extensive storage vacuoles in epithelial cells (Figure 3.11A). The storage was not altered by rhodamine B treatment (Figure 3.11C).

Normal cerebellum (Figure 3.12) showed 3 distinct layers; the molecular layer (Figure 3.12, labelled ML) containing few neurons and unmyelinated fibres, the granular layer (Figure 3.12, labelled GL) which was extremely cellular. Between these two layers was a single layer of huge neurons called Purkinje cells (Figure 3.12, labelled P). No difference was noted in the cerebellar structure of normal untreated (Figure 3.12B) and normal treated (Figure 3.12D) sections. Lysosomal storage was evident in sections from MPS IIIA
Figure 3.10: Lysosomal storage analysis in liver sections

Toluidine blue stained 5µm paraffin sections of liver samples taken at 7 months of age (6 months of Rhodamine B treatment). A = MPS IIIA Untreated, B = Normal Untreated, C = MPS IIIA Treated, D = Normal Treated. Sections taken at 1000x original magnification. Bar = 25 µm. Arrows indicate lysosomal storage in hepatocytes and arrowheads indicate lysosomal storage in endothelial cells.
Figure 3.11: Lysosomal storage analysis in kidney sections.

Toluidine blue stained 5μm paraffin sections of kidney samples taken at 7 months of age (6 months of Rhodamine B treatment). A = MPS IIIA Untreated, B = Normal Untreated, C, D = Normal Treated. Sections taken at 1000x original magnification. Bar = 25 μm. Arrows indicate lysosomal storage in the proximal convoluted tubule (PCT) epithelial cells as opposed to the distal convoluted tubule (DCT).
Figure 3.12: Lysosomal storage analysis in cerebellum sections.

Toluidine blue stained 5µm paraffin sections of cerebellum samples taken at 7 months of age (6 months of Rhodamine B treatment).  A = MPS IIIA Untreated, B = Normal Untreated, C= MPS IIIA Treated, D = Normal Treated. Sections taken at 1000x original magnification. Bar = 25 µm. Arrows indicate lysosomal storage in Purkinje cells (P) and arrowheads in neurons. ML indicates molecular layer and GL indicates granular cell layer.
untreated mice in Purkinje cells and also in a few neurons in the ML (Figure 3.12A). No alteration in the level of storage vacuoles was observed in MPS IIIA mice treated with rhodamine B; in either Purkinje cells or neurons (Figure 3.12C).

The cerebral cortex of normal untreated and treated mice (Figure 3.13B and D, respectively) had a predominance of pyramidal neurons and treatment with rhodamine B caused no abnormalities in neuronal structure. In contrast, cortical neurons from MPS IIIA untreated mice contained lysosomal storage vacuolation (Figure 3.13A). Sections from MPS IIIA treated mice showed similar levels of storage to the MPS IIIA untreated mice (Figure 3.13C).
**Figure 3.13: Lysosomal storage analysis in cerebral hemisphere sections.**

Toluidine blue stained 5µm paraffin sections of the cortical regions of the cerebral hemisphere samples taken at 7 months of age (6 months of Rhodamine B treatment).  A = MPS IIIA Untreated, B = Normal Untreated, C= MPS IIIA Treated, D = Normal Treated. Sections taken at 1000x original magnification. Bar = 25 µm. Arrows indicate lysosomal storage in neurons.
Chapter Three: Inhibition of gag synthesis in vitro and in murine MPS IIIA

3.4 Discussion

Pathology in MPS disorders arise from an imbalance between the synthesis and degradation of gag chains and subsequent gag storage. Traditional approaches to correct this imbalance rely on replacing the deficient enzyme by BMT, ERT or gene therapy to remove accumulated substrate (Peters et al. 1998, Neufeld and Muenzer 2001, Eto and Ohashi 2002, Krivit 2002, Desnick 2004, Harmatz et al. 2004, Wraith et al. 2004). More recently, novel approaches seek to reduce the synthesis of substrate and thus prevent or minimise its initial accumulation. The latter approach is known as substrate deprivation therapy (SDT). Pathology in MPS has been shown to correlate to the level of gag storage (Bunge et al. 1998), thus any treatment that reduces lysosomal storage is likely to have a positive effect. In MPS patients with detectable residual enzyme activity, decreased delivery of gag to the lysosome is expected to more closely match the capacity of residual enzyme activity and improve gag turnover rate, which should have a positive clinical outcome. Inhibitors of glycolipid synthesis have been used in vitro (Platt et al. 1994a, 1994b), and have been shown to have positive effects in in vivo animal models (Platt et al. 1997a, 1997b, Jeyakumar et al. 1999) and are currently in the clinic or clinical trials for a number of glycoplipid disorders (Cox et al. 2000, 2003, Butters et al. 2003). The aim of this chapter was to determine if this type of approach could be adapted to reduce the synthesis of glycosaminoglycans, which are the primary storage material in MPS disorders.

This study has demonstrated that rhodamine B and sodium chlorate reduced gag synthesis in normal cells. Furthermore rhodamine B was also able to reduce gag synthesis in MPS cells, including some tissues that display pathology in vivo. In MPS cells this decreased gag synthesis led to a reduction in the level of gag within the lysosome. The accumulation of both HS (MPS IIIA skin fibroblasts) and dermatan sulphate (MPS VI skin fibroblasts)
was reduced by rhodamine B treatment, confirming the general nature of this gag synthesis inhibitor.

This positive response \textit{in vitro} was extended to an \textit{in vivo} therapy trial in the MPS IIIA mouse. Both normal and MPS IIIA mice tolerated the 6 month treatment regimen of 1 mg/kg weekly intravenous injections of rhodamine B with no obvious physical side effects observed. No difference was noted in bodyweight, organ weight, organ gag content or urinary gag excretion in treated normal mice compared to untreated normal mice. This suggests that the natural level of gag may be either very low, tightly regulated or could be at the limit of detection of the assay system employed (Roberts \textit{et al.} 2006). In contrast, treated MPS IIIA mice exhibited a distinct reduction in urinary gag excretion, liver size, total gag and lysosomal gag content, as well as reduced bodyweight gain compared to untreated MPS IIIA mice. A reduction in lysosomal brain gag was also measured suggesting that rhodamine B is able to cross the blood-brain barrier and regulate brain gag synthesis, although there was persistent lysosomal storage observed by light microscopy. Further characterisation of CNS pathology in MPS IIIA is presented in chapter 4 including behaviour testing.

Despite the reduction in gag content, histological assessment of storage did not show a change in the number of lysosomal inclusions seen in liver, kidney and brain. This is in contrast to enzyme replacement therapy for MPS disorders where a reduction in the number of storage vacuoles under microscopic examination in animal models is observed (Sands \textit{et al.} 1994, Byers \textit{et al.} 2000, Gliddon and Hopwood 2004). Despite lack of effect on lysosomal inclusions, treatment with rhodamine B showed other effects such as normalisation of bodyweight, urinary gag excretion and total and lysosomal gag content in the liver, demonstrating a positive effect. A reduction in lysosomal gag content was
observed in both liver and brain of treated MPS IIIA mice suggesting that histological methods may not be able to detect these alterations in gag turnover. Another explanation is that the nature of the storage material is not of gag origin, but due to secondary storage of ganglioside, which has been reported in a number of MPS disorders including MPS IIIA (Bhaumik et al. 1999, Gliddon and Hopwood 2004, McGlynn et al. 2004, Savas et al. 2004, Walkley 2004). Most reports of this type are specific for CNS related pathology and little is known about secondary accumulation of glycolipids in somatic organs. In particular, the primary storage of heparan sulphate in both human and caprine MPS IIID (Jones et al. 1998) and human and canine MPS IIIA (Fischer et al. 1998) has been shown to be accompanied by abnormal storage of G\textsubscript{M2} and G\textsubscript{M3} gangliosides in the brain, but proof of this type of secondary storage has not confirmed in somatic organs. Rhodamine B is thought to decrease the amount of gag synthesised de novo, however, since lysosomal storage is present before birth (Greenwood et al. 1978), lysosomal storage may still be evident. This type of treatment may be halting/slowing the accumulation of recently synthesised gag targeted for degradation but will not affect gag already stored. In addition, gag metabolism is a dynamic system, and examination of lysosomal storage at a given time may have shown the presence of gag storage in sections analysed, when in fact the gag was in the process of being turned over.

The results of this chapter demonstrate that SDT can alter the progression of somatic disease in MPS IIIA. SDT with rhodamine B has the potential to affect other MPS disorders due to its non-specific inhibition of the synthesis of all gag types. Multiple small and large animal models of MPS are available and would be suitable to test the general applicability of this type of therapy (reviewed in Haskins et al. 2002, Suzuki and Mansson 1998, and Table 1.4).
Treatment with rhodamine B also reduced brain gag suggesting that this molecule is capable of crossing the blood-brain barrier. The effect of SDT on neurological function is currently unknown and Chapter 4 quantitates, via learning and memory tests, the effect of treating MPS IIIA mice with rhodamine B and the outcome on the associated CNS pathology.
CHAPTER 4

IN VIVO BEHAVIOUR PATHOLOGY
AND SDT IN MPS IIIA
4.1 Introduction

Neurological pathology is a feature of 7 of the 11 MPS types and any potential therapy must address brain storage and the resultant aberrations in neurological function. The observation that rhodamine B treatment reduced lysosomal gag levels in the brain (Section 3.3.5) suggests that this molecule is capable of crossing the blood-brain barrier. The aim of this chapter was to evaluate the effect of rhodamine B on neurological function by subjecting MPS IIIA mice to standard behaviour tests that measure learning and memory.

Behaviour paradigms to measure learning and memory have been applied to the study of human neurological disease and treatment efficacy. Each test has different advantages/disadvantages and needs to be carefully evaluated for any application for which they are considered. There are a number of variations of water mazes, cross mazes and arm mazes which test spatial learning and memory (reviewed in Crawley et al. 1997, Crawley 1999, 2000) that can be used in rodents. One of the most commonly used behaviour tests is the Morris Water Maze (Morris 1984). This tests spatial learning and memory in rodents by measuring their ability to locate a hidden platform in a pool of opaque water, using visual cues. This test involves a negative stimulus (escape from a pool of cold water), in order for mice to complete the task and receive the reward (escape platform). The time taken to reach the platform (escape latency) is a measure of learning ability. This test has been used in numerous rodent models, with early experiments being conducted in rats to investigate anatomical brain structures needed for spatial learning and memory (Morris 1984). The limitations of this test is that the primary measure of learning ability is escape latency, which has been shown to be a crude measure of learning ability in rodents since the same latency could result from very different spatial strategies (Gerlai 2001, Gerlai et al. 2002, Bartoletti et al. 2002). Escape latency can also be influenced by non-neurological factors such bodyweight, genetic background and degree of motivation. The Morris water
maze has an unrestricted area for the mice to swim in and a myriad of visual stimuli for the rodents to allow spatial orientation.

A modification of the Morris water maze, known as the cross maze or T-maze has been developed to improve performance by decreasing the available swimming area and restricting the visual stimuli to the immediate surroundings of the pool. (Dumas and Rabe 1994, Wahlsten et al. 2005, Summers et al. 2006) (Section 2.6.3 and Figure 2.4). In addition to escape latency, an advantage of the cross maze is the use of two non-time dependent parameters. Firstly, the number of incorrect arm entries/ re-entries is recorded. Secondly correct entries are also recorded and expressed as a proportion of total trials. A correct trial is recorded if the mouse goes directly into the arm containing the platform from the release point.

Water maze behaviour tests have been used to demonstrate that lysosomal storage in the brain results in a reduced capacity to learn and memorise tasks. MPS VII and MPS IIIA mice displayed a marked decline in spatial learning ability over a number of different training sessions (O'Connor et al. 1998, Gliddon and Hopwood 2004, Frisella et al. 2001, Sakurai et al. 2004, Crawley et al. 2006). Affected MPS IIIA and MPS VII mice treated with recombinant enzyme from birth had improved learning capacity compared to their untreated littermates and their performance approached that of normal mice (O'Connor et al. 1998, Gliddon and Hopwood 2004). Mice that were given recombinant enzyme starting at 6 weeks of age or later, when the blood-brain barrier had closed, did not display any improvement (O'Connor et al. 1998, Gliddon and Hopwood 2004). Similar improvements in behaviour function in the Morris water maze have been observed in MPS VII mice treated with direct brain injections using gene therapy or cell therapy systems (Frisella et al. 2001, Sakurai et al. 2004).
The repeated acquisition and performance chamber (RAPC) is gaining popularity as an alternative to the traditional water mazes. RAPC is a positive reward test, where mice navigate through a plastic box containing one-way lockable doors in order to consume a saccharin solution once they complete the task (Section 4.2.1). The 64 different possible door combinations provide a greater variety of learning sequences that can be changed with each session. Mice can thus be tested multiple times, which is useful for timecourse analysis. The assessment of both learning and rote performance within the same behavioural test session and within-subject is possible with RAPC testing. In the MPS VII knockout mouse model, untreated mice performed significantly worse than untreated normal suggesting that the RAPC is a good test for detecting learning deficits in this model. Specifically a decreased learning capacity was observed as increased latency to the goal box and increased numbers of errors within each testing session (Brooks et al. 2002). This learning deficit was corrected when MPS VII mice were treated with intra-cerebral gene therapy after which these mice showing similar learning capabilities to normal mice (Brooks et al. 2002).

In addition to changes in neurological function, CNS pathology can also result in changes in motor function. These can be assessed in tests such as open field locomotion, rotarod, balance beam, posture and gait to measure general motor co-ordination tasks in mice (Crawley et al. 1997, Crawley 1999, 2000). Mouse models of MPS I and MPS III have been shown to have altered exploratory activity as measured by the open field test (Li et al. 1999, Cressant et al. 2004, Hemsley and Hopwood 2005). In MPS III, this seems to correlate well with hyperactive behaviour in patients and may provide an additional measure of therapeutic efficacy in these models.
SDT with rhodamine B improved a number of physical and biochemical parameters of MPS IIIA in the mouse model, in particular a decrease in the level of gag storage in the treated mouse brain was observed. This encouraging result led to the expectation that rhodamine B treatment could affect behaviour in the MPS IIIA mouse. The aim of this chapter was to find the most applicable test for detecting differences in learning and memory abilities in mice. Behaviour was assessed in four different tests; the RAPC, Morris water maze, water cross maze and open field exploration in normal and MPS IIIA untreated mice. MPS IIIA mice treated with rhodamine B were evaluated in the water cross maze and open field tests.
4.2 Evaluation of different behaviour tests in MPS IIIA and normal mice

MPS IIIA mice and normal mice were evaluated in 3 different tests of learning and memory; the repeated acquisition and performance chamber (RAPC) (Section 2.6.1), Morris water maze (MWM) (Section 2.6.2) and water cross maze (Section 2.6.3). The open field test (Section 2.6.4) was used to assess locomotion and exploratory activity.

4.2.1 Repeated Acquisition and Performance Chamber

The RAPC is a spatial learning and memory test that has a large combination of different learning sequences. It is therefore possible to test the same animals on multiple occasions as they are not exposed to the same sequence more than once in the repeated acquisition component of the test. Testing was conducted at 12 weeks (sessions 1-4), 20 weeks (sessions 5-8) and 31 weeks (sessions 9-12). Both latency (the time to reach the goal) and total errors (the number of mistakes made) were measured. Testing protocols were split into two phases, performance (or memory) (Section 2.6.1.8) and repeated acquisition (or learning) (Section 2.6.1.9) which were distinguished by a static audio stimulus inside the chamber during performance testing.

4.2.1.1 Performance phase

At 12 weeks of age both MPS IIIA and normal mice (n=7 per group) showed a reduction in time to reach the goal box from the first to the last trial of each session, with a final trial time of approximately 75 seconds for both genotypes (Figure 4.1A). The number of errors also decreased from the first to the last trial of each session in both normal and MPS IIIA mice (Figure 4.1B). No significant difference between the two genotypes was observed in either latency or errors (p>0.05). At 31 weeks of age, normal mice showed an increased trend in latency compared to 12 week old normal mice but this did not reach significance...
Figure 4.1: RAPC analysis: Performance phase.

Performance latency and errors for adult male untreated normal (–■–) and MPS IIIA (–■–) mice was assessed in the RAPC at 12 and 31 weeks of age. Latency at 12 weeks (A) and 31 weeks (C) is presented as time taken to reach the goal chamber. Errors at 12 weeks (B) and 31 weeks (D) were recorded as an incorrect door entry. All mice were subjected to two trials (e.g. A1 and A2) for each session, where the results are expressed as the mean ± standard error of two trials within each session (A, B, C). Each session was performed on a different day (sessions 1-4 or 9-12), with the same sequence used for each session.
(p>0.05). MPS IIIA mice displayed similar latency to 12 week old MPS IIIA mice (Figures 4.1C and D). At 31 weeks of age the error rate for both groups was similar to that at 12 weeks. No difference was observed between the performance latency and error rates between the two genotypes at 31 weeks of age (p>0.05). Testing was also conducted at 20 weeks of age and consistent with the 12 and 31 week results, no difference was detected between normal and MPS IIIA mice (data not shown).

4.2.1.2 Repeated acquisition phase

At 12 weeks of age both the MPS IIIA and normal mice showed a reduction in time to reach the goal box within each individual sequence (i.e. from trial A to C) (Figure 4.2A) (p<0.001). The initial error rate (Figure 4.2B) was quite high, especially in session 1, while the mice were still ‘habituating’ to the new sequence, and the MPS IIIA mice made significantly more errors than normal (p<0.05), however, within each session the number of errors generally decreased with the MPS IIIA mice making the same number of errors as the normal mice (p>0.05). The number of errors was substantially higher in the repeated acquisition phase when compared with the performance phase. At 31 weeks normal mice showed an increased latency time compared to 12 weeks, with a plateau in learning ability seen in both genotypes with MPS IIIA mice having a faster latency time compared to the normal mice (p<0.05) (Figure 4.2C). The error rate of the MPS IIIA was slightly greater in the repeated acquisition phase at 31 weeks of age compared with the normal mice, however there was no statistical difference between the two groups (p>0.05) (Figure 4.2D). Testing was also conducted at 20 weeks of age and showed the same trend as that described at 12 and 31 weeks (data not shown).
Repeated acquisition (learning) latency and errors for adult male untreated normal (–Ŷ–) and MPS IIIA (Ŷ–) mice was assessed in the RAPC at 12 and 31 weeks of age. Latency at 12 weeks (A) and 31 weeks (C) is presented as time taken to reach the goal chamber. Errors at 12 weeks (B) and 31 weeks (D) were recorded as an incorrect door entry. All mice were subjected to two trials (e.g. A1 and A2) for each session, where the results are expressed as the mean ± standard error of two trials within each session (A, B, C). Each session was performed on a different day (12 weeks: sessions 1-4 or 31 weeks: sessions 9-12), with different learning sequences used for each session (Appendix I).
4.2.1.4 Incidence of failure

Mice were considered to have failed the test if they took longer than 360 seconds (6 minutes) to navigate through the correct door sequence and consume the saccharin solution. Mice that failed the test had an escape latency value of 360 seconds recorded. Normal mice had 46 failed trials out of total 576 trials (8%) in the performance phase, compared to MPS IIIA mice which had 14 failed trials out of 504 total trials (2.8%). This equates to approximately a three-fold increase in performance failure in normal mice compared to MPS IIIA mice. A higher failure rate was observed in the repeated acquisition phase, due to the changing learning sequences. Normal mice had 60 failed trials out of 576 total trials (10.4%), compared to 17 failed trials out of 504 total trials (3.4%) in the MPS IIIA mice, which is approximately a three fold higher failure rate over their MPS IIIA counterparts.

4.2.1.5 Auditory evoked brainstem response (ABR)

Auditory evoked brainstem responses are a well-recognised method to test the hearing ability of mice. This test was set up to test mice used for RAPC analysis to ensure that they were able to hear the auditory stimulus used to distinguish the two phases of the RAPC test (Section 2.6.1.10). Mice were tested at approximately 150 days (24 weeks) and 265 days (38 weeks) of age, with each ear being tested separately.

MPS IIIA mice at 150 days were found to have a threshold of 10 ± 10 dB for the LHS and 14 ± 5.5 dB for the RHS compared to normal mice that were found to have a threshold of 3.3 ± 5.8 dB for the LHS and 0 dB for the RHS (Figure 4.3). No statistically significant difference was found when normal mice were compared to MPS IIIA mice in the left ear (p>0.05, one-way ANOVA), however normal mice did show a significant difference in the right ear (p<0.05, one-way ANOVA). MPS IIIA mice of 265 days of age were found to
Figure 4.3: Auditory evoked brainstem response

Auditory evoked brainstem responses were determined in MPS IIIA (blue bars) compared to normal mice (green bars) at 24 weeks (normal, n=3 and MPS IIIA, n=5, Figure 4.3A) and 38 weeks (normal, n=6 and MPS IIIA, n=7, Figure 4.3B) to determine their hearing threshold. All mice were tested in LHS and RHS ears which were analysed separately. Results are expressed as the mean ± standard deviation for each genotype for each ear. * indicates p<0.05 a statistically significant difference in untreated MPS IIIA versus normal mice by one-way ANOVA.
have a threshold of $17.1 \pm 4.9$ dB for the LHS and $11.7 \pm 4.1$ dB for the RHS compared to normal mice which had a threshold of $2.9 \pm 4.9$ dB for the LHS and $10 \pm 8.2$ dB for the RHS. No statistically significant difference was found when normal mice were compared to MPS IIIA mice in the right ear ($p>0.05$, one-way ANOVA), however MPS IIIA mice did show a significant difference in the left ear ($p<0.05$, one-way ANOVA). This hearing range difference does not affect hearing or constitute a moderate or severe hearing deficiency, defined as 40-80 dB (Sands et al. 1995). One MPS IIIA mouse was found to have a unilateral ABR threshold of 65 dB which did not appear to affect either the performance or acquisition phase of the RAPC test.

4.2.2 Morris Water Maze

MPS IIIA mice and age-matched normal mice (n=7 per group) were tested at approximately 36 weeks in the Morris Water Maze, a time when their pathology is well developed (Section 2.6.2).

4.2.1.1 Acquisition phase

On day 1 of the acquisition phase (platform located in the SW quadrant) no difference was observed between MPS IIIA and normal mice, with the majority of mice failing trials and needing to be guided to the platform. On days 3-7 of the acquisition phase (Section 2.6.2.3) the search time for both the normal and MPS IIIA mice decreased ($p<0.001$), and the MPS IIIA mice had a higher escape latency, but this did not reach significance (Figure 4.4A, $p>0.05$ for all trials). A similar trend was observed in the relearning phase (Section 2.6.2.5) with an escape latency time comparable to the latter trials of the acquisition phase (data not shown).
Figure 4.4: Morris Water Maze analysis.

Male MPS IIIA (n=7, blue bars) and normal (n=7, green bars) mice were habituated to the pool and surroundings for days 1 and 2 and the acquisition phase was performed on consecutive days 3-7 (A), where the hidden platform was located in the SW quadrant. In the final phase the platform was moved to the NE quadrant in the reversal phase on days 13 and 14 (B). Results are expressed as mean ± standard deviation of 4 trials from the four cardinal points N, E, W, S and represent the search time spent in the pool locating to platform for each genotype. Figure C represents male MPS IIIA (n=7, blue bars) and normal (n=7, green bars) mice which were tested in the probe phase on Day 8. During this, the platform was removed from the pool and the time spent in each quadrant was determined. Results are expressed as the mean ± standard deviation of each genotype.
A

ACQUISITION

Day 3  Day 4  Day 5  Day 6  Day 7

Search time (seconds)

B

REVERSAL

Day 13  Day 14

Search time (seconds)

C

PROBE

NE  NW  SE  SW

Search time (seconds)

Quadrant
4.2.1.2 Reversal phase

In the reversal phase of the test (Section 2.6.2.6), both normal and MPS IIIA took longer to find the escape platform than in their previous learning trials due to the new location. (Figure 4.4C). The MPS IIIA mice were able to find the newly located platform in equal search time for both trials, whereas the normal mice took longer to find the platform initially but were able to decrease their search time in the second reversal testing. No difference was observed between the genotypes of mice (p>0.05).

4.3.1.3 Probe phase

In the probe phase (Section 2.6.2.4), the platform was removed from the pool and time spent in each quadrant was recorded for a 60 second time period (Figure 4.4C). Both the normal and MPS IIIA mice did not spend the majority of their time in the target quadrant, but chose to swim mainly in the NW quadrant where the experimenter was standing. Both groups of mice did show a preference for the target quadrant after the NW quadrant, followed by the NE quadrant then the SE quadrant. No statistical difference was observed between either group (p>0.05).

4.3.1.4 Failure measurements

Mice were considered to have failed in the Morris water maze test if they were unable to reach the escape platform within 90 seconds. Therefore these mice had an escape latency value of 90 seconds recorded. The percentage of failed trials in the acquisition phase was higher in normal mice than in MPS IIIA mice with an overall failure rate of 12.8% compared to 9.2% in the MPS IIIA mice (Figure, 4.5). Normal mice also had a higher percentage failure in the reversal phase compared to MPS IIIA mice, failing over 30% of trials on the first day compared to less than 5%.
Figure 4.5: Morris water maze failure measurements

During acquisition (days 3-7), relearning (days 9-12) and reversal training (days 13-14) failure measurements were recorded. If the mouse failed to reach the platform within the given 60 seconds, a fail was recorded. This represents the percentage of total trials which were failed by each genotype normal (green lines) versus MPS IIIA (blue lines).
4.2.3 Cross maze analysis

Cross maze analysis was carried out according to Summers et al. (2006) and Dumas and Rabe (1994) at 30 weeks of age in MPS IIIA untreated and MPS IIIA treated mice as well as age-matched normal littermates.

4.2.3.1 Learning phase

Both normal and MPS IIIA untreated mice learnt the place-learning task during the learning phase (Section 2.6.3.3), shown by a reduction in the time taken to find the hidden platform, a decrease in incorrect entries and an increase in number of correct trials across the 6 days period (p<0.0001, Figure 4.6A). By day 3 of the 6 day learning phase, mice had learnt to locate the platform and no further reduction in latency was observed for any group. Untreated MPS IIIA mice were significantly slower than normal mice at reaching the platform (p<0.001). Although the treated MPS IIIA mice displayed reduced escape latency compared to untreated MPS IIIA mice by day 6 this did not reach significance. No difference was observed between males and females (p>0.05).

An increase in the number of correct entries performed by normal, MPS IIIA mice across the 6 day learning period (p<0.001) as mice learnt the position of the platform (Figure 4.6B). By day 4 normal mice were able to locate the platform correctly on the first try 60-75% of the time compared to untreated MPS IIIA mice 35-40% of the time, which showed that MPS IIIA untreated mice thus made significantly less correct entries compared to normal. No difference in the number of correct entries was observed between males and females (p>0.05).

Conversely, the number of incorrect entries performed decreased over the learning period (Figure 4.6C) such that by day 4 normal mice were averaging 0.5 – 1 incorrect entry
Figure 4.6: Cross maze learning and memory phase analysis.

MPS IIIA (n=9, blue bars), normal (n=9, green bars) mice were habituated to pool and surroundings for day 1 and the learning phase was performed on consecutive days 2-6 where the hidden platform was located in the East arm. Figure A represents the escape latency (seconds) for mice to locate hidden platform. Figure B represents the mean number of incorrect entries/re-entries during trials. Figure C represents the mean number of correct entries by going straight to hidden platform measured as binary result. Results are expressed as mean ± SEM of 2 blocks of 3 trials from the three cardinal points N, W, S for each group separated by a 30 minute interval. Learning and memory timeframe is indicated by the solid lines on the X-axis.
compared to MPS IIIA untreated mice which had ~2 incorrect entries. In contrast to the previous 2 measured parameters, a sex effect was observed in the number of correct entries. Both male and female untreated MPS IIIA mice made significantly more incorrect entries than normal mice (p<0.05).

4.2.3.2 Memory phase

After the learning phase of testing, mice were given a 10 day rest and their ability to memorise the position of the hidden platform was evaluated (Section 2.6.3.4). Testing was conducted in the same format as for the learning phase; however it was only conducted over 1 day. Overall, the data was comparable to that observed on day 6 at the end of the learning phase. Untreated MPS IIIA mice displayed increased escape latency (p=0.004), a decreased number of correct entries (p<0.001) and an increased number of incorrect entries (p=0.003) compared to normal mice.

4.2.3.3 Failure measurements

Similar to the MWM, mice were considered to have failed the test if they did not find the escape platform within 60 seconds. Mice that failed the test had an escape latency of 60 seconds recorded. The MPS IIIA untreated mice had a higher incidence of failure compared to normal mice (Figure 4.7), however this was very low compared to other the previous two tests. MPS IIIA mice failed 4.76% of the time during the learning period. Importantly, normal mice performed well in this test with failures only observed on the first day of testing with an overall failure rate of 1.06%.
Figure 4.7: Water cross maze failure measurements.

To determine the incidence of failure in the water cross maze, the trials that were failed (i.e. time taken > 60 seconds) for MPS IIIA untreated (blue lines) and normal untreated (green lines) are presented as a proportion of the total trials. Data is expressed as the number of failed trials expressed as a percentage of the total trials for both the days 1-6 of learning and day 16 (memory).
4.2.3.4 Effect of SDT on learning and memory in cross maze

Of the three learning and memory tests conducted, the cross maze had the greatest discriminatory power to detect differences between normal and MPS IIIA untreated mice. Therefore, MPS IIIA mice treated with 1mg/kg weekly injections of rhodamine B were also tested for learning using the cross maze protocol (Section 2.6.3.3).

Compared to untreated MPS IIIA littermates, treated MPS IIIA mice displayed reduced escape latency by day 6, however this did not reach significance (Figure 4.8A, p>0.05). An increase in the number of correct entries performed by MPS IIIA treated mice across the 6 day learning period (p<0.001) was observed as mice learnt the position of the platform (Figure 4.8B), such that by day 4, treated MPS IIIA mice were able to locate the platform correctly on the first try 35-40% of the time. This was a significant improvement over untreated MPS IIIA mice (p<0.001). No difference in the number of correct entries was observed between males and females (p>0.05). Conversely, the number of incorrect entries measured in treated MPS IIIA mice decreased over the learning period (Figure 4.8C) such that by day 4 treated MPS IIIA mice were averaging 1 incorrect entry. In contrast to the previous 2 measured parameters, a sex effect was observed in the number of correct entries. Both male and female untreated MPS IIIA mice made significantly more incorrect entries than normal mice, but the decrease observed upon rhodamine B treatment reached significance only in male MPS IIIA mice (p<0.001).

Treated mice failed 2.38% of all trials which was improved compared to MPS IIIA untreated mice and approached the normal range.
Figure 4.8: Cross maze learning and memory phase analysis.

MPS IIIA (n=9, blue bars), normal (n=9, green bars) and MPS IIIA treated with rhodamine B (n=11, purple bars) mice were habituated to pool and surroundings for day 1 and the learning phase was performed on consecutive days 2-6 where the hidden platform was located in the East arm. Figure A represents the escape latency (seconds) for mice to locate hidden platform. Figure B represents the mean number of incorrect entries/re-entries during trials. Figure C represents the mean number of correct entries by going straight to hidden platform measured as a predicated proportion of total trials. Results are expressed as mean ± SEM of 2 blocks of 3 trials from the three cardinal points N, W, S for each group separated by a 30 minute interval. Learning and memory timeframe is indicated by the solid lines on the X-axis.
4.2.3.5 Analysis of eye pathology in MPS IIIA mice

Previous reports indicate that MPS IIIA mice develop corneal clouding at approximately 7 months of age, which is the same age as was used in this study. To determine if eyesight impairment was a confounding factor in performance in the cross maze, the degree of corneal clouding was examined as well as the characterisation of lysosomal storage within different regions of the eye under electron microscopy (Sections 2.8.2, 2.8.5).

4.2.3.5.1 Physical assessment

MPS IIIA treated, untreated and normal mice was assessed for presence of corneal clouding by an ophthalmologist under fibre optic lamps. No indication of corneal clouding was observed in any of the mice analysed (Figure 4.9).

4.2.3.5.2 Light and electron microscopic examination of eye pathology

Eyes were taken post-mortem and fixed (Section 2.8.2) and thin sections were cut and stained using toluidine blue (Section 2.8.5). No lysosomal storage was evident in normal sections of cornea (Figures 4.10A and B) or retina (Figures 4.10C and D) under light or electron microscopy. However, lysosomal storage was observed in all MPS IIIA sections using 1 μm toluidine blue stained sections (Figure 4.10E and 4.10H) and further examination was conducted on ultrathin section using electron microscopy (Section 2.8.8). Storage was noted in the corneal epithelium, stroma (Figure 4.10F), retinal pigmented epithelial cells (Figure 4.10I) and stroma and ciliary body in MPS IIIA sections, confirming the previous report of Bhaumik (Bhaumik et al. 1999). Treatment with intravenous injections of rhodamine B did not reduce lysosomal storage in the eye, which was still evident in all the areas observed in MPS IIIA untreated mice (Figures 4.10G and 4.10J).
Figure 4.9: Macroscopic eye examination.

To determine if normal (A) and MPS IIIA untreated (B) mice had corneal clouding as part of their pathology, normal and MPS IIIA mice examined under a Nikon SMZ-800 zoom stereomicroscope at 10x original magnification. Images were taken using a Motic Moticam 2000 camera and software. No evidence of corneal clouding was observed in either case. Scale bar indicates 1mm.
**Figure 4.10: Lysosomal storage analysis in eye sections**

1μm toluidine blue stained sections of eye cross-sections viewed under 1000x original magnification for normal cornea (A) normal retina (C) MPS IIIA cornea (E) and MPS IIIA retina (H) images were taken on an Olympus BX40 light microscope using an Olympus DP50 camera. Electron microscopic analysis of each section was conducted on a Phillips CM100 transmission electron microscope at a magnification of 4600x. B and D represent normal cornea and retina respectively with no evidence of lysosomal storage. F and I represent MPS IIIA untreated cornea and retina respectively with lysosomal storage evident in the corneal fibroblasts in the stroma and the retinal pigmented epithelial cells. G and J represent MPS IIIA treated cornea and retina respectively with lysosomal storage patterns unchanged compared to MPS IIIA untreated. Bar = 20μm from images taken at 1000x original magnification and electron microscope images bar = 5 μm.
4.2.4 Open field analysis

Open field analysis was conducted at 3 and 10 weeks of age in normal and age-matched MPS IIIA untreated and treated mice. The number of times each mouse reared (Figures 4.11A and 4.11C) and the number of lines they crossed (Figures 4.11B and 4.11D) within a 3 minute period was recorded. At three weeks of age MPS IIIA (both treated and untreated) mice had more line crossings than their normal counterparts with MPS IIIA untreated MPS IIIA mice statistically different to normal mice (p<0.05, one-way ANOVA). The number of rearing events was also significantly increased in MPS IIIA compared to normal (p<0.05, one-way ANOVA) and the MPS IIIA untreated were significantly different to the MPS IIIA treated which displayed less rearing (p<0.05, one-way ANOVA).

At 10 weeks of age, MPS IIIA mice had slightly less line crosses than their normal counterparts with MPS IIIA treated mice significantly lower than both normal mice and MPS IIIA untreated mice (p<0.05, one-way ANOVA). A similar statistical trend was evident in the number of rearing events observed with MPS IIIA untreated having significantly more rearing events than their treated counterparts and treated mice were also different to normal mice.

Both normal and MPS IIIA treated mice showed significant differences at 3 versus 10 weeks with normal mice having less line crosses and rearing events at 3 compared to 10 weeks (p<0.001) and MPS IIIA treated mice having more line crosses and rearing events at 3 compared to 10 weeks (p<0.001).
Figure 4.11: Open field analysis.

Analysis of MPS IIIA untreated (blue bars), MPS IIIA treated (purple bars) and normal mice (green bars) aged 21 days (3 weeks) and 70 days (10 weeks). Mice were tested for exploratory activity within a 3 minute trial period. The numbers of rears (A) and number of line crosses (B) in 3 minutes were recorded. Data is expressed as the mean ± standard deviation of each measurements for each genotype at the two time points. * indicates p<0.05, One-Way ANOVA, normal versus MPS Untreated, ** indicates p<0.05, One-way ANOVA, MPS Untreated versus Treated, # indicates p<0.05, One-way ANOVA, normal versus MPS Treated. Solid arcs indicate significant difference within normal and MPS Treated groups at 3 compared to 10 weeks (p<0.001).
**Diagram A**

- **Y-axis:** Number of line crosses in 3 mins
- **X-axis:** 3 weeks vs. 10 weeks
- **Notes:**
  - p<0.001
  - * indicates a significant difference

**Diagram B**

- **Y-axis:** Number of rear in 3 mins
- **X-axis:** 3 weeks vs. 10 weeks
- **Notes:**
  - p<0.001
  - * indicates a significant difference
4.3 Discussion

CNS pathology affects a large number of MPS patients and is refractory to current treatments. The development of any new therapy must address CNS associated pathology and functional outcome in a quantifiable manner. The availability of MPS mouse models similar to their human disease counterparts allows the investigation of a number of different behaviour paradigms and the effect of therapy on disease outcome (O'Connor et al. 1998, Brooks et al. 2002, Cressant et al. 2004, Gliddon and Hopwood 2004, Sakurai et al. 2004, Hemsley and Hopwood 2005, Crawley et al. 2006, Frisella et al. 2001). The mouse models of MPS diseases are amenable to standard behaviour tests and specifically MPS IIIA mice have been shown to display quantifiable learning and memory deficits. (Gliddon and Hopwood 2004, Hemsley and Hopwood 2005, Crawley et al. 2006). These types of tests have been useful in validating and characterising disease progression in MPS mouse models.

Two of the behaviour tests analysed did not discriminate between the ability of normal and MPS IIIA mice to learn and memorise a task. Both of these tests, the RAPC and Morris water maze, have been used previously for assessing pathology in MPS mouse models and also in induced hippocampal lesions in normal mice (O'Connor et al. 1998, Brooks et al. 2002, Cressant et al. 2004, Gliddon and Hopwood 2004, Sakurai et al. 2004, Hemsley and Hopwood 2005, Crawley et al. 2006).

Genetic background of mice is crucial to their performance in the RAPC. Studies comparing the performance of inbred C57Bl/6 and 129SvJ mouse strains in the RAPC demonstrated an increased latency and error rate in the 129SvJ mice compared to the C57Bl/6 (Brooks et al. 2000). The 129SvJ mice lacked motivation to perform and were unable to complete as many trials as the C57Bl/6 mice. The MPS IIIA and age-matched
normal mice used in this study were on a mixed strain of predominantly 129SvJ and C57Bl/6 with some CD1 and SJL strain combinations (Bhaumik et al. 1999). The competing influence of background strain may have had a confounding effect on behaviour on the performance in the RAPC, leading to an inability to discriminate between normal and MPS. The increased failure rate observed in normal animals suggests that different levels of motivation exist in order to perform in the test. In particular, the normal mice displayed a reduced preference for the saccharin reward solution compared to MPS IIIA mice. Other solutions besides saccharin could be used in the reward system to see if this improves the performance and learning in the task. Testing normal mice with a hippocampal lesion could be included to determine if the test is set up with the correct parameters to confirm the differences in learning demonstrated in the Brooks study (Brooks et al. 2000). This could be an important internal control for the RAPC setup and should be considered in future experiments.

Hearing and eyesight are also important factors for successful completion of the RAPC task. Hearing was determined using the auditory evoked brainstem response. In contrast to some Sanfilippo patients who display hearing loss (Zafeiriou et al. 2001), only one MPS IIIA mouse was found to have a hearing deficiency and this was in one ear only. All other mice (normal and MPS IIIA) tested were able to hear at an adequate level, which is important for a behaviour test which relies on detecting an auditory stimulus to detect the different phases of the test. This suggests that all mice were able to hear the stimulus to detect the different phases of the testing protocol. Eyesight is also not affected in MPS IIIA mice at the age of testing and will be discussed later for the water maze task.

Since no behavioural abnormalities were detected using the RAPC testing schedule, the same mice were tested in the Morris water maze. This was conducted as previously
described (Gliddon and Hopwood 2004) and was composed of four distinct phases: acquisition (Days 3-7), probe phase (Day 8), relearning phase (Days 9-12) and a reversal phase (Days 13 and 14). MPS IIIA and normal mice were released from the four cardinal points designated north, south, east and west and the average of these trials was used in the analysis. Both MPS IIIA and normal mice had a decreased search time as testing progressed, suggesting they were capable of locating the hidden platform. The search time for the normal mice is comparable to that shown by Gliddon and Hopwood (2004) with mice spending 20 ± 10 seconds in the final acquisition session. However, in contrast, the MPS IIIA mice were able to locate the platform almost as quickly as the normal mice spending 30 ± 10 seconds in the final acquisition session. Unlike the MPS IIIA mice in Gliddon’s study who were not able to locate the platform as quickly and showed search times of approximately 40 ± 5 seconds (Gliddon and Hopwood 2004). Again, different genetic background contributions over successive generations between the two studies could have subtly altered the performance in the MPS IIIA mouse. The generation of MPS IIIA mice on a stable genetic background is likely to resolve some of these issues associated with genetic drift and changing phenotype due to mixed strains.

Similar to the RAPC, normal mice showed a lack of motivation to perform with an overall failure rate of 12.8% compared to 9.2% in MPS IIIA mice. However, both normal and MPS IIIA mice were prone to wall hugging (thigmotaxis) and passive floating, behaviours which affect performance but are not dependent on hippocampal function.

In the probe phase, both normal and MPS IIIA mice spent the majority of their time in the north-west quadrant which had the experimenter as a visual cue, raising the possibility that the escape platform is not adequate reward for the mice as they have learnt over the sessions that the experimenter is the one to remove them from the pool. Recent evidence
has shown that the probe trial may be confounded since it is run considerably longer than the length of a training trial. This means that subjects with good hippocampal function may actually stop searching in the target quadrant sooner after having found no platform in it and appear to show a decrease in spatial memory performance (Gerlai 2001).

In contrast to the previous two tests, a variant of the Morris water maze, the cross maze was able to distinguish between the two genotypes. A significant difference in learning, but not memory, was detected in MPS IIIA mice, which was particularly evident in male mice. MPS IIIA mice were considerably slower at locating a hidden platform than normal mice and they made a greater number of incorrect entries/re-entries than normal mice. Correct entries, as measured as a predicted proportion were also greatly reduced in MPS IIIA untreated mice. This test was chosen to evaluate the effect of substrate deprivation therapy in this mouse model. MPS IIIA mice treated with 1mg/kg intravenous weekly injections of rhodamine B showed improved ability to learn compared to their untreated littermates in all parameters tested. Escape latency decreased as did the number of incorrect entries while the number of correct entries increased.

Treated MPS IIIA mice maintained their ability to locate the platform after a 10 day rest period and had significantly fewer incorrect entries and more correct entries than untreated MPS IIIA mice. The long-term effects of treatment on memory were not tested in this study and it remains to be seen if SDT has halted or merely slowed down CNS deterioration. The water cross maze is amenable to repeated testing of memory over long periods of time and could be used to characterise the progression of neurological pathology in MPS IIIA and its response to treatment. The improvement in performance by the treated mice indicates that SDT can positively affect neurological pathology in MPS animals with a mature blood-brain barrier. This is in contrast to systemic ERT, which has proven
effective only when administered in the neonatal period before blood-brain barrier maturation in mice (O'Connor et al. 1998, Gliddon and Hopwood 2004).

One of the likely factors in the lack of detectable differences between the two genotypes of mice in the two different water maze tests is the additional external stimuli present in the Morris water maze setup. Visual cues which were posters and interior doors, walls etc, as well as an obvious cue of the observer were prominent in this test. In contrast, the external stimuli were restricted in the water cross maze by the black plastic which surrounded the pool. The visual cues were a lot closer to the pool edges, so they were easily visible to the mice and the observer was obstructed from view, whereas the cues in the Morris water maze were located on the walls further away from the pool edges making the cues harder for the mice to see and the observer was easily visible for the mice.

In addition, the restricted swimming area in the water cross maze test compared to the Morris water maze may be more conducive to performance as both normal and MPS IIIA mice displayed a reduced incidence of wall hugging and floating in the water cross maze.

However, the most likely reason for detecting differences between MPS IIIA and normal mice was the fact that more observational parameters were measured. These factors were the incorrect arm entries/re-entries and the proportion of correct trials. These are important non-time dependent parameters, which enabled the confirmation of differences between MPS IIIA untreated, treated and normal mice and helped indicate the presence of a learning deficit. Escape latency is a poor measure of learning ability due to the different spatial strategies that can give similar escape latency scores. This can be overcome by the inclusion of sophisticated image-tracking systems to measure the proximity to the escape platform, path length and swimming speed which inform the observer about learning
strategies to find the escape platform (Gerlai 2001). Additional parameters in the water cross maze are similar and easy to measure and do not require expensive equipment.

Corneal opacity was not impaired in MPS IIIA mice so the differences in learning capacity observed are more likely to be attributable to hippocampal dysfunction rather than impairment in visual acuity. Gag storage in MPS IIIA mice was present in corneal stromal epithelium, retinal pigmented epithelium and retinal stroma as well as ciliary bodies under electron microscopic examination of eyes, which was unchanged with treatment. In order to conclusively rule out a subclinical effect of storage, a visible platform test could be conducted in future analyses to determine if the mice can see the platform. Mice that are able to see the platform should locate it immediately since this is their escape from the water. However, this test is normally conducted at the end of the testing protocol where mice have been previously exposed to the location of the hidden platform and may not be testing visual acuity. Ideally, the visual platform should be conducted as part of the habituation protocol before learning acquisition commences.

The open field test has been used to detect exploratory activity and locomotion in MPS models. MPS IIIA mice have been reported to have increased exploratory activity (hyperactivity) at 3 weeks of age and then have reduced activity from 6 weeks of age (hypoactivity) (Hemsley and Hopwood 2005). Similar results to the Hemsley study were observed at 3 weeks of age, with MPS IIIA untreated mice having increased activity compared to age-matched normal untreated mice. However, at 10 weeks of age, MPS IIIA untreated mice displayed a similar trend in reduced activity compared to normal mice in the previous Hemsley study, however this did not reach significance at 10 weeks for either line crosses or rearing events. To determine the outcome of SDT, MPS IIIA treated mice were subjected to open field analysis at 3 and 10 weeks of age. Treated mice showed a
reduction in activity at both 3 and 10 weeks compared to MPS IIIA untreated mice which were similar to the previous Hemsley study. Both MPS IIIA untreated and treated mice display the same hyperactivity as previously reported (Hemsley and Hopwood 2005) suggesting that treatment has not affected this behaviour activity in MPS IIIA mice.

It is important to point out that the MPS IIIA mouse retains 3-4% of normal sulphamidase activity. Because the concept behind SDT is a re-balancing of gag synthesis and degradation, it is more likely to be effective as a therapy in animal models, and in patients, that retain a degree of residual enzyme activity, capable of turning over the reduced level of substrate delivered to the lysosome. Thus efficacy is likely to be proportional to residual enzyme activity. The most common and severe human MPS IIIA mutations, R245H, R74C and S66W, possess significantly less sulphamidase activity than observed in the murine model; with 0.05%, 0.16% and 0.21% of normal enzyme levels reported respectively (Perkins et al. 1999, 2001). While successful in this murine model of MPS IIIA, the general applicability of SDT to the range of clinical severity observed in human MPS IIIA awaits the generation of additional models with low residual enzyme activity. Having a range of mouse models with varying degrees of residual enzyme will be important in the application of learning and memory tests and the outcome of SDT on the development of CNS pathology.

Overall, it was found that the water cross maze was the most useful for detecting differences in MPS IIIA mice compared to age-matched littermates. As treatment of MPS IIIA mice with 1 mg/kg rhodamine B was shown to improve learning deficits associated with MPS IIIA, substrate deprivation appears to be a viable treatment for CNS pathology in MPS IIIA and that small molecules able to cross the blood-brain barrier are able to have an effect on the neurological pathology caused by lysosomal storage.
Since using SDT had positive outcomes on somatic pathology as described in chapter 3 and also on neurological pathology as demonstrated using the water cross maze in this chapter, it is important to determine the long-term effects of suppressing gag synthesis in MPS IIIA. Chapter 5 addresses the issues of toxicity; firstly by a multi-generational study over 4 generations and secondly by administering a higher dose of rhodamine B.
CHAPTER 5
SDT MULTI-GENERATIONAL STUDY
AND TOXICITY ANALYSIS
5.1 Introduction

If SDT is to be a potential therapy for MPS, it would need to be administered long-term potentially over the life of the patient. This chapter addresses the issues of toxicity of rhodamine B over an extended timeframe and examines the long-term effects of decreasing gag synthesis. A multi-generational study was initiated to evaluate the continuous exposure of 1 mg/kg rhodamine B in MPS IIIA mice over 4 generations and effect on pathology. An increased dose of rhodamine B at 5 mg/kg was evaluated for toxicity and efficacy in MPS IIIA mice.

There is only limited information in the literature on toxicity of rhodamine B, with a few reports published on the administration of rhodamine B via different routes, to investigate acute exposure to rhodamine B in animals and humans. Rhodamine B has been shown to be metabolised by de-alkylation and in vitro studies have shown that this occurs in the liver (Webb et al. 1961). The LD$_{50}$ of rhodamine B is 89.5 mg/kg with death usually occurring within 2 minutes of intravenous administration (Webb et al. 1961).

Oral administration of rhodamine B (17 mg per mouse per week for 52 weeks) in rodent drinking water results in stomach polyps and intestinal tumours (Bonser et al. 1956). Oral feeding studies (1% dietary level rhodamine B fed over 3 months) results in significant growth retardation, liver damage, heart, spleen and kidney abnormalities and ultimately mortality (Hansen et al. 1959, Webb et al. 1961). Subcutaneous administration of a high dose of rhodamine B (800 mg over 10 weeks) results in sarcoma, hepatoma, intestinal polyps and lacerated skin (Bonser et al. 1956, Gangolli et al. 1967). Intravenous injection of rhodamine B between 50 and 200 mg/kg/week in rodents led to tumours, liver atrophy, hyperaemia and increase in Kupffer cells and 76% mortality within 5 months (Umeda 1952, 1955, 1956).
Overall, rhodamine B has toxic side-effects and significant mortality when administered to rodents at high doses over either short or extended periods of time. Little information is available on the administration of the significantly lower doses of rhodamine B investigated in Chapters 3 and 4.

Two cases of overexposure to rhodamine B in food products have been reported (Kelner 1985). Both patients reported red coloured urine, which contained fluorescent compounds, however, no adverse effects from the rhodamine B were observed. Another report involved exposure of people to an unknown amount of aerosolised rhodamine B (Dire and Wilkinson 1987), resulting in transient mucous membrane and skin irritation with all patients having resolution of their symptoms within 24 hours.

A multi-generational study in rats, fed the equivalent of 210 mg of pure rhodamine B in their diet at 1g/3kg dry diet for 12 months over six generations (Nainggolan-Sihombing 1984) resulted in growth retardation and decreased reproductive ability, as seen by successive reduction in litter size with each generation (Nainggolan-Sihombing 1984). Changes in behaviour such as itching, irritability, aggressiveness and cannibalism were observed as well as increased susceptibility to infection and the development of tumours. This raises concerns about the long-term administration of rhodamine B as an SDT agent.

To determine if SDT is to become a viable therapy for MPS IIIA, 1 mg/kg rhodamine B, as previously reported (Chapters 3 and 4) (Roberts et al. 2006), was administered over 4 generations of MPS IIIA mice with pregnant dams being treated during pregnancy. Two aspects were addressed in this study: firstly, hepatotoxicity and liver function, and
secondly, the effect on MPS IIIA pathology. A higher dose of rhodamine B (5mg/kg) was administered to MPS IIIA mice for 6 months to determine if improved efficacy could be observed.
5.2. Multi-Generational Study

MPS IIIA mice were treated with intravenous weekly injections of 1 mg/kg rhodamine B (Section 2.4.7). At 10 weeks of age, treated females were paired with age-matched treated males (Section 2.4.3). Once a female was determined to be pregnant the male was removed from the cage. The female continued to receive weekly intravenous injections of 1 mg/kg during pregnancy to observe the effect of low dose treatment on in utero outcome (Section 2.4.7). The offspring continued to receive weekly intravenous 1 mg/kg rhodamine B injections starting at 3 weeks of age until time of sacrifice (Section 2.4.7). This was conducted over 4 generations of MPS IIIA mice with pregnant dams being treated during pregnancy and until offspring were weaned at 21 days of age. MGS1 was designated as the first litter obtained from treated dams, with offspring treated weekly until sacrifice. Select offspring were bred for an additional 3 generations, with MGS4 designated as the final generations, treated continuously with rhodamine B.

5.2.1 Litter size

Litter size was determined to evaluate any toxic effects of rhodamine B treatment in utero. MPS IIIA (untreated and treated) had more pups in their litters than normal mice, however this was not statistically different (Figure 5.1, p>0.05). No adverse effects on the dams’ health were observed when treated with rhodamine B throughout pregnancy with all dams conceiving and carrying their pregnancy to term.
Figure 5.1: Multi-generational study litter size.

MPS IIIA untreated (blue bars) and MPS IIIA treated with rhodamine B (purple bars) were used for breeding to determine any toxic effects of rhodamine B injections in utero. Total litter size was determined and results are expressed as the mean ± standard deviation of breeding pairs for each genotype. The green solid line represents the mean normal litter size ± 1 standard deviation indicated by the dashed green lines.
5.3 Toxicity of rhodamine B

Initial characterisation of liver cell morphology with rhodamine B treatment was conducted using H&E staining. In addition to this, the special stains Sirius Red, Perl and PAS-digest were used to determine if treatment with rhodamine B over 4 generations was causing toxic effects on the liver. Analysis was conducted at either generation 1 (MGS1), at the beginning of the multi-generation study, or at generation 4 (MGS4), after extended exposure to low dose rhodamine B treatment. Liver function tests were also conducted on MGS4 since hepatic damage is reported as a toxic side effect of rhodamine B treatment (Bonser et al. 1956, Hansen et al. 1959).

5.3.1 Liver histology: H&E: MGS1 and MGS4

H&E staining of liver sections from normal untreated mice from MGS1 (Figure 5.2A) and MGS4 (Figure 5.2B) showed normal cell architecture with predominantly hepatocytes being seen in sections analysed from all groups (Figure 5.2A-F). An increase in hepatocyte nuclear size was a common feature of normal and MPS IIIA sections, which was particularly evident in MPS IIIA sections (Figure 5.2). A small amount of mononuclear infiltrate was observed (MNI) in MPS IIIA untreated (Figure 5.2D) and also treated mice from MGS4 (Figure 5.2F) which was not observed in MGS1 mice, which is likely to be related to MPS IIIA pathology during that generation, since it was evident in the untreated MPS IIIA mice as well as the treated mouse group.

5.3.2 Liver histology: Special stains: MGS1 and MGS4

Sirius red staining for connective tissue and bile ducts showed fine staining around portal tracts, with no staining observed in the parenchyma of the liver in normal mice from both MGS1 (Figure 5.3A) and MGS4 (Figure 5.3B). MPS IIIA pathology did not alter the
Figure 5.2: MGS 1 and MGS4 Haematoxylin and Eosin staining.

Liver sections from normal mice from MGS1 (A) and MGS4 (B) showed predominantly normal hepatocytes with some binucleate cells evident (arrows). MPS IIIA mice from MGS1 (C) showed binucleate cells and increased nuclear size in hepatocytes (arrowheads). Treatment with rhodamine B at MGS1 (E) or MGS4 (F) showed similar staining to MPS IIIA untreated mice. MPS IIIA mice untreated (D) and treated (F) from MGS4 also displayed evidence of mononuclear infiltrate (MNI). Images taken at 200x original magnification using an Olympus BX51 light microscope with an Olympus DP12 camera attachment. Bar = 100 μm.
Figure 5.3: MGS 1 and MGS4 Sirius Red staining.

Liver sections from normal mice from MGS1 (A) and MGS4 (B) showed positive Sirius Red staining around the portal tracts (arrows). MPS IIIA mice from MGS1 (C) and MGS4 (D) showed similar staining pattern. Treatment with rhodamine B at MGS1 (E) or MGS4 (F) showed similar phenotype to other groups analysed. Images taken at 200x original magnification using an Olympus BX51 light microscope with an Olympus DP12 camera attachment. Bar = 100 μm.
staining pattern observed with Sirius red in either MGS1 (Figure 5.3C and F) or MGS4 (Figure 5.3D). MPS IIIA mice treated with rhodamine B did not display an altered staining pattern in either MGS1 (Figure 5.3E) or after extended exposure to treatment in MGS4 (Figure 5.3F) compared to the normal and MPS IIIA untreated liver sections.

Perl staining to analyse iron content of the liver showed small blue granules evident in the hepatocytes in normal liver sections in MGS1 (Figure 5.4A) and multiple granules seen in MGS4 (Figure 5.4B). MPS IIIA untreated showed weak staining of granules in hepatocytes with no evident disruption to the cellular iron transport system. A few positively stained granules were seen in MGS1 (Figure 5.4C) with multiple granules seen in MGS4 (Figure 5.4D). Treated MPS IIIA sections displayed a reduction compared to normal and MPS IIIA untreated in MGS1 (Figure 5.4E) and MGS4 (Figure 5.4F). Even though multiple granules were seen in some sections, overall the amount of staining in all sections analysed was low.

PAS digested staining to analyse mucins showed a small amount of PAS-positive material evident in hepatocytes with less evident staining in sinusoids of normal liver sections in MGS1 (Figure 5.5A) and MGS4 (Figure 5.5B). MPS IIIA untreated sections showed increased staining in the sinusoids in MGS1 (Figure 5.5C) and MGS4 (Figure 5.5D) with staining in hepatocytes as well. Similar to their untreated counterparts, MPS IIIA treated sections also showed PAS-positive material evident in the hepatocytes and sinusoids in MGS 1 (Figure 5.5E) and MGS4 (Figure 5.5F).
Figure 5.4: MGS 1 and MGS4 Perl staining.

Liver sections from normal mice from MGS1 (A) and MGS4 (B) showed positive Perl staining shown as blue granules in hepatocytes (arrows). MPS IIIA mice from MGS1 (C) and MGS4 (D) showed similar staining pattern. Treatment with rhodamine B at MGS1 (E) or MGS4 (F) showed similar staining to other groups analysed. MGS4 mice show slightly more positive granules in untreated groups with minimal positive staining in treated groups. Images taken at 200x original magnification using an Olympus BX51 light microscope with an Olympus DP12 camera attachment. Bar = 100 μm.
Figure 5.5: MGS 1 and MGS4 PAS-digest staining.

Liver sections from normal mice from MGS1 (A) and MGS4 (B) showed positive PAS-digested staining shown as magenta staining in hepatocytes (arrowheads) and sinusoids (arrows). MPS IIIA mice from MGS1 (C) and MGS4 (D) showed similar staining pattern. Treatment with rhodamine B at MGS1 (E) or MGS4 (F) showed similar staining to other groups analysed. Images taken at 200x original magnification using an Olympus BX51 light microscope with an Olympus DP12 camera attachment. Bar = 100 μm.
5.3.3 Liver function tests

Liver function tests (Section 2.4.11) were used to determine the status of liver health and as an indicator of rhodamine B toxicity, and were analysed in MGS4. These tests were conducted on mouse plasma and included total protein, albumin, globulin, bilirubin and liver enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltranspeptidase (GGT) as per section 2.4.11. Aminotransferases, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury. Alkaline phosphatase (ALP), 7-glutamyl transpeptidase (GGT), and bilirubin act as markers of biliary function and cholestasis and albumin reflects liver synthetic function (Harris 2005).

All liver function tests were similar in normal and MPS IIIA untreated mice (Figure 5.6) except for globulin concentration which was significantly decreased in MPS IIIA untreated mice (Figure 5.6, p<0.05), for an unknown reason, however this is likely to be associated with MPS IIIA pathology. MPS IIIA mice treated with rhodamine B showed similar liver function test scores to normal and MPS IIIA untreated (p>0.05). Levels of alkaline phosphatase (ALP) in treated mice were slightly lower than normal and untreated MPS IIIA mice but this was not significantly different (p>0.05).

5.4 Effect of treatment on MPS IIIA pathology

Results in Chapters 3 and 4 demonstrate that intravenous rhodamine B at 1 mg/kg improves a number of somatic and neurological parameters of MPS IIIA Primary parameters such as bodyweight, tissue weights and lysosomal storage were analysed in the different generations of mice with treatment with 1 mg/kg rhodamine B as previously described in Sections 2.4, 2.5, 2.7 and 2.8.
Figure 5.6: Liver function tests

Liver function tests parameters of total protein, albumin, globulin, bilirubin and liver enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltranspeptidase (GGT) were measured using plasma from MGS4 mice. Units for each parameter measured are indicated in brackets. Results are expressed as the mean ± standard deviation for each group. MPS IIIA untreated (blue bars), MPS IIIA treated (purple bars), normal untreated (green bars). * indicates p<0.05, one-way ANOVA, normal versus MPS IIIA untreated.
5.4.1 Mouse bodyweights

Mouse bodyweights were recorded on a weekly basis. No difference in bodyweight was observed over 4 generations of rhodamine B treatment in either males (Figure 5.7, p>0.05) or females (data not shown).

5.4.2 Tissue weights

One of the primary indications of MPS IIIA pathology is enlargement of liver and spleen. MPS IIIA mice treated over 4 generations with rhodamine B had no decrease in liver size (Figure 5.8A, p>0.05) compared to MPS IIIA untreated mice. Treatment of MPS IIIA mice with rhodamine B over 4 generations had not effect on spleen size compared to untreated MPS IIIA mice (Figure 5.8B, p>0.05). Therefore, treatment over an extended timeframe had no improved benefit compared to 6 month treatment at the same dose as reported in chapter 3.

All other organ weights fell within the normal range and did not change with treatment of rhodamine over the 4 generations of mice reported (Table 5.1).

5.4.3 Lysosomal storage by light microscopy

Treatment of MPS IIIA for 4 generations with rhodamine B had no effect on lysosomal inclusions as observed by light microscopy (Figure 5.9). Widespread lysosomal storage was evident in liver hepatocytes (Figure 5.9A and B), the distal convoluted tubule epithelium of the kidney (Figure 5.9C and D), cerebellar Purkinje cells (Figure 5.9E and F) and pyramidal neurons in the cerebral hemisphere (Figure 5.9G and H) similar to MPS
Figure 5.7: Multi-generational study male bodyweights.

Bodyweight measured at 6 months of treatment was compared in MPS IIIA mice treated with rhodamine B over 4 generations (MGS1-4, purple bars). MPS IIIA untreated (blue bars), MPS IIIA treated with 1 mg/kg rhodamine B (purple bars) and normal untreated (green bars) are shown from non-MGS mice. All data is presented as the mean ± standard deviation for each group at each generational timepoint. * indicates statistically significant difference, p<0.05, one-way ANOVA, MPS IIIA untreated versus normal untreated and ** indicates statistically significant difference, p<0.05, one-way ANOVA, MPS IIIA untreated versus treated.
Figure 5.8: Multi-generational study tissue weights.

Liver (A) and spleen (B) weights measured at 6 months of treatment were compared in MPS IIIA mice treated with rhodamine B over 4 generations (MGS1-4, purple bars). MPS IIIA untreated (blue bars), MPS IIIA treated with 1 mg/kg rhodamine B (purple bars) and normal untreated (green bars) shown from non-MGS mice. All data is presented as the mean ± standard deviation for each group at each generational timepoint. * indicates statistically significant, p<0.05, one-way ANOVA, MPS IIIA untreated versus normal untreated and ** indicates p<0.05, one-way ANOVA, MPS IIIA untreated versus treated.
Table 5.1: Multi-generational study tissue weights

<table>
<thead>
<tr>
<th></th>
<th>Kidney wt (g)</th>
<th>Heart wt (g)</th>
<th>Lung wt (g)</th>
<th>Brain wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Untreated</td>
<td>0.50 ± 0.17</td>
<td>0.18 ± 0.03</td>
<td>0.25 ± 0.04</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>MPS IIIA Untreated</td>
<td>0.55 ± 0.11</td>
<td>0.20 ± 0.03</td>
<td>0.23 ± 0.05</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>MGS1 MPS IIIA Tx</td>
<td>0.63 ± 0.13</td>
<td>0.22 ± 0.05</td>
<td>0.23 ± 0.03</td>
<td>0.44 ± 0.03</td>
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<tr>
<td>MGS2 MPS IIIA Tx</td>
<td>0.57 ± 0.10</td>
<td>0.20 ± 0.02</td>
<td>0.24 ± 0.03</td>
<td>0.48 ± 0.02</td>
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<tr>
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<tr>
<td>MGS4 MPS IIIA Tx</td>
<td>0.56 ± 0.09</td>
<td>0.18 ± 0.03</td>
<td>0.24 ± 0.05</td>
<td>0.46 ± 0.05</td>
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</tbody>
</table>

Post-mortem tissue weights (grams) recorded for low dose rhodamine B treated MPS IIIA mice from multi-generational study mice generations 1 to 4. Data is expressed as the average ± standard deviation for each group. A combined average for normal untreated and MPS IIIA untreated is presented. No difference was observed between any group for any tissue (p>0.05, one-way ANOVA)
Figure 5.9: Multi-generational study lysosomal storage.

Toluidine blue stained 5μM paraffin sections showed extensive lysosomal storage as indicated by arrows. Liver sections from MGS1 (A) and MGS4 (B) showed storage in hepatocytes, kidney sections from MGS1 (C) and MGS4 (D) showed storage in the distal convoluted tubule (DCT) epithelium. In the brain, the cerebellum of MGS1 (E) and MGS4 (F) showed storage in Purkinje cells (P) and also pyramidal neurons from the cerebral hemisphere of MGS1 (G) and MGS4 (H). All samples were taken at 7 months of age (6 months of Rhodamine B treatment). Scale bars indicate 25 μm.
III A untreated as previously shown in chapter 3 (Figures 3.10-3.13). Treated mice from all generations showed no alteration in lysosomal inclusions.

## 5.5 High dose rhodamine B study

MPS III A mice were injected with weekly intravenous tail injections of 5 mg/kg rhodamine B to determine if a higher dose was firstly more effective at preventing MPS III A disease pathology and secondly if the mice were able to tolerate a higher dosing treatment regime.

### 5.5.1 Measurement of physical parameters

In general, high dose treatment of 5 mg/kg did not show any reduction in MPS III A pathology. High dose treated animals had similar bodyweight range, tissue organ weights to MPS III A low dose treated mice, suggesting that the treatment was having minimal effect on pathology. No difference was observed between mice treated at 1 mg/kg rhodamine B compared to 5 mg/kg rhodamine B in regards to bodyweight, liver and spleen weight (Figure 5.10, p>0.05), which were in the same weight range as untreated MPS III A mice (data not shown).

### 5.5.2 Effect of high dose on lysosomal storage

No reduction in lysosomal storage was observed in MPS III A mice (Figure 5.11). This is similar to that seen with low dose rhodamine B treatment. Liver hepatocytes (Figure 5.11A), the distal convoluted tubule epithelium of the kidney (Figure 5.11B), cerebellar Purkinje cells (Figure 5.11C) and pyramidal neurons in the cerebral hemisphere (Figure 5.11D) showed extensive lysosomal storage.
Figure 5.10: Comparison of 1 mg/kg and 5 mg/kg bodyweight and liver and spleen weights.

Bodyweight of MPS IIIA male mice (A) treated with 1 mg/kg (open bars) or 5 mg/kg rhodamine B (solid bars). Liver weight (B) and spleen weight (C) of 1 mg/kg (open bars) or 5 mg/kg rhodamine B (solid bars). Data is expressed as the mean ± standard deviation for each group.
Figure 5.11: Effect of high dose treatment on lysosomal storage.

Toluidine blue stained 5μm paraffin sections of sections from MGS1 high dose treated MPS IIIA mice showed lysosomal vacuolation similar to untreated and low dose levels. Liver sections (A) showed lysosomal storage in hepatocytes (arrows), kidney sections (B) showed lysosomal storage in distal convoluted tubules (DCT) epithelium but minimal storage in proximal convoluted tubules (PCT). In the brain, the cerebellum (C) showed lysosomal storage in Purkinje cells (P) and pyramidal neurons in the cerebral hemisphere (D). Sections taken at 1000x original magnification. Bar = 25μm.
5.5.3 High dose toxicity study by histology

Since the high dose treated animals did not display physical improvement in parameters previously reported with treatment of rhodamine B, a toxicity study was carried out to determine if the high dose rhodamine B was having hepatotoxic effects since there are reports of death due to hepatic damage in rhodamine B treated animals (Bonser et al. 1956, Hansen et al. 1959). Liver sections were stained with haematoxylin and eosin (H&E), Sirius Red, Perl and PAS-D and examined for evidence of hepatotoxicity.

H&E staining showed large, binucleate hepatocytes which were common in all sections analysed (Figures 5.12A and B). Sections from the high dose treatment showed areas of mononuclear inflammation (MNI) (Figure 5.12A) which were not observed in the low dose treated mice (Figure 5.12B) in MGS1 but were evident in MGS4 MPS IIIA untreated and treated mice.

Sirius red staining for connective tissue, as a measure of liver fibrosis, showed no change in staining pattern with high dose treatment, with fine staining around the portal tracts (Figure 5.12C) and minimal staining in the parenchyma when compared to either MPS IIIA untreated or low dose treated mice (Figure 5.12D).

Perl staining to examine the cellular iron transport machinery showed weak staining in hepatocytes in high dose treatment similar to the other groups analysed with minimal positively stained blue areas (Figure 5.12E).

PAS digested sections to analyse mucins did not show any difference in PAS-positive material with high dose treatment (Figure 5.12G) compared to low dose treated mice (Figure 5.12H) or MPS IIIA untreated mice.
Figure 5.12: Histopathological analysis of high dose rhodamine treatment.

5μm paraffin embedded liver sections were stained with haemotoxylin and eosin (H&E) (A, B), Sirius Red (C, D), Perl (E, F) and PAS-digest (G, H) to examine hepatotoxicity. Arrows indicate binucleate hepatocytes (A, B) and positively stained cells for each section for C-H. MNI indicates mononuclear infiltrate (A) Mice are as follows MPS IIIA high dose rhodamine B treated mice (A, C, E, G) and MPS IIIA low dose rhodamine B treated mice (B, D, F, H). H&E and Sirius Red were taken at 200x original magnification and Perl and PAS-digested images were taken at 400x original magnification. Images were taken on an Olympus BX51 microscope using an Olympus DP12 camera. Scale bars indicate 100 μm (A-D) and 50 μm (E-H) respectively.
5.6 Discussion

Since rhodamine B has been shown to positively affect MPS IIIA disease pathology in both somatic (Chapter 3) and neurological systems (Chapter 4), the toxicity of rhodamine B was investigated. This was achieved using a multi-generational study over 4 generations to determine the outcome of extended exposure to low dose treatment and also to evaluate any toxic effects on offspring of dams treated with low dose rhodamine B in utero. The outcome on MPS IIIA disease pathology was also evaluated during the MGS. In addition to this, a high dose of 5 mg/kg rhodamine B was administered to MPS IIIA mice to determine if this dose was tolerated and if it had any improvement on clinical disease outcome.

Overall, the rhodamine B injections were tolerated well over 4 generations. A reduction in liver size was not evident in any of the generation analysed. This is in contrast to a multi-generational study conducted in rats (Nainggolan-Sihombing 1984) and could be due to a much lower dose administered to the mice in this study. This is unlikely to be due to toxicity, since normal mice that received rhodamine B treatment did not display a reduction in liver size (Chapter 3).

Studies of rhodamine B toxicity have shown that oral administration in mice and rats was reported to cause death due to hepatic damage (Bonser et al. 1956, Hansen et al. 1959). Since the liver has been shown to be the primary site of metabolism of rhodamine B in a number of animal models (Webb and Hansen 1961, Webb et al. 1961) liver sections were further analysed for effects of hepatotoxicity using histopathological methods.

Paraffin sections were stained with H&E, Perl, Sirius Red and PAS-digested special stains to determine if the rhodamine B treatment was having any toxic effects on the liver. No
obvious differences were noted in the H&E stained normal, MPS IIIA untreated and MPS IIIA treated (either high or low dose) liver sections with similar staining characteristics for each group. Mononuclear inflammation was noted in MGS1 high dose treated and MGS4 untreated and treated MPS IIIA mice, which is likely to be associated with MPS IIIA pathology. Overall, the treatment did not have any detrimental effects on liver histopathology with no alterations in connective tissue fibrosis (Sirius red), no disruption to the iron cellular transport system observed (Perl) and no alteration in mucin staining (PAS-D), with the latter being slightly increased in MPS IIIA mice. This is important since rat studies conducted by Umeda in 1956, showed that the livers from rats treated with subcutaneous rhodamine B at varying high concentrations were generally atrophic, hyperaemic (excess blood in organs), displayed haemosiderosis (excess iron accumulated), had increased Kupffer cells, had amyloidosis, nuclear degenerations and vacuolar degeneration of liver cells (Umeda 1956). The doses used in Umeda’s study were considerably higher (50 and 200 mg/kg/week) which showed toxic effects. In this study no significant abnormalities were observed post-mortem in mice treated over an extended timeframe of low dose treatment for 4 generations or at a higher dose.

In addition to histopathological analysis, liver function tests were carried out on generation 4 mice treated with rhodamine B. The tests included total protein, albumin, globulin, bilirubin and liver enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltranspeptidase (GGT). All liver function test parameters were similar in normal and MPS IIIA untreated mice with a slight reduction in globulin levels in MPS IIIA untreated mice observed, likely to be related to MPS IIIA pathology. Apart from this reduction in globulin, no alterations were observed in MPS IIIA mice, suggesting that pathology does not cause aberrations in liver function. Furthermore, when treated mice were analysed, they demonstrated similar liver function test scores to normal and MPS
III untreated with an increase in globulin concentration compared to MPS IIIA untreated mice. Altered liver function tests as a marker of hepatic injury can show an increase in transaminase activity (Navarro and Senior 2006) which was not observed in this case. Liver function tests have been used as a marker of hepatocyte injury and no alteration was observed with the extended dosing throughout the multi-generational study with low dose rhodamine B treatment.

The effect of a higher dose of intravenous rhodamine B was conducted in generation 1, with MPS IIIA males treated with weekly injections of 5 mg/kg rhodamine B. A reduction in liver weight and bodyweight was not observed with high dose treatment having a similar liver weight and bodyweight range to low dose treated and untreated MPS IIIA males. It is possible that the higher dose or extended timeframe of rhodamine B led to an increased liver weight due to toxicity, which would have counteracted the decrease in liver size shown in Chapter 3 since some studies have shown hepatomegaly when toxicity was observed12. However, toxicity studies using histopathology and liver function tests showed no alteration in pathology or liver health, so this is unlikely to be the case. The spleen size did not appear to be reduced with high dose treatment of rhodamine B, with a similar range observed in both treatment groups. This measurement is not constantly elevated in the MPS IIIA phenotype and the effect of low dose treatment has not been previously observed. It is possible that 5 mg/kg was not a high enough dose to see additional benefit of the treatment on MPS IIIA pathology and higher doses could be administered to determine these effects. The LD50 for rhodamine B is 89.5mg/kg, which is considerably higher than the 1 mg/kg, or the 5 mg/kg rhodamine B administered in this study. However, caution should be taken since a natural level of gag synthesis is needed for normal cell functioning (Templeton 1992). Another possible explanation is that the mice have become

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12 [Link to Pesticides website](https://www.pesticides.gov.uk/uploadedfiles/Liver%20paper%20post%20ACP(1).doc)
resistant to treatment and are no longer showing the reduction of physical phenotype observed in chapter 3. Other factors that may influence the susceptibility of this type of treatment include genetic background. Having mice on an unstable genetic background such as the mixed strain New York mice may introduce unknown variables on physical weights and response to treatment. This means that it is more difficult to discriminate between strain variation and differences due to the MPS IIIA mutation. The generation of congenic strains will be able to address some of these problems (Crawley et al. 2006) since they are bred onto a stable genetic background. Finally, the process of gag synthesis is a tightly controlled metabolic pathway. By interfering with synthesis of gags over an extended timeframe, a positive outcome on pathology may no longer be evident. The biosynthetic machinery may compensate over an extended timeframe of inhibition of gag synthesis.

Overall, the multi-generational study showed that treatment of pregnant dams with rhodamine B did not have any deleterious side-effects on litter size and that reducing gag synthesis over 4 generations of mouse breeding (approximately 2 years in total) was not detrimental to MPS IIIA mouse health. A high dose rhodamine B treatment was tolerated well by MPS IIIA mice, with no improvement on MPS IIIA pathology. A toxicity study was found that treatment (at either the low or dose) was not hepatotoxic in the different groups with a number of histopathological markers seen in higher dose studies in rats not observed in any case.
CHAPTER 6
DISCUSSION AND CONCLUSIONS
6.1 Discussion and Conclusions

Lysosomal storage disorders and in particular the mucopolysaccharidoses are chronic and progressive clinical syndromes with a wide range of clinical severity. Over 43 lysosomal storage disorders have been identified (Gieselmann 1995, Futerman and van Meer 2004), with an overall Australian prevalence of approximately 1 in 7000-8000 live births (Meikle et al. 1999). The clinical symptoms vary with each disorder, due to the effects of the protein defect and the nature of the stored material (Winchester et al. 2000). ERT and BMT are the only multi-tissue treatments available, however ERT does not address a number of sites including the brain and BMT has a high mortality associated with it. Currently, only the somatic system can be targeted and intravenous enzyme replacement is available clinically for MPS I (approved for use in Japan, EU and USA13), MPS II (approved for use in USA by FDA in July 2006 and awaiting approval in early 2007 in the EU14) and MPS VI (approved for use in EU and USA in 200615).

Even though ERT is the treatment of choice for non-neurological MPS disorders, as it affects the life-threatening symptoms of MPS, there are a number of problems associated with this type of therapy. Firstly, targeting the CNS still remains a major challenge. The blood-brain barrier prevents access of the recombinant enzyme to the CNS, and unless administered by direct injection into the brain (Savas et al. 2004), shows little effect. Similarly, access to the cartilage and cornea is restricted and pathology continues to worsen despite treatment (Crawley et al. 1997, Byers et al. 2000). It is likely that MPS patients on ERT regimes, even though they show improvement in mobility and other clinical markers, will exhibit subtle changes in disease progression from an acute disease where patients die in the second or third decade to one of chronic degenerative joint...
disease, blindness and mental retardation. In addition to problems of enzyme access, cost estimates for ERT in Fabry patients is between AUD $200,000 and AUD$400,000 per patient per year\(^\text{16}\) (Sawkar *et al.* 2002) with similar expenditure expected to be associated per annum for ERT in other LSDs. This, along with the fact that enzyme has to be infused on a weekly basis, usually in a hospital setting, turns the focus to alternative treatment strategies such as gene/cell therapy and substrate deprivation therapy.

Treatment for lysosomal storage disorders can be achieved in two ways. Firstly, treatments aimed at increasing the amount of deficient enzyme, such as enzyme replacement therapy, bone marrow transplantation and gene therapy directly target gag for degradation. An alternative way to approach therapy is by slowing down the initial synthesis of substrate, known as substrate deprivation therapy. This type of therapy has been shown to be effective in a number of glycolipid storage disorders, where an inhibitor of the synthesis of a precursor ceramide, interferes with the production of glycolipids. In turn this decreases the amount of substrate reaching the lysosome, reduces the burden on the cell and corrects patient pathology. The drug, *NB*-DNJ (Zavesca) has been used clinically in type I Gaucher patients (Cox *et al.* 2000, 2003) with phase III clinical trials in progress for juvenile Tay-Sachs and juvenile Sandhoff,\(^\text{17}\) Niemann-Pick C and type III Gaucher disease\(^\text{18}\). SDT has been used successfully to reduce lysosomal storage by inhibition of glycolipid synthesis in a number of animal models and is used clinically to treat type I Gaucher patients. This thesis aimed to determine if this approach could be applied to MPS disorders by inhibiting the synthesis of gag substrate. A reduction in gag synthesis is expected to have a flow-on effect on lysosomal gag storage and ultimately on somatic and CNS disease progression in MPS.


\(^{17}\) http://ntsad.org/S05/S05research_init.htm

Chapter Six: Discussion and Conclusions

Substrate deprivation therapeutics can be formulated into orally administered drugs which have been shown to have improved patient compliance, especially in paediatric patients (Pawar and Kumar 2002). The use of small inhibitor molecules opens the door for access to the CNS, which is yet to be successfully targeted. Most inhibitors fall below the molecular weight cut off of 500 Daltons, so are therefore able to penetrate the blood-brain barrier, by passive diffusion. This is important, since reduction of storage in the CNS is likely to have a positive outcome on reducing CNS pathology.

For SDT to be effective, some residual enzyme needs to be present to turnover gag. Antibody analysis has revealed that most MPS patients have reduced but detectable levels of protein, which in general are associated with detectable residual enzyme activity (Brooks 1993). Detailed genotype/phenotype correlations are needed in order to determine the predicted outcome of a specific mutation. Of interest is that the three most common and severe mutations identified in MPS IIIA, R245H, S66W and R74C, still possess some residual enzyme activity (Perkins et al. 1999, 2001) which is important for SDT to be an efficacious therapy. In infantile-onset patients where residual enzyme activity is likely to be lower, this can potentially be enhanced by using SDT as an adjunct to enzyme replacement therapy. The combination of SDT and ERT is likely to reduce the dose of enzyme required and the frequency of enzyme administration for patients.

It has been demonstrated that in vitro treatment of normal and MPS patient skin fibroblasts resulted in a dose dependent decrease in gag synthesis when treated with rhodamine B. This was shown to have a flow-on effect on lysosomal gag storage in vitro with a non-specific inhibition observed against dermatan and heparan sulphate. This positive response in vitro was extended to an in vivo therapy trial in the MPS IIIA mouse. In vivo treatment with 1mg/kg weekly injections of rhodamine B, reversed weight gain, urinary gag
excretion and elevated tissue gag levels characteristic of MPS IIIA pathology as reported in chapter 4. The effect of SDT on behaviour was tested in a 4-arm water cross maze, which measures spatial learning and memory (Chapter 5). MPS IIIA untreated mice did not perform to the same level as normal littermates having increased escape latency, increased incorrect entries and decreased correct entries. Treatment with rhodamine B significantly improved MPS IIIA performance towards normal with treated mice having decreased escape latency, decreased incorrect entries and increased correct entries when compared to MPS IIIA untreated littermates. This shows that SDT with a general inhibitor of gag synthesis, rhodamine B, is able to slow down the progression of MPS IIIA disease and improve neurological outcome as measured using the cross maze. This type of therapy could be applicable to MPS patients and be developed as a stand alone therapy.

This study demonstrates “proof of concept” that reducing the level of gag synthesis has a positive effect on delaying pathology in an animal model of MPS. Mouse models are useful in that they have a low maintenance cost, short breeding cycles and sufficient numbers can be generated quickly for testing therapeutic principles. However, to fully understand the potential of SDT as a human therapy capable of addressing all sites of MPS pathology, long-term studies of SDT must be undertaken, preferably in large animal models. Smaller animal models have a limited lifespan and may not display the slower developing pathologies such as skeletal disease, which can only be seen in larger animal models and are more indicative of the human disease. Numerous animal models are available encompassing the range of pathology, CNS, skeletal, and otherwise, observed in human MPS (Ellinwood et al. 2004). In particular the MPS IIIA dog models available provide invaluable tools for evaluation of this type of therapy (Fischer et al. 1998, Jolly et al. 2000) before translation can be achieved in a human setting. The long-term (>6 months) chronic effect of depressing gag synthesis showed no deleterious effects as demonstrated in
MPS IIIA mice by the multi-generational study. Larger animal studies such as in the MPS IIIA dog, and subsequent treatment with SDT should uncover any associated problems with depressing gag synthesis that more closely match the human situation. If successful in these larger models of MPS IIIA, the same type of therapy could be applied to MPS I and IIIB dogs and MPS VI cats to determine effectiveness of reducing gag synthesis in alternative models of storage disease.

Other aspects for further investigation would be to test this type of inhibitor in models of combined storage. Of particular interest is the MPS VII knockout mouse model, which stores multiple gags and displays both CNS and skeletal pathology. There are a number of models of MPS VII that have been generated that have various levels of residual enzyme activity ranging from 1-3% (Birkenmeier et al. 1989, Sands and Birkenmeier 1993, Watson et al. 1998, Tomatsu et al. 2002). All of these models of MPS VII would be useful for trialling rhodamine B treatment to determine the relationship between residual enzyme activity, the outcome on pathology and effect of treatment. It has been shown in chapter 4 that rhodamine B treatment of MPS IIIA mice was able to improve their learning capability in a cross maze test suggesting that the rhodamine B was likely to pass through the blood-brain barrier. The advantage of this type of therapy is that it relies on small molecules which are able to target tissues which have previously been shown to have no effect with replacement enzyme by ERT or gene therapy. The effect of SDT treatment skeletal development and prevention of skeletal dysplasia is currently unknown and requires further investigation.

As discussed, SDT has the potential to be used in MPS conditions as a stand-alone therapy such as currently employed for NB-DNJ in Gaucher disease (Jeyakumar et al. 1999, Cox et al. 2000, 2003). The potential of SDT may be more fully realised when considered as an
adjunct to other forms of therapy, especially in patients with juvenile onset who lack residual enzyme activity. The efficacy of SDT combined with BMT has been demonstrated in Sandhoff mice (Jeyakumar et al. 2001). Mice on combined therapy survived significantly longer than those on either treatment alone, indicating the potential for combined therapies in patients with a low level of residual enzyme activity. Similarly, combined SDT and ERT or gene therapy can have a number of advantages. Administration of enzyme will have an immediate effect to reduce systemic storage and continuation of patients on SDT could prevent or slow down re-accumulation of storage. This could have a number of flow-on effects including reducing the dose of enzyme required, frequency of enzyme administration and altering the mode of administration. Together, these could be expected to lower the financial burden of treatment and increase patient compliance (Tifft and Proia 2000). In addition, SDT can be expected to impinge on “difficult to treat” tissues. By nature of their small size, chemical inhibitors can be expected to distribute throughout the body reaching tissues and cells not accessible to large recombinant lysosomal enzymes.

Another type of combination therapy could be applied to HS-storing MPS disorders, which simultaneously targets the primary and secondary storage products stored in the lysosome. An inhibitor of gag synthesis could be used in conjunction with an inhibitor of secondary glycolipid or ganglioside accumulation, such as miglustat (NB-DNJ), which is able to target inhibition of glycolipids thought to be the cause of most CNS associated pathology. By targeting both types of substrates, this is likely to have positive effects on clinical outcome, especially in the CNS, where NB-DNJ has been shown to alleviate pathology in GSL models (Platt et al. 1997a, Jeyakumar et al. 1999). It is thought that the ganglioside storage is a strong contributor to the CNS associated storage in MPS disorders (Bhaumik et al. 1999, Glidddon and Hopwood 2004, McGlynn et al. 2004, Savas et al. 2004, Walkley
2004) and that targeting both of these lysosomal substrates simultaneously, is likely to have a synergistic effect.

Another important consideration is the choice of SDT agent. Of the available gag synthesis inhibitors, only rhodamine B has been tested for toxic effects \textit{in vivo}. Reports of an acute exposure to rhodamine B, at a larger dose than used here, resulted in mucous membrane and skin irritation in humans (Dire and Wilkinson 1987), with no adverse effects. It was shown that long-term exposure to low dose rhodamine B in mice was well tolerated; however, the effect of this type of inhibition when translated to humans as a therapy is currently unknown. Although it may be feasible to use rhodamine B as a SDT agent \textit{in vivo}, other inhibitors may be more practical. Of particular consideration should be the fluorinated analogues of glucosamine and galactosamine respectively as reported by Berkin and colleagues (Berkin \textit{et al.} 2000a, 2000b).

SDT for glycolipid storage disorders have been targeted using non-specific inhibitors. The same approach targeting gags for MPS disorders has been shown to be effective for a non-specific inhibitor of gag synthesis. Treatment of normal mice with low dose rhodamine B had no deleterious side-effects and suppression of gag synthesis in MPS IIIA over 4 generations did not show any signs of toxicity. However, non-specific inhibition of gag synthesis in cells may also have undesirable effects, since gags have important roles in normal cell function and targeting specific gags is more appropriate. Inhibition of specific gag synthesis can be achieved using gene silencing of the biosynthetic machinery, which will reduce the production of gags or sulphation of gags within cells. By targeting specific gags, this type of therapy will be applicable to groups of storage disorders which have the same lysosomal substrate accumulated. RNA interference for the transferases and sulphatases involved in gag biosynthesis are ideal targets for gene silencing.
In conclusion, SDT can be used to target the gag substrate. No obvious side effects to administering rhodamine B were noted in an extended timeframe study suggesting that this treatment is safe. SDT targeted to MPS disorders reduced gag synthesis, had a flow-on effect on gag storage and improved the clinical progression in a mouse model of MPS IIIA. Of particular interest is that SDT proved effective in reducing CNS pathology as detected by reduced lysosomal brain gag and improvement in a behavioural learning and memory test. This is promising since the CNS has so far resisted all attempts at correction. Transfer of this concept from animal studies to clinical trials should be relatively straightforward, since the framework has already been set up for the use of SDT agents in glycolipid storage disorders. The search for safer and more effective agents than those used in preliminary studies is ongoing and it is anticipated that this type of therapy may be a viable treatment option for children with MPS especially those with CNS pathology.
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References


References


References


References


References


References


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APPENDIX I
Appendix I: RAPC Repeated Acquisition Sequences

List of doors and their corresponding sessions that remained open during repeated acquisition trials. Note: all other doors not listed remain locked during testing.

<table>
<thead>
<tr>
<th>Session</th>
<th>A1-B2-C1-D3</th>
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</thead>
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<tr>
<td>Session 2</td>
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<td>A1-B2-C3-D1</td>
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</tr>
<tr>
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<td>A2-B3-C1-D2</td>
</tr>
<tr>
<td>Session 12</td>
<td>A3-B2-C1-D3</td>
</tr>
</tbody>
</table>
Appendix II: Publications arising from this work:


Publications arising from collaborative work:

Addenda

Chapter 1
p10, line 18: Insert words “and produce” after breed (add and produce)
p12, line 5: Change “relative permeable” to “relatively permeable”
p14, line 1: Remove comma after blood-brain barrier
p16, line 18: Insert after co-administration of antihistamine... "or by prolonging the infusion time or by a range of other drugs including those with anti-inflammatory and immunosuppressive properties such as corticosteroids (Schiffman and Brady 2002, Drugs; 62(5):733-742; Beck et al. 2004, Eur. J. Clin. Invest. 34:838-844).
p17, line 6: Add word “function” before reference.
p18, line 13: Replace words “Recent evidence has” with “It has been”
p19, line 14: Remove the word “outcome”
p21, line 19: Insert underlined words “In another study of MPS mice were treated... and 12 month...”
p22, line 21: Insert “and cross-correction via mannose-6-phosphate mediated uptake following cell engraftment” at end of sentence.
p23, line 4: Replace word “production” with “localisation”
p24/25, Figure 1.7: Figure 1.7 A-D have been replaced. Legend line 9: replace “though” with “thought”
p26, line 1-3: Sentence should read “These patients showed a reduction of mean liver and spleen volumes and also an increased...”
p28, line 5 and line 8: Replace “Twitcher” with “twitcher” to indicate strain of mice
p28, last line: Replace sentence with “These compounds can be formulated to be administered orally, which is a major advantage”
p30, line 3: Add word “white” after “New Zealand”
p32, line 12: Add comma after “isoflavones”
p33, line 24: Replace “beta-glucuronidase” with “beta-glucosidase”
p34, lines 3, 4, 5, 8: Replace “beta-glucuronidase” with “beta-glucosidase”
p34, line 15: Insert word “the” after “stop codons in”
p34, line 20: After “preclinical studies” insert “in MPS I”
Figure 1.9 legend, line 15 Replace “structure” with “structures”
p40, line 11: Replace “2-3” with “two to three”
p41, line 6: Replace “uncovered a role in TGF-β regulation in adult cells via protein-protein interaction” with “uncovered a role of syndecan-2 and its direct binding to TGF-β which regulates TGF-β signalling in adult cells”

Chapter 2
p71, lines 4-6: Replace sentence with “If a mouse failed to reach the escape platform within the allocated 60 seconds, the mouse was place manually onto the platform”

Chapter 3
p85, line 11 and Figure 3.5: Insert: “Female mice included in this study were not used for colony breeding and therefore were not pregnant or had litters.” A power analysis to determine how many female mice would be needed to confirm the difference in bodyweight was conducted and to achieve a power analysis of 80% with a difference in weight of 15% a sample size of 4 mice would be needed and 2-5 mice were analysed for each group at 4 months of treatment.
Table 3.1: Insert “(n=3 per group)” after “normal treated mice” in table legend.
p86, line 22: Insert “A reduction in total gag was seen in MPS IIIA treated mice, which on average was reduced to 74% of MPS IIIA untreated total gag levels in the liver (Figure 3.8A). This would incorporate total gag including a lysosomal component, and this decrease is likely to be due to reduced
lysosomal gag. To confirm this, lysosomal gag was also analysed. Analysis of lysosomal gag showed a greater reduction compared to total gag levels, with MPS IIIA treated mice on average reduced to 60% of MPS IIIA untreated lysosomal gag levels (Figure 3.8B). This was expected since MPS IIIA is a lysosomal storage disorder and most of the gag reduction was lysosomal gag.”

Chapter 4

p91, line 2: “si” should read “is”
p91, line 11: Add word “been” after “has not”

Chapter 5

p103, 2nd last line of 2nd last paragraph: Missing full stop at end of the sentence.
p114, insert before 3rd paragraph: “Qualitative changes in lysosomal storage in the brain or somatic tissues have not been observed in either study reported in chapters 3 and 4 using light microscopy analysis. However, a small decrease in brain lysosomal gag was seen when a quantitative assay was employed. The discrepancy between the biochemical measurement and visual assessment of storage may be due to the relative insensitivity of light microscopy to detect small changes in lysosomal storage. SDT acts to slow down the accumulation of gag substrate in the lysosome, providing the opportunity for residual lysosomal enzyme activity to slowly turn over resident and newly acquired substrate. Thus conceptually SDT will not have the dramatic effect on gag storage observed with other types of treatment such as enzyme replacement therapy.”

Chapter 6

p128, line 12: Remove “had” after “Kupffer cells”
p129, line 24: Replace “natural level” with “minimal level”
p130, line 18: Remove “was” after “a toxicity study”

Chapter 6

p132, 2nd paragraph: Reword first sentence to clarify: “Even though ERT has been used to treat a large number of patients with non-neurological MPS pathology, there are still a number of problems associated with this type of therapy. Firstly, targeting....”