Stem Cell Therapy for Treatment of Central Nervous System Pathology in α-Mannosidosis Guinea Pigs

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Thesis submitted for the degree of Doctor of Philosophy

in

The University of Adelaide (Faculty of Medicine)

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May 2004
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**Abbreviations:**

1°: primary  
2°: secondary  
3°: tertiary  
ART: Assisted reproductive technology  
ASM: Acid sphingomyelinase  
BBB: Blood brain barrier  
bFGF: Basic fibroblast growth factor  
βME: β-Mercaptoethanol  
BMT: Bone marrow transplantation  
CHO: Chinese hamster ovary  
CNS: Central nervous system  
CSA: Cyclosporin A  
CY3: Indocarbocyanine  
CY5: Indodicarbocyanine  
Da: Daltons  
DMSO: Dimethyl sulfoxide  
EB: Embryoid body  
EBM: Embryoid body cultured in MEDII  
EG: Embryonic germ  
EGF: Epidermal growth factor  
EPL: Primitive ectoderm-like  
ERT: Enzyme replacement therapy  
ES: Embryonic stem  
et al.: et alius  
FACS: Fluorescence-activated cell sorting  
FCS: Fetal calf serum  
FGF: Fibroblast growth factor  
FITC: Fluorescein  
g: gravity  
G: gauge  
GFAP: Glial fibrillary acid protein  
GSK-3β: Glycogen synthase kinase-3β
GVHD: Graft versus host disease
IVF: In vitro fertilisation
kb: kilobases
kDa: kilo Daltons
L: litres
LIF: Leukaemia inhibitory factor
LSD: Lysosomal storage disease
μ: micro
μD: micro Farads
μg: micrograms
μl: microlitres
M: moles/litre
M6P: mannose-6-phosphate
MFI: Median fluorescence intensity
min: minutes
ml: millilitres
mol: moles
MPR: mannose-6-phosphate receptor
MPSIIIA: Mucopolysaccharidosis type IIIA
MSC: Marrow stromal cell
MSCV: Mouse stem-cell virus
n: nano
NDS: Normal donkey serum
NF200: Neurofilament protein, 200kDa subunit
NGF: Nerve growth factor
NPD: Niemann-Pick disease
NSC: Neural stem cell
Oct4: Octamer 4
O.D.: Optical density
OsO₄: Osmium tetroxide
PBS: Phosphate buffered saline
PEG: Polyethylene glycol
Pfa: Paraformaldehyde
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<tr>
<td>PMP</td>
<td>1-Phenyl-3-methyl-5-pyrazolone</td>
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<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>rhαM</td>
<td>Recombinant human lysosomal α-mannosidase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>Sfrp2</td>
<td>Secreted frizzled-related protein-2</td>
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<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
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<td>U</td>
<td>units</td>
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<td>V</td>
<td>Voltage</td>
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<td>WCH</td>
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Thesis abstract:

Lysosomal storage disorders (LSD) are a group of heritable genetic diseases resulting from a deficiency of one or more lysosomal enzyme activities, with broad pathological consequences. One of the most difficult aspects of these diseases to treat is central nervous system (CNS) pathology. Numerous strategies are being pursued in order to develop effective therapies for CNS pathology in LSD. One of these strategies involves the implantation of stem cells for the in vivo secretion of deficient enzyme in the brain, to be taken up by host cells, to therapeutic effect.

α-Mannosidosis is a LSD resulting from a functional deficiency of lysosomal α-mannosidase. This deficiency results in the accumulation of various oligosaccharides in the lysosomes of affected individuals, to cause progressive neurological degeneration and other somatic pathology. We have a guinea pig model of this disease that closely models human α-mannosidosis. Although enzyme replacement therapy has shown great promise for treatment of somatic pathology in α-mannosidosis guinea pigs, it is not effective for treatment of brain pathology. Thus, this disease model was chosen as an appropriate disorder for the evaluation of intra-cranial stem cell implantation as a therapeutic approach.

α-Mannosidosis guinea pigs display significant neurological abnormalities as part of the course of their disease. We postulated that the development of tests to quantitate the loss of neurological function underlying these characteristics would be useful for evaluation of therapies in this model. The first aim of this study was thus to establish such tests.

The Morris water maze has been used to evaluate therapies for neurological disease in mouse models of LSD, and its use in guinea pigs has more recently been described for analysis of memory and learning difficulties arising from prenatal ethanol exposure. A pilot study was first carried out to determine the feasibility of using this test to investigate cognitive deficits in α-mannosidosis guinea pigs. Following the observation that neurological pathology develops to a readily quantifiable extent by three months of age, a larger study was undertaken using naïve (not previously tested) three month old α-mannosidosis and normal guinea pigs, to provide a background against which cell implantation could later be evaluated.

Previously, gait changes have been observed in α-mannosidosis guinea pigs.
(relative to normal animals) at two and three months of age (Dr. Kim Hemsley, unpublished observation). This thesis included further investigation of these changes. A previously developed general neurological examination protocol (originally established by Dr. Allison Crawley) was also further modified (in collaboration with Dr. Allison Crawley) and used to test all of the animals in this study, in order to chart the progression of pathology observed in α-mannosidosis.

The establishment the Morris water maze along with further characterisation of gait changes and neurological abnormalities further expands the existing battery of histological and biochemical tests available for the analysis of pathology in the α-mannosidosis guinea pig. The availability of such tests thus adds to the potential utility of this animal model to evaluate CNS treatment options in LSD.

Another aim of this study was to construct an embryonic stem (ES) cell line for the over-expression of recombinant human lysosomal α-mannosidase (rhαM), and to evaluate the potential of this cell line for therapeutic, supra-physiological expression of α-mannosidase in the α-mannosidosis brain. With a view to achieving sustained over-expression of rhαM in mouse ES cells and their differentiated progeny, expression vectors were constructed before generating transfected mouse ES cell clones, and isolating and characterising these clones for rhαM expression and differentiation potential. Further characterisation of the highest expressing clone included a cross-correction experiment, which showed that enzyme produced by transfected cells was able to be endocytosed by α-mannosidase-deficient human skin fibroblasts and mediate a reduction in stored oligosaccharides. These results suggest possible therapeutic utility of mouse ES cell clones expressing rhαM for ES cell therapy in the α-mannosidosis guinea pig brain. Additionally, constructing this cell line using pluripotent mouse ES cells allows the flexibility to take advantage of future development of techniques for the manipulation and implantation of embryonic stem cells and their differentiated progeny. Future developments in this area should thus open up further avenues of investigation for stem cell therapies in the guinea pig model and other animal models of α-mannosidosis.

The implantation of pluripotent ES cells into sites of neonatal or adult animals carries the risk of teratoma formation. In order to facilitate site-specific differentiation of implanted pluripotent cells based on local environment (in contrast to continued
division and teratoma formation), implantation of low cell numbers has previously been utilised. Whilst characterisation of the transfected cell lines was ongoing, preliminary studies using this approach were carried out in order to develop the methods required for this therapy. This involved the implantation of untransfected differentiated mouse ES cells into the dentate gyrus of neonatal α-mannosidosis and normal (or heterozygous) guinea pig brains. Animals were sacrificed at various time points to determine survival and engraftment of implanted cells, as well as a group being taken through to 13 weeks post-implantation and tested with various behavioural tests.

Surviving graft derived cells were detected only out to eight weeks post-surgery. No graft-derived cells were observed, however, in any of the animals at 13 weeks post implantation. Further studies suggested that the lack of cell survival may be due to an immune response against the implanted cells despite the immunosuppression of implanted animals, or perhaps be due to a sub-optimal phenotype of implanted cells. There was no effect on behavioural pathology in α-mannosidosis guinea pigs as a result of this treatment. Histological analysis confirmed that these animals had no surviving cells, and no apparent reduction in lysosomal storage in the hippocampus. Thus, further refinement of differentiation and implantation protocols would be required for the development of a potentially effective therapy in this model.

The following review introduces the general field of LSD and specifically α-mannosidosis, beginning with the biology of the lysosome and the deficiencies that result in LSD. This review also discusses historical, current and future therapeutic approaches to treatment of LSD, including stem cell therapies.
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 is made in the text,

I give consent to this copy of my thesis being made available for photocopying and loan if accepted for the award of the degree.

SIGNED:.....  DATE:14/5/04
Acknowledgements:

Firstly I would like to thank my supervisors John Hopwood and Allison Crawley for their exceptional support, inspiration and guidance throughout my PhD. The last few years have been an incredible learning experience, on every level.

Thanks also to all the people I have worked with, without whose support this project would not have been possible; Kim Hemsley for her exceptional guidance, input and support, and for effectively acting as a third supervisor; Thomas Berg for his guidance in the early stages of this project; Tom Litjens (one of the most patient and organised people I have met) for his guidance with the molecular biology side of things; Dyane Auclair for all her invaluable assistance and guidance with the guinea pigs, (particularly for helping me to "think like a guinea pig"); Barbara King for her assistance with cyclosporin treatment and enzyme assays; Lynn Scarman, Hanan Elmassih, and Loren Ellis in the animal house for care of the guinea pigs and help with cyclosporin treatment; Adeline Lau for sharing the (occasional) pain of ES cell culture; and everyone else in the Allan Campbell lab- Colleen Bindloss, Litsa Kargeorgos, Briony Gliddon, Tony Pollard, Rowani Mohd Rawi, Peter Savas, Dan Toop, and Mike Bawden for always having the right widget.

Also from Women’s and Children’s Hospital, I’d like to thank Maria Fuller for her assistance with the mass spectrometry; Vivienne Muller and Paul Nelson for their help with enzyme assays and getting the PCR working again; Tony Ferrante, James Irvine and Greg Hodge for their assistance and guidance with FACS; Tony Bourne and the staff at the Department of Histopathology for the stains and use of their microscope; Craig Hirte and Janine Jones of the Public Health Research Unit for their invaluable and enthusiastic (!) help with statistics; Sophie Lazenkas for all her help with the bureaucratic side of things; and the WCH Core Lab for running all the cyclosporin (and other biochemical) assays.

From the University of Adelaide, I’d like to thank Dan Peet and Steve Wood for providing the basic vectors used in this study and for the helpful discussions; Joy and Peter Rathjen for their guidance and training with ES cells and helpful discussions; Ken Lang also for the useful discussions; and Rob Moyer for the helpful discussions, occasional antibody, and for splitting a hotel room at the ISSCR conference in Washington DC. I’d also like to thank Meredith Wallwork (Adelaide Microscopy) for assistance with confocal microscopy and Lyn Waterhouse (also Adelaide Microscopy) for her assistance and guidance with sample processing for electron microscopy.

Special thanks are due to BresaGen Ltd. for allowing the use of their lab space, equipment and expertise for the bulk of the histology part of this project, and providing support in the form of accommodation (and beer, through Bruce) at the Stem Cells conference in San Diego. Specifically, I would like to thank Peter Wigley, Jackie Zanetti and Chris Juttner for facilitating the collaboration; Natalie Hulbert for her crucial assistance and guidance with stereotaxic surgery (I know where that next decimal is now); Bruce Davidson and Dallas Grasby for the many helpful discussions; Adrian Meedeniya for his assistance and guidance with histology and image analysis, and for all the invaluable discussions on science, culture, politics, scotch whisky and everything else; Kate Pensa for her help and guidance with all the in situ; and Paul Bello for his prompt first aid when I impaled my hand on a rack of flame-polished Pasteur pipettes. I’d also like to thank Grant Hennig (Centre for Neuroscience, Flinders University) for his invaluable assistance and expertise with confocal microscopy and image processing, especially into the early hours of the morning.
Finally, I'd like to extend thanks to Miles Davis, John Zorn, Bill Hicks and the countless other avatars of the last few centuries for making the long hours of graphs and data processing more bearable; my friends and especially family for their love and support (particularly Danica Goode for her supreme patience, love and understanding), and my various housemates throughout the last few years: Luke for his innovative take on blended food; Ben for all the music, fragging and refreshing “unsanity”; Paul for the Project Mayhem backyard renovations; Heidi for “caning it hardcore” and Annie just for being Annie. Last but by no means least, I’d like to give thanks to the universe for an interesting first quarter of the ride...
Chapter 1:
Introduction
1.1.0 The Lysosome

The lysosome was first identified as a cytoplasmic particle containing acid hydrolases (DeDuve et al., 1955). These particles were further shown (by electron microscopic characterisation) to be membrane-bound vacuoles containing one or more acid hydrolases (Novikoff et al., 1960). The enzymes contained in these acidic organelles (for example, proteases and glycosidases) act to degrade substrates such as proteoglycans and glycoproteins (Hopwood and Brooks, 1997). Lysosomes can be distinguished from other intracellular organelles by their high density on Percoll gradients, their acidification properties, and the presence of known lysosomal proteins (Hopwood and Brooks, 1997).

In addition to degradative enzymes, lysosomes consist of membrane proteins such as structural proteins, proton ATPase for maintaining the acidic environment within the lysosomes (Van Dyke, 1996), transporters involved in the export of degradation products, ion channels, molecules involved in organelle traffic and fusions, proteins involved in interacting with the cytosol for regulation of the degradation process or lysosomal function, and molecules designed to resist or contain the degradative enzymes themselves (Hopwood and Brooks, 1997). Thus, effective lysosomal function requires not only the effective expression of a number of degradative enzymes, but also a whole plethora of other proteins.

Macromolecules destined for degradation in the lysosomes first undergo endocytosis at the cell surface via clathrin-coated pits, delivering them into endosomes. Cleavage of substrates such as proteoglycans begins in the endosomal compartment, before these partially degraded products are delivered to the lysosome for further digestion.

1.1.1 Lysosomal biosynthesis

Synthesis of lysosomal proteins for delivery to the lysosomes in normal cells involves a number of steps (see Figure 1.1 for a diagrammatical representation of these processes; Grabowski and Hopkin, 2003). Lysosomal proteins are first synthesised on membrane-bound polysomes in the rough endoplasmic reticulum (ER; Kornfeld, 1986). Each of these proteins contains a hydrophobic amino terminal signal peptide that interacts with a signal recognition molecule, an 11S ribonucleoprotein. This interaction facilitates transport of the newly synthesised protein across the ER membrane into the
lumen of that organelle (Erickson et al., 1981, 1983, Rosenfeld et al., 1982). Lysosomal enzymes then undergo glycosylation of selected Asn residues, involving the transfer of a preformed oligosaccharide from a lipid-linked intermediate to the newly synthesised polypeptide (Kornfeld and Kornfeld, 1985). The signal peptide is then cleaved within the ER, before further processing of the Asn-linked oligosaccharide, involving the excision of three glucoses and one of the mannose residues from the oligosaccharide. The proteins are then packaged into vesicles referred to as the trans Golgi network (TGN; Griffiths et al., 1988).

Shortly after this packaging, during passage through the TGN, these proteins undergo a variety of post-translational modifications, and are sorted for targeting to their final destination. The characteristic modification for most lysosomal enzymes is the addition of phosphomannosyl residues, which allows binding to high affinity mannose-6-phosphate (M6P) receptors, facilitating efficient translocation of these proteins to the lysosome (Kaplan et al., 1977).
Figure 1.1: Schematic representation of the lysosomal/endosomal system.
Synthesis of lysosomal proteins occurs in the rough endoplasmic reticulum (RER), before post-translational modification in the lumen of the smooth endoplasmic reticulum (ER) and the Golgi. In the trans-Golgi network (TGN), lysosomal enzymes are sorted for either secretion or passage to the lysosome. Lysosomes can fuse with other vesicles such as lysosomes, endosomes, and the multivesicular body (MVB).
Figure from Grabowski and Hopkin, 2003.
Figure 1.1
The M6P moiety on lysosomal enzymes is generated in two steps, involving the sequential action of two different Golgi enzymes (Kornfeld, 1986). The enzyme N-acetylglucosaminylphosphotransferase first transfers N-acetylglucosamine-1-phosphate from the sugar uridine diphosphate-N-acetylglucosamine to the 6-hydroxy position of one or more residues of N-linked oligosaccharide chains on the lysosomal enzymes, giving rise to a phosphodiester intermediate. The enzyme N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase then removes the N-acetylglucosamine residue, leaving the M6P moiety on the lysosomal enzyme. (Kornfeld, 1986, Kornfeld and Mellman 1989). The M6P moiety can then bind to M6P receptors in the TGN, allowing efficient targeting of the enzyme to the lysosome (Pfeffer, 1988).

In addition to the oligosaccharide processing described above, lysosomal enzymes can also undergo further proteolytic processing by proteinases in the prelysosomal and lysosomal compartments, including fragmentation and trimming at the amino and carboxyl termini of the protein (Kornfeld, 1986).

1.1.2 Mannose-6-phosphate receptors

Two distinct types of M6P receptors (MPRs) have been isolated: a 46kDa and a 300kDa form (reviewed by von Figura and Hasilik, 1986, Pfeffer, 1988, Dahms et al., 1989, and Pfeffer, 1991). The 46kDa MPR requires the presence of divalent cations for efficient binding, and is thus referred to as the cation-dependent MPR. This receptor is a type I integral membrane glycoprotein, comprised of a 257 amino acid extracellular domain, and a 67 amino acid cytoplasmic domain. Additionally, the dimerisation of this receptor may be necessary for correct intracellular transport between the cell membrane, TGN and endosome (Pfeffer, 1988). In contrast, the 300kDa MPR demonstrates binding activity independent of divalent cations, and is thus referred to as the cation-independent MPR. Also a type I integral membrane glycoprotein, this receptor has been shown to be identical in structure and function to the insulin-like growth factor receptor and is therefore also referred to as the M6P/IGF-II receptor (Goda and Pfeffer, 1988). This receptor is also comprised of two domains, a 226.5kDa extracellular domain and a 164 amino acid cytoplasmic domain.

Both the 46kDa and 300kDa MPRs have been shown to be involved in the sorting of proteins to the lysosome (Goda and Pfeffer, 1988), although only the 300kDa (cation-independent) receptor is involved in the endocytosis of extracellular lysosomal
enzymes at the cell surface (Kornfeld, 1992). Both receptors are found primarily within endosomes and the Golgi, and to a lesser extent, at the cell surface. These receptors are readily transported between these compartments, irrespective of whether they are bound to a mannose-6-phosphorylated lysosomal enzyme (Pfeffer, 1987, Duncan and Kornfeld, 1988).

1.1.3 Mannose-6-phosphate dependent and independent transport

Following mannose-6-phosphorylation of newly synthesised lysosomal enzymes, the majority of these proteins are targeted directly to the lysosome via a direct intracellular route. These enzymes bind to either a 46kDa or 300kDa MPR in the TGN, at pH 7.0. This complex then exits the Golgi and is transported in clathrin-coated vesicles to the prelysosomal compartment (Goda and Pfeffer, 1988). This compartment is also often referred to as the "intermediate compartment", as it links endocytosed material destined for degradation and newly synthesised lysosomal enzymes (Duncan and Kornfeld, 1988). The low pH (less than 6) in this compartment facilitates dissociation of the MPR from the phosphorylated mannose residue of the lysosomal enzyme or proteins (Griffiths et al., 1988). The lysosomal enzymes then continue their transport to the lysosome, while the free MPR is either recycled back to the Golgi for further targeting of newly synthesised lysosomal enzymes (Duncan and Kornfeld, 1988) or transported to the plasma membrane in order to participate in the endocytosis of exogenous lysosomal enzymes (Dahms et al., 1989).

A proportion of mannose-6-phosphorylated lysosomal enzyme (usually 5-20%) escapes lysosomal targeting via this pathway, resulting in secretion from the cell (Dahms et al., 1989). Secreted enzyme may then be recaptured by binding MPRs present on the cell surface, resulting in internalisation and delivery to the lysosome via an endocytic pathway (Willingham et al., 1981). This secretion-recapture mechanism has been shown in fibroblasts to target approximately 5-10% of lysosomal enzymes to the lysosome (Vladutiu and Rattazzi, 1979). This endocytic pathway is highly fortuitous for therapeutic approaches to lysosomal storage disorders (discussed further below), since all cells require an active lysosomal system and appear to be able to take up lysosomal enzymes from surrounding cells and body fluids. This endocytic pathway thus provides an efficient mechanism for targeting lysosomal enzymes provided by
various replacement therapies (discussed later in this thesis) to the lysosome, where they are needed to correct the cellular defect.

Lysosomal enzymes may also be targeted to the lysosome independently of the MPR pathway (Kornfeld, 1986). Evidence for the existence of this alternative pathway came from characterisation of cells from patients with I-cell disease (mucolipidosis II). Affected cells in this disease are unable to synthesise the 6-phosphomannosyl marker on their lysosomal enzymes, resulting in these enzymes being unable to bind to the MPR for lysosomal targeting (Kornfeld, 1986). Fibroblasts from I-cell patients contain normal levels of some lysosomal enzymes (such as β-D-glucuronidase and acid phosphatase), but significantly reduced activities of other enzymes, such as arylsulphatase A. However, lysosomal storage in I-cell disease is limited to only certain tissue types (primarily connective tissue), with no storage observed in the liver, spleen, kidney or brain, which all contain near normal levels of lysosomal enzymes (Kornfeld, 1986). The M6P-independent pathway for transport of proteins to the lysosome is thought to involve targeting via an 11 amino acid cytoplasmic domain on these proteins (Pfeffer, 1991), or via receptors specific for mannose, galactose, fucose and N-acetylgalactosamine (Koster et al., 1994).

1.2.0 Lysosomal storage disorders

The concept of a "lysosomal storage disorder" (LSD) was first introduced by Hers in 1965, to explain how the absence of an enzyme (α-glucosidase) could lead to the fatal condition known as Pompe disease. Since then, LSD have been recognised as a group of genetic disorders (each resulting from a different genetic defect) characterised by accumulation of various macromolecular substrates in the lysosomes of affected individuals. Lysosomal storage disorders occur due to a functional and/or quantitative deficiency of an enzyme or enzymes required for the degradation of these substrates, or transport of the degradation products out of the lysosome (Hopwood and Brooks, 1997). Storage of these compounds results in distended lysosomes visible by electron microscopy, as can be seen in Figure 1.2. Lysosomal storage disorders collectively occur in approximately 1 per 7700 live births in Australia (Meikle et al., 1999).

Lysosomal storage disorders usually follow an autosomal recessive mode of inheritance, with the exceptions being Hunter syndrome, Fabry disease and more
recently, Danon disease, which display an X-linked mode of inheritance (Clarke, 1997, Nishino et al., 2000). Lysosomal storage diseases can be classified into different subtypes based on the stored material or the nature of the substrate, with different pathological consequences (Clarke, 1997). To date, there are over 45 LSD known (see Table 1.1).

Lysosomal storage disease patients present with a variety of different clinical symptoms. The development of this pathology is progressive, at a rate depending on the nature and severity of the storage disease. Clinical presentations of different LSD can vary depending on whether the disorder involves somatic storage, CNS storage, or both (reviewed by Neufeld, 1991; Clarke, 1997; Hopwood and Brooks, 1997).

Facial dysmorphia is commonly observed in disorders with somatic storage, resulting in a characteristic facial appearance in LSD patients. Hepatosplenomegaly (enlarged liver and spleen) is also a common feature in LSD with somatic storage, with extreme cases reporting enlargement of up to 75 to 80 times normal volume for these organs (Clarke, 1997). Skeletal involvement (the main features of which are commonly referred to as dysostosis multiplex) is also often seen with somatic storage. This feature involves intrinsic abnormalities in the structure of growing bone, resulting in morphological and structural changes in the skeletal system of LSD patients. These changes can include a narrowing of the vertebral ends of the ribs in combination with a flattening and broadening at the sternal end, an enlargement of the diaphyses and an irregular appearance to the metaphyses of long bones, and a “roughened” appearance of the epiphyseal centres. Dysostosis is often most pronounced in the vertebrae, with anterior beaking and complete vertebral dislocation (Clarke, 1997).

Another common feature of LSD is CNS dysfunction, present in approximately two-thirds of all LSD (Professor John Hopwood, personal communication). This dysfunction is thought to occur as a result of lysosomal storage within the brain, but the exact mechanisms underlying much of the CNS dysfunction are at present poorly understood (Clarke, 1997). The pathogenesis of neurological disease will be discussed in further detail below. Clinically, neurological involvement usually involves presentation with cognitive deficits, behavioural abnormalities and delayed developmental milestones (Clarke, 1997).
Figure 1.2: Electron micrograph showing lysosomal storage
Neurons from the cortex (A) and cerebellum (B) of an α-mannosidosis guinea pig (Crawley et al., 1999). Arrows indicate lysosomal storage.
Table 1.1: Summary of the lysosomal storage disorders and present patient treatment strategies.
Table reproduced with permission from Lysosomal Diseases Australia (Newsletter, November 2000).
<table>
<thead>
<tr>
<th>Disease</th>
<th>Clinical Phenotype</th>
<th>Enzyme Deficiency</th>
<th>Chromosome Location</th>
<th>Bone Marrow Transplantation</th>
<th>Animal Model</th>
<th>Human Enzyme Replacement Therapy</th>
<th>Australian Prevalence</th>
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1.3.0 α-Mannosidosis

First described specifically in humans in 1969 by Kjellman et al., α-mannosidosis is one of two disorders (along with β-mannosidosis) of glycoprotein catabolism (part of a group of disorders collectively referred to as the glycoproteinoses) that result in abnormal levels and excretion of mannose-rich oligosaccharides (reviewed by Thomas and Beaudet, 1995 and Michalski and Klein, 1999). This autosomal recessive disease is caused by a mutation(s) in the α-mannosidase gene that result in a decrease or loss of α-mannosidase activity and/or protein in the lysosomes of affected individuals. α-Mannosidosis occurs in approximately 1 per 1 million live births in Australia (Meikle et al., 1999).

1.3.1 Clinical description

Desnica and others originally suggested that α-mannosidosis could be separated into two groups based on clinical severity: infantile (type I) and juvenile-adult (type II) α-mannosidosis, with the infantile form resulting in more rapid progression of the disease (Desnica et al., 1976, Letson and Desnica, 1978). In reality a continuum of clinical severity probably exists, depending on the effect of the mutation on the α-mannosidase protein, and thus the residual activity (Thomas and Beaudet, 1995). Others have reported that most patients present with no residual α-mannosidase enzyme activity, even when displaying a mild phenotype (Berg et al., 1999). As a result, it has been suggested that other genetic or environmental factors might have a significant impact on the disease severity (Michalski and Klein, 1999).

Various mutations, including splicing, missense and nonsense have been characterised in α-mannosidosis patients, although at present there appears to be no direct correlation between types of mutation and severity of disease presentation (Nilssen et al., 1997; Riise et al., 1997; Gotoda et al., 1998, reviewed in Michalski and Klein, 1999). The most common mutation observed in human patients involves an amino acid substitution (R750W) and accounts for approximately 21% of disease alleles in European patients (Michalski and Klein, 1999).

Lysosomal storage can be observed in somatic cells such as hepatocytes and Kupffer cells by use of electron microscopy. Storage is also observed in numerous regions of the CNS, along with significant ballooning of the neurons. Lysosomal
storage has been found to develop progressively in most tissues, with the rate of progression depending on the severity of disease phenotype (Thomas and Beaudet, 1995; Michalski and Klein, 1999).

Affected patients present with hepatosplenomegaly, coarse facial features, dysostosis multiplex, and rapid, progressive mental retardation (Thomas and Beaudet, 1995). Patients also present with delayed development including psychomotor retardation and progressive neurological degeneration. Skeletal malformities involving an ovoid configuration, flattening and beak-like appearance of the vertebral bodies are also observed (Thomas and Beaudet, 1995).

Most α-mannosidosis patients suffer from recurrent pulmonary infections within their first two years of life (Clarke, 1997). Patients can also present with corneal clouding and hearing loss. Deafness is thought to occur as a result of both repeated early infections and neurological degeneration (Dorland et al., 1988). Additionally, patients can present with decreased serum IgG levels and a decreased PR interval on EKG has been previously reported (Mehta and Desnick, 1978). Patients with type I α-mannosidosis usually die between three and 12 years of age (Thomas and Beaudet, 1995), while type II patients often survive into adulthood.

1.3.2 Diagnosis of α-mannosidosis

α-Mannosidosis patients secrete increased amounts of oligosaccharides in their urine, a characteristic which can be used for diagnosis in patients presenting with clinical symptoms consistent with a LSD. This is achieved by separating these oligosaccharides using thin-layer chromatography (Sewell, 1979). If the resulting oligosaccharide pattern indicates α-mannosidosis, the diagnosis can be confirmed by measurement of acid α-mannosidase activity in fibroblasts or white blood cells from the patient (Kolodny and Mumford, 1976). Prenatal diagnosis is also possible in families at risk (Poenaru et al., 1979). Detection of carriers of α-mannosidase mutations is possible by measurement of cellular enzyme levels, although it is often necessary to correlate α-mannosidase levels with other lysosomal enzyme levels (such as β-hexosaminidase) and to age-matched controls, due to the overlap in α-mannosidase levels in normal and heterozygous individuals (Dr. Thomas Berg, personal communication).
Neonatal screening programmes (such as that at the Women's and Children's Hospital) are currently carried out on all newborns in South Australia, to screen for a number of genetic diseases. Research is currently underway to develop screening in the newborn to detect selected LSD. In conjunction with the development of effective therapies for LSD (including α-mannosidosis), newborn screening would allow therapy to begin early in life, thereby affording the best prognosis for an affected child.

1.3.3 Animal models of α-mannosidosis

A number of α-mannosidosis animal models exist, including cattle, cats, mice and guinea pigs. The first of these discovered was the α-mannosidosis cow, (in Angus cattle) which was first described in Australia (Whitten and Walker, 1957). Characterised by head tremor, aggressive tendency, ataxia, lack of coordination, failure to thrive and premature death, it was originally described as pseudolipidosis, due to the similarity observed to the lipidoses in children (Whitten and Walker, 1957). Following further characterisation, this disease was found to result from a deficiency of lysosomal α-mannosidase, and was thus renamed α-mannosidosis (Hocking et al., 1972). Affected Angus cattle usually died before 18 months of age as a result of their disease (Jolly, 1978; Healy et al., 1990). A more severe phenotype of α-mannosidosis has additionally been reported in Galloway cattle, whose affected calves either aborted, were stillborn or died shortly after birth (Borland et al., 1984; Healy et al., 1990).

A number of other naturally occurring animal models of α-mannosidosis have since been described. α-Mannosidosis is particularly well characterised in cats, where it has been reported in a seven month old Domestic Shorthaired cat (Burditt et al., 1980), in Domestic Longhaired cats aged seven to 15 months (Cummings et al., 1988), and in two independent families of Persian kittens (Vandevelde et al., 1982; Jezyk et al., 1986; Maenhout et al., 1988). Following histological analysis, extensive vacuolation was observed in the neurons and glial cells of α-mannosidosis cats, throughout the CNS (Cummings et al., 1988). Poor myelination of the cerebral white matter has been observed (particularly in Persian kittens; Jezyk et al., 1986), and notable axonal spheroid formation was observed both in Persian kittens and in Domestic Longhaired cats (Cummings et al., 1988). Extensive Purkinje cell loss was also noted in Domestic Longhaired cats (Cummings et al., 1988). The presence of meganeurites, secondary
neurites, and dendritic changes have also been observed in feline α-mannosidosis (Walkley et al., 1981). Although there is considerable heterogeneity with regard to the onset, progression, and nature of pathology, all α-mannosidosis cats display neurological pathology in the cerebellum, resulting in cerebellar dysfunction, which manifests as ataxia-dysmetria and intention tremors. Additionally, some cats show other abnormal behavioural features, such as running in circles, jumping without provocation and standing in the water bowl, along with progressive dementia and apathy (Jezyk et al., 1986). Other findings include corneal changes, hepatomegaly, and radiographic abnormalities of the spine and long bones (Jezyk et al., 1986; Cummings et al., 1988).

A knockout mouse model of α-mannosidosis has also been generated by targeted disruption of the lysosomal α-mannosidase gene (Stinchi et al., 1999). These mice displayed lysosomal storage in liver, pancreas, kidney, thyroid gland, smooth muscle cells, osteocytes, and various cells of the central and peripheral nervous system. Axonal spheroids were also observed in the brains of α-mannosidosis mice (Stinchi et al., 1999). This model exhibits a relatively mild phenotype, however, as despite these histological observations, no clinical symptoms were evident in mice studied up to the age of 12 months (Stinchi et al., 1999).

A naturally occurring case of α-mannosidosis in domestic guinea pigs was first described in a preliminary report by Muntz et al. (1999). Following this initial description, this model was extensively characterised (see Figure 1.5; Crawley et al., 1999, Berg and Hopwood, 2002). α-Mannosidosis in the guinea pig occurs as a result of a missense mutation leading to a substitution of arginine by tryptophan at amino acid position 227 (R227W), resulting in a loss of α-mannosidase activity (Berg and Hopwood, 2002). The guinea pig represents an excellent model for the study of CNS pathology due to the similarity of disease pathology and progression to that observed in humans (Crawley et al., 1999), along with the convenience of working with a naturally occurring relatively small animal model.

α-Mannosidosis guinea pigs display stunted growth compared to normal animals, and notable behavioural abnormalities. These include excessive time spent eating, subdued activity, abnormal posture, and abnormal hind limb positioning and gait (Crawley et al., 1999). Only subtle differences in skeletal appearance are observed
in α-mannosidosis guinea pigs, which include irregular long bone cortical thickening, and shorter long bones and vertebrae compared to age- and sex-matched controls (Crawley et al., 1999). Upon histological examination of the guinea pig brain and spinal cord, it was found that neuronal vacuolation was observed throughout the CNS (including the cerebral cortex, hippocampus, thalamus, cerebellum, midbrain, pons, medulla, and the dorsal and ventral horns of the spinal cord), similar to that observed in humans (Crawley et al., 1999). Additionally, axonal spheroids were observed in the α-mannosidosis guinea pig, containing dense bodies, empty vesicles, mitochondria, membranous whorls and flocculent material (Crawley et al., 1999). Loss of Purkinje neurons of the cerebellum has also been reported (Crawley et al., 1999; Dr. Steven Walkley, personal communication).

The pattern of storage compounds observed following thin layer chromatography of tissue and urinary oligosaccharides was almost identical in humans and guinea pigs, and contrasted with that observed in cattle and cats (Crawley et al., 1999). Specifically, stored oligosaccharides in cattle and cats display terminal di-N-acetylcchitobiose, compared to the single terminal N-acetylglucosamine seen in humans and guinea pigs. This is due to a lack of chitobiase activity in cattle and cats, which is common to both guinea pigs and humans (Aronson and Kuranda, 1989).
Figure 1.5: α-Mannosidosis guinea pig and normal littermate.
The α-mannosidosis guinea pig (right) is homozygous for the nucleotide substitution mutation of thymidine to cytosine at position 679 (Thomas Berg, personal communication). This results in an amino acid change from arginine to tryptophan at position 227 (R227W). The normal littermate (left) is heterozygous for this mutation, and thus does not display clinical symptoms. Note the smaller size and less elongated facial features of the α-mannosidosis guinea pig. Photograph provided by Dr. Allison Crawley.
Figure 1.5

Normal  \(\alpha\)-Mannosidosis
1.3.4 Stored material in α-mannosidosis

Lysoosomal storage in the tissues of α-mannosidosis individuals consists of a number of different compounds. A total of 17 different types of oligosaccharides were found in the urine of affected humans (Yamashita et al., 1980; Matsuura et al., 1981) and cats (Abraham et al., 1986; Hard et al., 1991). These storage compounds all contain between two and nine mannose residues, with the majority of compounds being unbranched and containing between two and five mannose residues, and result from the incomplete catabolism of high-mannose and hybrid-type glycans (Michalski and Klein, 1999). The major compound stored in α-mannosidosis is the trisaccharide Man(α1-3)Man(β1-4)GlcNAc. Both Man(α1-6)Man(β1-4)GlcNAc as well as the trimannose structure Man(α1-3)[Man(α1-6)]Man(β1-4)GlcNAc are not seen as stored compounds, which could be due to the presence of residual α1-6 mannosidase activity (De Gaspieri et al.; 1992, Daniel et al., 1992). Additionally, a number of the stored oligosaccharides consist of linear structures with α1-2 linked mannose on one of the branches. These oligosaccharides may arise as a result of previous cytosolic catabolism by the soluble α-mannosidase activity, since complementary action of cytosolic and lysosomal α-mannosidase activities has been demonstrated (Shoup and Touster, 1976; Haeuw et al., 1991; al Daher et al., 1992; reviewed in Michalski and Klein, 1999).

Despite having no theoretical relevance as a substrate for lysosomal α-mannosidase, gangliosides (such as GM2 and GM3 ganglioside) have been shown to accumulate in a proportion of neurons in various animal models of α-mannosidosis, such as the cat (Siegel and Walkley, 1994) and guinea pig (Dr. Steven Walkley, personal communication). At present, the mechanism by which primary lysosomal storage of mannose-containing oligosaccharides leads to secondary ganglioside storage is unknown. It has been suggested however, that primary storage results in a generalised lysosomal derangement that impacts the function of the enzymes required for ganglioside degradation (Walkley, 1998).

1.3.5 Glycoprotein degradation

As mentioned previously, α-mannosidosis is a disorder of glycoprotein catabolism. Glycoproteins are highly prevalent molecules commonly found both
intracellularly and on the surface of normal cells (Berger et al., 1982). Thus, normal cellular metabolism involves turnover of large amounts of glycoprotein. These molecules consist of oligosaccharide chains covalently linked to a peptide backbone. These peptides can be linked with oligosaccharides through the hydroxyl groups of serine or threonine, or through the free amino acid group of asparagine (Thomas and Beaudet, 1995). Lysosomal degradation of glycoproteins involves degradation of the protein backbone from both the ends and internal points of the molecules, by a number of proteases and peptidases, as shown in Figure 1.3 (Figure adapted from Aronson, 1997 by Thomas Berg). In contrast, the oligosaccharide portions of the molecules are degraded in a stepwise manner from both the non-reducing and (to a lesser extent) the reducing ends (Thomas and Beaudet, 1995). Cleavage from the non-reducing end involves the sequential removal of single sugars from the oligosaccharide branches by a number of lysosomal exoglycosidases (Thomas and Beaudet, 1995). These enzymes include neuraminidase (sialidase), β-galactosidase, β-N-acetylhexosaminidase, α-fucosidase, β-mannosidase and α-mannosidase (Thomas and Beaudet, 1995). Degradation at the reducing end of the molecule involves the ordered removal of asparagine, fucose and N-acetylglucosamine from the protein-oligosaccharide linkage region (Thomas and Beaudet, 1995). Phosphatases and sulphatases are also required for the catabolism of glycoproteins, due to the presence of phosphorylated and sulphated residues within these molecules (Kornfeld, 1992, Smith et al., 1993).

1.3.6 α-Mannosidase

α-Mannosidases are a class of enzymes involved in one part of the stepwise degradation of glycoprotein-linked oligosaccharides. Multiple forms of α-mannosidase exist in a normal cell, broadly classifiable into three groups: acid lysosomal, intermediate/Golgi and neutral/cytosolic (reviewed in Daniel et al., 1994). For the purposes of this thesis, α-mannosidase discussed henceforth will refer to the lysosomal form of this enzyme (unless specifically stated otherwise).

Lysosomal α-mannosidase is an exoglycosidase that acts to cleave α-linked mannose residues specifically from the non-reducing end during the sequential degradation of N-linked glycoproteins (Aronson and Kuranda, 1989). Lysosomal α-mannosidase has a relatively broad natural substrate specificity, hydrolysing α(1-2),
α(1-3) and α(1-6) mannosyl linkages in high mannose and hybrid type glycans (reviewed in Michalski and Klein, 1999). The mannose-containing compounds that α-mannosidase degrades include N-linked glycans, dolichol intermediates, and glycosylphosphatidylinositol (GPI) membrane anchors (Daniel et al., 1994). This enzyme is distinguished from other cellular α-mannosidases by a combination of a low pH optimum (pH 4.5), Zn$^{2+}$ dependence, a broad natural substrate specificity, and inhibition by the indolizidine alkaloid swainsonine (Winchester, 1984; Michalski et al., 1990; Moremen et al., 1994; Daniel et al., 1994; reviewed by Michalski and Klein, 1999).
Figure 1.3: Structural example of a complex N-linked glycoprotein.
The lysosomal enzymes required for the degradation of this glycoprotein are indicated. Reactions 1-6 and I-IV act independently in this process: 1-6 remove oligosaccharides stepwise from the non-reducing end, while I-IV hydrolyse the protein and protein-to-carbohydrate linkage region. The glycoproteinoses resulting from a deficiency of the specific enzymes are shown in parentheses. Diagram provided by Dr. Thomas Berg, modified from Aronson (1997).
Figure 1.3

1. α-Neuraminidase (Sialidosis)
2. β-Galactosidase
3. β-Hexosaminidase
4. α-Mannosidase (α-Mannosidosis)
5. β-Mannosidase (β-Mannosidosis)
4. α-Mannosidase (α-Mannosidosis)
6. β-Hexosaminidase

IV. Chitobiase or Chitobiose

III. Glycosylasparaginase (Aspartylglucosaminuria)

II. Fucosidase (Fucosidosis)

I. Cathepsins

N-Acetylneuraminic acid (Sialic acid)
Galactose
N-acetylglucosamine
mannose
fucose
The gene encoding α-mannosidase has been localised close to the centre of human chromosome 19 (19p13.2-q12) (Champion and Shows, 1977). Upon assembly of the human cDNA for this gene it was found that the amino acid sequence codes for a signal peptide of 48 amino acids, followed by a polypeptide sequence of 962 amino acids (Nilssen et al., 1997). The α-mannosidase enzyme is initially synthesised as a single chain precursor, before processing into three glycopeptides, 15, 42, and 70kDa in size. The 70kDa peptide is then partially proteolysed to form three more peptides joined by disulphide bridges (Nilssen et al., 1997). Bovine α-mannosidase is found in the cell as a 250kDa dimer (Tollersrud et al., 1997), as is human α-mannosidase (Berg et al., 2001). The recombinant human α-mannosidase dimer (produced in Chinese hamster ovary cells) was found to be made up of two monomers, each consisting of two associated fragments of α-mannosidase, approximately 55kDa and 72kDa in size (Berg et al., 2001). Figure 1.4 shows the structure of the α-mannosidase precursor protein (Nilssen et al., 1997).
Figure 1.4: Schematic representation of the lysosomal α-mannosidase precursor.
Peptides are shown as boxes designated a-e according to their order in the precursor polypeptide. The black filled box represents the signal peptide. The amino acid position at each cleavage site is listed below the diagram. Potential N-glycosylation sites are shown as “lollipops” above the diagram: open circles represent high mannose type oligosaccharides, filled circles represent complex type oligosaccharides. The nature of the oligosaccharides linked to the “e” peptide are presently unknown. Diagram from Nilssen et al., 1997.
Figure 1.4
1.3.7 Pathogenesis of neurological disease in α-mannosidosis

Despite the deficiency of only a single enzyme in α-mannosidosis, a broad range of pathological features is observed in multiple tissues. Central nervous system pathology in particular represents the most devastating aspect of this disease. At present, the mechanisms by which lysosomal storage leads to the genesis of such complex neurological pathology in α-mannosidosis are poorly understood. In addition to mechanical distension due to storage affecting lysosomal function, the biochemical effects of secondarily stored compounds such as gangliosides may play a critical role in the development of neurological pathology (Walkley, 1998). It has been suggested that ganglioside storage in particular may be responsible for many of the toxic effects on the CNS seen in certain LSD (Walkley, 2003).

Ganglioside storage may have toxic effects on cells in a number of ways. Functional relationships have been demonstrated between specific types of gangliosides and growth factor receptors, such as epidermal growth factor (EGF) receptor associating with GM3 ganglioside (Bremer, 1994). Following binding, this complex is accumulated in clathrin-coated pits, then early and late endosomes, and ultimately the lysosome, for degradation. Gangliosides and other glycosphingolipids have also shown modulatory effects on receptors for NGF, FGF, PDGF, p60c-src and insulin (Yates, 1986; Chakraborthy et al., 1993; Bremer, 1994; Ferrari et al., 1995; Mutoh et al., 1995; Rabin and Mochetti, 1995; reviewed in Walkley, 1998), and interactions with growth factor molecules (Rusnati et al., 1999). Defective lysosome function may thus disrupt the normal regulation of these signalling processes, either through interaction with relevant receptors or signalling molecules, with subsequent effects on the cell.

A number of morphological changes have been observed in α-mannosidosis neurons at a microscopic level in addition to the distension reported as a result of lysosomal storage. The formation of ectopic dendrites has been reported in the α-mannosidosis cat, particularly in pyramidal neurons of the cortex (Walkley et al., 1981), although ectopic dendrites were not found in the α-mannosidosis guinea pig (Walkley, 2003). Further studies implicated GM2 ganglioside in the development of this feature of pathology in the α-mannosidosis cat, as GM2 ganglioside was not found to be stored in all neurons, but all neurons observed with ectopic dendrites were found to store this compound (Goodman et al., 1991). This correlation was additionally
confirmed following studies in other LSD, implicating GM2 ganglioside in a common pathological mechanism resulting in ectopic dendritogenesis in different LSD (Siegel and Walkley, 1994; Walkley et al., 1995). Subsequently, GM2 ganglioside was found to play a significant role in the initiation of dendritic sprouting in normal development (Goodman and Walkley, 1996). It was thus proposed that GM2 ganglioside may be a component of a regulatory mechanism controlling dendritic sprouting (Walkley et al., 2000). The sprouting of new primary neurites from mature neurons is a highly unusual event not reported under normal circumstances. Ectopic dendrites and their associated aberrant synapses could thus be responsible for some of the neurological dysfunction and cognitive deficits observed in LSD, by disrupting normal signalling in various mature neuronal circuits (Purpura and Suzuki, 1976; Walkley and Wurzelmann, 1995; Walkley, 1998; Walkley, 2003).

Another morphological change in neurons observed in α-mannosidosis (and other LSD) is the formation of axonal spheroids—swellings of significant size along the length of the axon. These structures have been reported in the cat (Walkley et al., 1981, Cummings et al., 1998), mouse (Stinch et al., 1999) and guinea pig (Crawley et al., 1999) models of this disease, and have been found to consist of dense bodies, mitochondria, empty vesicles, membranous whorls, and flocculent material (Walkley, 1998; Crawley et al., 1999). Previous studies have reported similar structures occurring distal to axonal lesions, indicating these structures to be characteristic of a block in retrograde transport (Smith, 1980; Tsukita and Ishikawa, 1980; Parton et al., 1992). It has thus been suggested that axonal spheroids in LSD may also arise from a block in retrograde transport (Walkley, 1998). The reasons for this occurring in LSD such as α-mannosidosis are unclear. Lysosomal dysfunction arising from primary storage could deprive the axon of a critical component for normal organelle transport (Walkley, 1998), or perhaps inhibit the endosome-lysosome fusion that may ordinarily occur in the axon, resulting in a “backing-up” of endosomal traffic, and consequently, enlarged endosomes (Walkley, 1998).

More recently (from studies with various LSD animal models) it has been found that the vast majority of these spheroids are found in GABAergic neurons (Walkley et al., 1991; March et al., 1997). The presence of axonal spheroids is thought to detrimentally influence neurological function in LSD by inhibiting action potentials (in terms of either efficacy or timing) in these inhibitory circuits (Walkley et al., 1991;
Walkley, 1998). Indeed, correlations found between the location and incidence of axonal spheroids, and the nature and severity of neurological disease in different LSD (Walkley et al., 1991; March et al., 1997) suggest that axonal spheroids may be a primary contributor to clinical neurological disease in LSD (Walkley, 1998).

Although the exact causes of neurological disease in LSD such as α-mannosidosis are not yet known, a greater understanding of the morphological changes within neurons of affected brain tissue will no doubt lead to rapid advances in the understanding of these diseases. The fact that such distinct changes exist has significant implications for therapy, due to the fact that the degree to which these features (primarily ectopic dendrites and axonal spheroids) can be reversed following therapy is presently unknown. Studies involving an induced (caused by the administration of the α-mannosidase inhibitor compound swainsonine) feline model of α-mannosidosis have supported this concern. In these studies, six months after the withdrawal of swainsonine treatment (and thus restoration of enzyme activity), lysosomal storage in neurons was found to be cleared (Walkley et al., 1987). However, ectopic dendrites (along with their synaptic connections) and axonal spheroids still persisted (Walkley et al., 1987). More recently, axonal spheroids have been reported to persist even years after swainsonine withdrawal in this model, accompanied by an apparent continuation in the death of Purkinje neurons, possibly as a result of the continued presence of axonal spheroids (Walkley, 1998). Development of effective therapies must thus also be accompanied by the development of effective strategies for early diagnosis, in order to allow early initiation of therapy (ideally before presentation of symptoms), and thus the best prognosis for the affected child.
1.4.0 Strategies for treatment of pathology in α-mannosidosis

As discussed above, α-mannosidosis patients present with both somatic and CNS pathology. Strategies such as enzyme replacement therapy (ERT) and bone marrow transplantation (BMT) have shown promise for treatment of somatic pathology in α-mannosidosis (although with certain caveats, discussed in more detail below). However, the CNS pathology represents the most devastating aspect of this disease in addition to being the most difficult aspect to effectively treat.

The ideal goals of a treatment strategy for CNS pathology in α-mannosidosis would be twofold. Firstly, functional α-mannosidase must be effectively delivered throughout the CNS, and secondly, this treatment must be sustainable (with minimal adverse effects for the patient), possibly involving either long-term in vivo protein production, or regular systemic administration of a therapeutic protein that could reach the CNS. New therapies for brain disease cannot be investigated directly in humans, but naturally occurring animal models of α-mannosidosis show great potential for the development of effective therapies for eventual clinical application.

1.4.1 Current approaches to patient management

Presently, therapeutic approaches to management of pathology in α-mannosidosis patients are based on treatment of specific sites of pathology, as is the case with many other LSD. Loss of eyesight due to corneal opacities could possibly be resolved by corneal transplantation, as has been achieved with other LSD (Bergwerk et al., 2000), while orthopaedic problems affecting mobility can be alleviated surgically. Sinopulmonary problems, when life-threatening, have also been treated surgically. To date, the only therapy investigated in humans for treatment of global disease pathology in α-mannosidosis is BMT (Will et al., 1987, Wall et al., 1998).

1.4.2 Bone marrow transplantation

Bone marrow transplantation was the first strategy investigated (and to date, the only therapy that has been trialed in humans) for treatment of global pathology in α-mannosidosis patients (Will et al., 1987). This procedure involves transplantation of bone marrow from a closely-matched, unaffected donor into an α-mannosidosis patient. Enzyme is transferred from donor cells to deficient cells by direct cell-to-cell contact.
(Margollicci et al., 1990), or through release into plasma (Hoogerbrugge et al., 1995). Exogenous enzyme is taken up by the MPRs on the cell surface and targeted to the lysosome (Pohllmann et al., 1995). The fact that the CNS contains cells such as microglia derived from bone marrow (Hickey and Kimura, 1988; Eglitis and Mezey, 1997) suggests BMT is also useful for treatment of CNS pathology in α-mannosidosis.

The first patient to be treated using this therapy received BMT at seven years of age (well into the clinical progression of this disease), and died 18 weeks following treatment (Will et al., 1987). More encouraging results were obtained, however, following studies of BMT in the feline model of α-mannosidosis. Animals treated at two to three months of age were shown to have little or no progression of neurological symptoms after one to two years, in comparison to untreated cats, which reach end stage disease by six months of age (Walkley et al., 1994). Additionally, functional α-mannosidase enzyme was found in neurons and glial cells of treated cats (Walkley et al., 1994). However, two of the three cats in this study also developed evidence of cerebellar disease, despite BMT. It was suggested that this brain region may be particularly sensitive to α-mannosidase deficiency, resulting in the initiation of pathological changes in this model even before transplantation at two to three months of age (Walkley et al., 1994).

Studies investigating BMT in other large animal models of LSD with CNS pathology also showed positive results. Transplantation of fucosidosis (another glycoprotein storage disorder) dogs at two to four months of age resulted in almost complete prevention of the development of clinical signs, compared to age-matched controls, while treatment six months later resulted in a definite slowing (but not prevention) of progression of neurological pathology (Taylor et al., 1992). However, transplantation in a 30 month old fucosidosis dog had previously shown almost no improvement in the CNS (Taylor et al., 1989). Collectively, these results indicate that BMT should be performed as early as possible for the best prognosis, ideally before presentation of clinical symptoms.

Bone marrow transplantation (using HLA-identical bone marrow from a non-affected sibling) has more recently been performed in a 22 month old α-mannosidosis patient (Wall et al., 1998), with promising results. Transplantation in this patient resulted in normalisation of white blood cell enzyme levels, as well as complete resolution of organomegaly and chronic sinopulmonary disease. Neurocognitive
functioning was thought to be stabilised, but it was too early to determine if 
neurological deterioration had ultimately been prevented (Wall et al., 1998). Other α-
mannosidosis patients have since been transplanted, resulting in successful engraftment 
(Krivit et al., 1999).

Although BMT shows promise for treatment of α-mannosidosis in humans, it 
also carries considerable risk as a procedure, due to the fact that the patient’s bone 
marrow is destroyed with cytotoxic agents prior to transplantation, to allow engraftment 
of donor bone marrow (Hoogerbrugge, 1997). Additionally, following transplantation 
there is the possibility of graft versus host disease (GVHD). Hoogerbrugge et al. (1995) 
reported that in a study of 40 patients receiving BMT for treatment of LSD, mortality 
was 10% among recipients of marrow from an HLA-identical sibling, and 20-25% 
among recipients of mismatched tissue. However, along with immunosuppressive 
drugs such as cyclosporin, alternative strategies are being investigated to prevent 
GVHD, such as the use of monoclonal antibodies to target T cells and cytokines 
(Hiscott and McLellan, 2000). Additionally, not all α-mannosidosis patients would be 
suitable candidates for BMT, as this therapy requires a compatible donor, ideally an 
HLA-matched sibling, only available for approximately 25-30% of patients 
(Hoogerbrugge, 1997). Thus, although BMT has shown promise for treatment of CNS 
pathology in α-mannosidosis, the limitations are such that BMT is not always a feasible 
treatment strategy.

1.4.3 Enzyme replacement therapy

With the development of recombinant techniques for protein production, the 
possibility of ERT for single gene LSD arose. Since it has been shown that α-
mannosidase can enter cells via MPR-mediated endocytosis in a similar fashion to 
other lysosomal enzymes (Sun et al., 1999), enzyme delivered to affected tissues could 
in theory be taken up by the cells to therapeutic effect. Recombinant human lysosomal 
α-mannosidase (rhαM, produced using Chinese hamster ovary (CHO) cells) has since 
been shown to be endocytosed by α-mannosidosis skin fibroblasts in a similar fashion, 
resulting in reductions in the levels of stored oligosaccharides (Berg et al., 2001). This 
study provided important confirmation, since not all recombinant lysosomal enzymes 
have been shown to be efficiently phosphorylated during production (Weber et al., 
51
The mannose-6-phosphorylation of lysosomal enzyme is particularly important for all therapeutic approaches to LSD involving recombinant enzyme production, due to the importance of this mechanism for delivering deficient enzyme to affected tissues.

Studies involving ERT in a guinea pig model of α-mannosidosis (by systemic administration of rhαM) showed widespread distribution of recombinant enzyme in somatic tissues and a resultant reduction of levels of stored oligosaccharide (King et al., in preparation). However, no recombinant enzyme was found in the CNS of treated animals, nor were the levels of stored oligosaccharides reduced (King et al., in preparation). This is to be expected due to the presence of the blood brain barrier (BBB).

1.5.0 The blood brain barrier

Due to the fact that α-mannosidosis (as with many other LSD) results from a functional deficiency of a single enzyme, therapy could in theory be effected by delivery of the deficient enzyme to sites of disease pathology (Brady et al., 1982). One of the major obstacles impeding systemic drug administration as a method for effective therapy in α-mannosidosis (and other LSD with CNS pathology) is the BBB, as this barrier acts to significantly limit delivery of enzyme to the brain.

The BBB is a functional barrier present in the cerebral microvasculature that acts to separate the bloodstream from the brain parenchyma, preventing the entry of significant amounts of most larger molecules from the bloodstream, whilst selectively transporting other molecules (for example glucose and certain amino acids) into the brain parenchyma (Robert and Robert, 1998). Brain capillaries consist of a single endothelial layer surrounded by a basement lamina, as with other capillaries. However, neighbouring endothelial cells are connected by tight junctions (Reese and Karnovsky, 1967). These tight junctions make up the anatomical element of the BBB, because they prevent macromolecules from entering into the capillaries from the blood, through the interendothelial spaces (Brightman and Reese, 1969). The capillaries are surrounded by the foot processes of astrocytes in the brain, through which molecules transported from the blood across the BBB enter the brain parenchyma (see Figure 1.6).

Most molecules are excluded from crossing the BBB based on both size and hydrophobicity. Only lipid-soluble molecules under a molecular size threshold of 400-600Da would be expected to cross the BBB without being selectively transported.
Thus, most systemically administered proteins or peptides would not cross the BBB in pharmacologically significant amounts (Pardridge, 1998).

1.5.1 Strategies for circumventing the blood-brain barrier: delivery of lysosomal enzymes to the LSD brain.

1.5.1.1 Direct injection

Delivery of therapeutic proteins to the brain parenchyma could also in theory be achieved by intra-cranial injection. Studies involving direct injection of sulphamidase enzyme into the brain of mucopolysaccharidosis type IIIA (MPSIIIA, another LSD with CNS pathology) mice have shown that injection of enzyme six weeks prior to sacrifice at 24 weeks of age results in significant clearance of lysosomal storage in the brain of treated animals (Savas et al., accepted for publication). Direct injection of enzyme 12 weeks or 18 weeks before sacrifice at 24 weeks of age also resulted in reduced lysosomal storage, with the degree of reduction related to the time since treatment (Savas et al., accepted for publication). Additionally, ERT performed in MPSIIIA mice from birth (thus only providing enzyme to the brain for the first two-to-three weeks, before the BBB has fully developed in mice) showed a significant reduction in lysosomal storage, along with improvements in cognitive function in treated mice at 20 weeks of age (Gliddon and Hopwood, 2004). These studies provide proof of principle and show that even a single dose of recombinant enzyme delivered to the brain can potentially have a therapeutic effect. However, due to the highly invasive nature of direct injection procedures, repeated therapy of this type would not be feasible for human patients in the long term. Additionally, this approach may require multiple injection sites to achieve sufficient protein distribution for therapeutic effect, further increasing the risk of this procedure. Other strategies have thus been pursued to increase the delivery of systemically administered therapeutic proteins across the BBB.

1.5.1.2 Blood-brain barrier modification

One of the methods investigated for delivery of therapeutic molecules to the brain is modification of the BBB. This involves increasing the permeability of the BBB in order to facilitate crossing by large molecules. This can be achieved by intra-aortic injection of osmotic agents such as arabinose or mannitol, or the use of other drugs
such as bradykinin, which acts as a peripheral vasodilator (reviewed in Kroll and Neuwelt, 1998).

Although disruption of the BBB has shown promise for the delivery of therapeutic enzymes to the brain, including α-mannosidase derived from human placenta (Barranger et al., 1979; Neuwelt et al., 1984), there are concerns about the safety of repeated BBB disruption (Culver et al., 1998). Disruption of the BBB allows not only the therapeutic compound access to the brain parenchyma, but also numerous other serum compounds, with potential side effects. Serum albumin in particular has been shown to be toxic to astrocytes in the brain (Nadal et al., 1995).

1.5.1.3 Pro-drugs

In contrast to the modification of the BBB, another strategy that has been investigated for delivery of molecules across the BBB is the use of pro-drugs. Pro-drugs are molecules designed to exploit the endogenous transport mechanisms of the BBB in order to cross from the bloodstream into the CNS. Systems exploited have included the transferrin receptor system (Friden, 1994), the carrier protein melanotransferrin (P97; Demeule et al., 2002), the insulin transport system (Fukuta at al., 1994), and the human immunodeficiency virus (HIV) TAT protein (Nagahara et al., 1998; Schwarze et al., 1999).

Studies involving the transferrin receptor system have usually involved the linking of an antibody to the human transferrin receptor to the molecule to be delivered. This has been successfully demonstrated with delivery of nerve growth factor (NGF) in pharmacologically active amounts to the brain (Friden, 1994), and more recently with delivery of a 6-7kb expression plasmid encoding a reporter gene encapsulated in liposomes with the anti-transferrin antibody on the surface (Shi and Pardridge, 2000).

The carrier protein melanotransferrin (P97) possesses a high level of sequence homology to human serum transferrin, chicken transferrin, and human lactoferrin (Brown et al., 1982, Rose et al., 1986). Following intravenous injection and in situ brain perfusion in mice, P97 was found to accumulate in brain (Demeule et al., 2002). Additionally, these studies suggested that P97 accumulation in brain may be a result of receptor-mediated endocytosis, suggesting the possible use of P97 as a delivery system to target drugs to the brain (Demeule et al., 2002).
Insulin beta fragments have also been used conjugated to other peptides (Fukuta et al., 1994), or more recently, chimeric antibodies to the human insulin receptor (Coloma et al., 2000). These studies have also demonstrated effective delivery of molecules across the BBB.

Recently, another strategy has been investigated, involving the creation of fusions between a therapeutic protein and the HIV TAT protein, in order to facilitate transduction across the BBB rather than receptor mediated transcytosis (Nagahara et al., 1998; Schwarze et al., 1999). Although the successful delivery even of large proteins such as the 120kD β-galactosidase was reported, the need to denature the fusion proteins before administration could exclude this strategy from use for some more sensitive or structurally complex therapeutic enzymes.

Although still in their infancy, pro-drug strategies may be particularly promising for delivery of smaller therapeutic (and less complex) molecules across the BBB. The feasibility of these strategies for treatment of various LSD would thus depend on the characteristics of each individual lysosomal enzyme required. The lack of exposure to the cells of the CNS using these strategies could also allow much lower toxicity to the brain than that observed with BBB modification strategies. However, the nature of these delivery strategies is such that repeated administration would be required for treatment of LSD.
Figure 1.6: The anatomical elements of the blood-brain barrier (BBB).
The BBB is the component of the brain microvasculature comprised of a single endothelial cell layer surrounded by a basement membrane. Interendothelial spaces contain tight junctions. Astrocyte foot processes abut the basement membrane. The insert shown is an enlargement of the cell membrane constituting the luminal surface of the BBB. Diagram from Audus et al., (1992).
Figure 1.6
1.6.0 Gene therapy

Gene therapy is the treatment of a disease by transfer of genetic material into the cells of a patient, which enables the cells to produce a therapeutic protein. For treatment of diseases with CNS pathology, this could be achieved by use of episomally expressed vectors such as adenoviral vectors (Günzberg and Salmons, 1995), or integrating vectors such as retroviral vectors (Kim et al., 1998) and adeno-associated virus (AAV) vectors (Watson et al., 1998). Gene therapy in the CNS would allow expression of a therapeutic protein in vivo, potentially avoiding the need for invasive repeated administration (Mulligan, 1993). Expression of the protein at the specific site of disease pathology could also potentially avoid side effects associated with systemic exposure to therapeutic doses of protein (Evans and Robbins, 1994).

Gene therapy has shown promise for treatment of CNS pathology in other LSD. In mucopolysaccharidosis type VII (MPS VII, another LSD showing CNS pathology) mice, adenovirus-mediated transfer of β-glucuronidase (the deficient enzyme in this disease) to the brain was shown to result in high levels of expression of the deficient enzyme, and resolution of storage in the brain (Ghodsi et al., 1998). Further studies involving intra-cranial injections of AAV to facilitate expression of β-glucuronidase in the brain have shown widespread enzyme distribution, reduction in lysosomal storage, and improvement in cognitive function in MPS VII mice (Frisella et al., 2001). Additionally, this vector was also found to be effective in preventing systemic clinical disease following intravenous injection in MPS VII mice (Daly et al., 2001).

Episomally expressed vectors such as the canine adenovirus vector (Kremer et al., 2000) have also shown promise for treatment of CNS pathology in LSD. These vectors have demonstrated preferential and high efficiency transduction of neurons in vivo, along with retrograde axonal transport (Soudais et al., 2001), and high level transgene expression in transduced neurons in vivo, sustained for at least one year after injection (Soudais et al., 2003). These properties suggest that these vectors may be useful for achieving widespread expression of therapeutic enzymes (such as α-mannosidase) in the brain.

Preliminary studies involving the transduction of affected human and feline fibroblasts with the α-mannosidase cDNA have shown that the transduced cells expressed functional α-mannosidase and could cross-correct other non-transduced affected cells (Sun et al., 1999). This study has demonstrated proof of principle for the
use of viral vectors to facilitate α-mannosidase expression, resulting in the cross-correction of affected cells *in vitro*. Further studies using animal models would thus be required to evaluate the long-term efficacy and safety of gene therapy approaches for α-mannosidosis.

1.6.1 Limitations of gene therapy

Gene therapy has a number of limitations, depending on the vector used. Firstly, gene expression may decline over time, as in the case of transient episomally expressed vectors (Günzberg and Salmons, 1995), and to a lesser extent with integrating retroviral vectors incorporating strong viral promoters due to the phenomenon of promoter extinction (Palmer et al., 1991). Secondly, random integration of genetic material into the cell genome carries the risk of insertional mutagenesis (Donahue et al., 1992) with possible carcinogenic effects if a tumour suppressor gene were to be disrupted, or an oncogene activated (Crystal, 1995). This has been shown to be more than just a theoretical possibility in a clinical trial for treatment of X-linked severe combined immune deficiency using a retroviral gene therapy approach. Despite immune reconstitution and significant clinical benefit, two patients in this trial developed leukaemia almost three years following treatment, as a result of insertional mutagenesis (Hacein-Bey-Abina et al., 2003; Kohn et al., 2003).

Other studies have revealed other potential safety issues relating to the use of vectors such as AAV. In evaluating the efficacy of AAV vectors for treatment of MPS VII mice in long-term studies, it was found that treated mice had a significantly increased incidence of liver tumours (Donsante et al., 2001). Furthermore, it was suggested that these tumours may not have occurred simply as a result of insertional mutagenesis (Donsante et al., 2001). Further investigations into the safety of these vectors thus need to be carried in order to evaluate their ultimate therapeutic potential (Monahan et al., 2002).

The generation of replication competent virus is also a possibility (Donahue et al., 1992), although this risk has been greatly minimised by deleting genes for viral packaging from the vectors and expressing them in *trans* from a packaging cell line (Miller, 1990).

A number of these limitations can be overcome by the use of an *ex vivo* gene therapy strategy involving transduction of the cells outside the patient before re-
implantation. This allows the transduced cells to be analysed for gene expression and to ensure that no replication competent vector is present. The majority of *ex vivo* gene therapy strategies have previously been investigated for use with terminally differentiated cells such as fibroblasts (Taylor and Wolfe, 1997) or macrophages (Eto and Ohashi, 2000). However, recent advances in stem cell biology have introduced the possibility of using pluripotent stem cells as a substrate for therapeutic gene expression in LSD (stem cell therapy).

### 1.7.0 Stem cells

“Stem” cells are a group of cell types that are functionally defined as being self-renewing (under certain conditions), that is, able to undergo multiple rounds of cell division to give rise to undifferentiated daughter progeny. Additionally, under different conditions stem cells can differentiate to form multiple mature cell phenotypes. Stem cells are defined as either pluripotent (able to form all the mature cell phenotypes in the body, in the case of embryonic stem cells or embryonic germ cells; Rathjen et al., 1998; Shamblott et al., 1998; NIH stem cell report, 2001) or multipotent (able to differentiate into multiple mature phenotypes, in the case of most adult stem cells; Flax et al., 1998; NIH stem cell report, 2001; Mayani, 2003). Stem cells are also defined by their source, either from the adult (in the case of many different types of adult stem cells; Mayani, 2003), fetus (in the case of embryonic germ cells and various tissue stem cells; Flax et al., 1998; Shamblott et al., 1998), or embryo (in the case of embryonic stem cells; Rathjen et al., 1998; Thomson et al., 1998).

### 1.7.1 Adult stem cells

Adult stem cells (also sometimes referred to as tissue stem cells) collectively are a group of rare cell types present in various tissues and organs of an animal, maintained throughout the entire life of the animal (Watt and Hogan, 2000). Adult stem cells are present in many tissues, such as bone marrow stromal stem cells and haematopoietic stem cells in the bone marrow and blood (Owen et al., 1988; Morrison et al., 1997), pancreatic stem cells in the pancreas (Zulewski et al., 2001), and neural stem cells (NSCs) in the dentate gyrus of the hippocampus and subventricular zone underlying the lateral ventricles (Gage, 2000). Adult (tissue) stem cells can also be derived from various fetal tissues, where they are thought to exist in higher concentrations than in
adulthood and play an integral role in development of the tissues and organs in question (Temple, 2001; NIH stem cell report, 2001).

Adult stem cells (or tissue stem cells) differ from pluripotent stem cells such as embryonic germ (EG) cells or embryonic stem (ES) cells in that they are thought to be somewhat more restricted in their differentiation potential. That is, they have the capacity to differentiate only to a restricted set of cell phenotypes depending on their tissue of origin, rather than all the cell phenotypes present in an adult (that is, they are multipotent, not pluripotent). Additionally, when cultured in vitro, although adult stem cells have been shown to have the potential for long term expansion (Zhou et al., 2000) they are not thought to have as extensive a proliferative capacity as ES cells (Amit et al., 2000; Carpenter et al., 2003).

Although adult stem cells initially appeared to be committed to a given set of cell phenotypes relevant to their tissue of origin, a number of studies have shown that adult stem cells may be more plastic than originally thought. That is, under the right conditions they may be able to form other cell phenotypes unrelated to the mature cell types in the tissue or organ from which the stem cells were originally derived (Bjornson et al., 1999; Kopen et al., 1999; Brazelton et al., 2000).

1.7.1.1 “Plasticity” and “transdifferentiation” of adult stem cells

Although adult stem cells are considered more restricted in their differentiation potential than pluripotent cells such as ES cells, studies into adult stem cell biology have apparently demonstrated that adult stem cells may be less committed to a particular phenotype (and indeed, lineage) than was previously thought. Neural stem cells have been reported to differentiate (“transdifferentiate”, to a different lineage) into a number of different blood cell types when injected into irradiated mice (Bjornson et al., 1999). Similarly, stromal cells derived from mouse bone marrow were reported to differentiate into astrocytes when injected into neonatal mouse brains (Kopen et al., 1999). Cells derived from adult mouse skeletal muscle have also been reported to have the potential to differentiate into a number of different blood cell types (Jackson et al., 1999). These studies raised many exciting prospects for therapy because they suggest that ultimately, a relatively easily accessible cell population such as autologous bone marrow could possibly be used for treatment of diseases such as brain disease, rather than necessarily using NSCs isolated from primary cultures of CNS tissue. More
recently, many of these observations have been attributed to fusion events occurring between host and graft cells, resulting in cells displaying the properties of each (Ying et al., 2002, Terada et al., 2002). Other studies have cautioned that the very low frequency of “transdifferentiation” events observed in these implantation studies place them in a range that could possibly be attributed to “false positives” or experimental error (Daley et al., 2003).

In 2002, Jiang and others reported the isolation of multipotent adult progenitor cells (MAPCs), a pluripotent adult stem cell found after culture of bone marrow stromal elements for extended periods under specific conditions. These cells have been isolated from both human and mouse bone marrow, and are reported to generate most cell types \textit{in vitro} and following injection into mouse blastocysts (Jiang et al., 2002). These cells may prove to be of great benefit for a variety of therapeutic approaches, but the highly specialised culture conditions they appear to require are at present a major constraint on their wider application to various disease models (Daley et al., 2003). Further studies into the manipulation and culture of these interesting cells may in future prove them to be useful for a range of therapies, along with providing further insight into mammalian development and the true nature of stem cell “plasticity”. Depending on the outcome of these studies, adult stem cells may in future prove to be a valuable source of cells for a wide range of different therapies.

1.7.2 Embryonic germ cells

Embryonic germ cells are derived from primordial germ cells, present in the gonadal ridge of the post-implantation embryo (Shamblott et al, 1998). These cells normally develop into mature gametes (oocytes and sperm), but under certain culture conditions have the capacity to form embryoid bodies (aggregates of undifferentiated as well as partially and fully differentiated cells) containing cells that proliferate rapidly in culture and display gene expression patterns representative of multiple tissue lineages (Shamblott et al, 1998; Shamblott et al., 2001). Although these studies have demonstrated that EG cells are pluripotent as per the definitions for both ES and EG cells (Shamblott et al., 2001), EG cells have been less extensively studied with \textit{in vitro} and \textit{in vivo} studies in comparison to ES cells. However, preliminary implantation studies involving derivatives of these cells suggest their potential use as a source of cells for stem cell therapies (Kerr et al., 2003).
1.7.3 Embryonic stem cells

Embryonic stem cells are a pluripotent cell type derived from the inner cell mass of the pre-implantation embryo (NIH stem cell report, 2001). Mouse ES cells have been extensively studied for almost 20 years, largely with a view to investigating the mechanisms involved in differentiation and early embryogenesis, as a tool for germline transgenesis and as a vector for gene therapy (Evans and Kaufman, 1981; Doetschman et al., 1985; Bradley et al., 1992; reviewed by Rathjen et al., 1998). Mouse ES cells can be maintained indefinitely in an undifferentiated (pluripotent) state in culture through the addition of gp130 agonists such as leukaemia inhibitory factor (LIF, Smith, 1992).

Human ES cells were first isolated in 1998 (Thomson et al., 1998), stimulating much excitement about the prospects of ultimately using these cells for treatment of a wide range of diseases. Human ES cells are presently derived from embryos generated during assisted reproductive technology (ART) protocols such as in vitro fertilization (IVF), surplus to the requirements of these procedures, and obtained with the informed consent of the couple involved (reviewed in NIH stem cell report, 2001). Following fertilisation of the oocyte, the zygote (fertilised egg) divides for the first time at around day two to produce a two-cell embryo. By day three the embryo reaches the eight cell stage referred to as the morula, and then at day four, the cells of the embryo adhere tightly to each other (this process is called compaction) such that by day five, the cavity of the blastocyst is completed (NIH stem cell report, 2001). At this stage, the inner cell mass has begun to separate from the outer cells which become the trophectoderm that surround the blastocyst. This day five embryo represents an optimum stage for implantation in an IVF procedure, and also for the derivation of ES cells.

In order to derive ES cells for culture, the inner cell mass (the cells of which will be used to establish the ES cell culture) must be separated from the trophectoderm (see Figure 1.7 for a graphical description of the inner cell mass of the blastocyst). This is done either through microsurgery (mechanical isolation...
Figure 1.7: Mammalian embryogenesis

A: The early stages of mammalian embryogenesis, from zygote to blastocyst (pre-implantation) and gastrula (post-implantation). The ultimate differentiation potential of cells of the early embryo is indicated by the range of cell types present in the mature animal (shown below). Arrow indicates the inner cell mass of the pre-implantation embryo. Inset (B) is a photograph of a pre-implantation blastocyst, with inner cell mass indicated by the arrow. Diagram modified from NIH stem cell report, 2001.
Figure 1.7

A

B

Zygote

Blastocyst

Gastrula

Ectoderm (external layer)

Mesoderm (middle layer)

Endoderm (internal layer)

Skin cells of epidermis

Neuron of brain

Cardiac muscle cells

Skeletal muscle cells

Tubule cell of the kidney

Red blood cells

Smooth muscle (in gut)

Pancreatic cell

Thyroid cell

Lung cell (alveolar cell)

Germ cells

Sperm

Egg
of the inner cell mass) or immunosurgery (involving the use of antibodies directed against the trophectoderm to break it down, leaving the inner cell mass (reviewed by Trounson, 2002)). The isolated inner cell mass can then be grown in culture to establish the ES cell lines.

Unlike adult stem cells, ES cells are relatively unrestricted in their differentiation potential, being ‘pluripotent’ and thus able to differentiate into any of the cell types present in mammals (reviewed in Rathjen et al., 1998; NIH stem cell report, 2001). This is illustrated in Figure 1.7, a cartoon representing mammalian embryogenesis, illustrating the ultimate differentiation potential of cells of the embryo post-implantation. Because ES cells are derived from the inner cell mass of the pre-implantation embryo, they also demonstrate similarly broad differentiation potential (NIH stem cell report, 2001). This characteristic, along with their potential for theoretically limitless expansion in culture (Amit et al., 2000; Carpenter et al., 2003), has led to much basic and applied research into ES cell biology. One of the goals of this research has been to understand the signals and conditions required for differentiation into various cell types, with a view ultimately to the derivation of specific cell phenotypes for cell replacement strategies (Daley et al., 2003).

Despite significant technical advances in the culture of human ES cells since their initial isolation (Xu et al., 2001), the culture of human ES cells is still significantly more involved than for mouse ES cells. Due to the many similarities in the biology of human and mouse ES cells, mouse ES cells thus represent an ideal system for evaluating ES cell-based approaches to therapy in small animal models.

1.7.3.1 Directed differentiation of ES cells

As further investigations into the properties and potential of stem cells have been carried out, much work has focused on the development of protocols for the directed differentiation of ES cells towards specific cell phenotypes. The development of protocols for the reliable manipulation and differentiation of pluripotent ES cells opens up further possibilities for the study of specific signals relating to commitment and lineage specification at various stages of early development, as a source of pharmacological assays for screening the activities of various drugs and other compounds, and for the generation of potentially therapeutically useful cell types for transplantation (Guan et al., 2001). The aim of these in vitro differentiation protocols is
essentially to recapitulate the conditions and signals that result in the formation of specific lineages of differentiated cells from pluripotent ES cells. For neural differentiation, these protocols are generally separated into three stages: induction, selection, and in some cases, survival promotion. Various protocols have been reported that involve one or more of these different stages in the derivation of the final cell population. Figure 1.8 provides an overview of these different stages of directed differentiation.

1.7.3.1a Induction

Induction phases of ES cell differentiation focus on the early events in embryogenesis that govern differentiation to various cell lineages, starting with the three germ layers (see Figure 1.8). Differentiation to mature neural phenotypes thus requires early induction to neurectoderm, the early embryonic precursor of the mature CNS. This has been reported using a number of different approaches. One of the most commonly used methods is induction mediated by the vitamin A derivative retinoic acid (RA, reviewed by Guan et al., 2001). This involves culturing ES cells in suspension as aggregates called embryoid bodies, or EBs, in the presence of high concentrations of RA (10$^6$ to 10$^7$M), resulting in an increased frequency of neurectodermal differentiation and ultimately neuronal differentiation in these EBs, compared to EBs grown in the absence of added RA (Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995; Bain et al., 1996; Guan et al., 2001). The mechanism by which RA induces neuronal differentiation is at present unclear, but studies have shown a simultaneous upregulation of neural-specific genes and a downregulation of mesodermal genes following RA treatment (reviewed by Guan et al., 2001).

Other compounds have also more recently been shown to have neural inducing activity in ES cells, such as the Wnt antagonist Sfrp2 (secreted frizzled-related protein-2; Aubert et al., 2003), the broad spectrum protein kinase inhibitor staurosporine (Schumacher et al., 2003), and the 4,6-disubstituted pyrrolopyrimidine TWS119, found to modulate the activity of GSK-3β (glycogen synthase kinase-3β; Ding et al., 2003). Additionally, neural inducing activity has been reported following co-culture with the stromal cell lines PA6 (Kawasaki et al., 2000; Kawasaki et al., 2002), MS5 and SI7 (Barberi et al., 2003).
Figure 1.8: Directed differentiation of ES cells in vitro

The three main stages of in vitro differentiation protocols are indicated; Induction, selection and further differentiation. Blue arrows indicate the aim of the “induction” stages of these protocols- to increase the frequency of neural differentiation through induction of ES cells to early neural phenotypes. Red arrows and crosses indicate the aim of the “selection” stages of these protocols- to encourage proliferation of neural lineages and restrict and/or eliminate pluripotent cells and cells from non-neural lineages, respectively. Green arrows indicate the aim of the “further differentiation” stages of these protocols- to encourage the survival of and/or differentiation to specific mature neural phenotypes. Different cell fates following differentiation are indicated. Cell types described by a semi-circular arrow have been shown to be self-renewing. ES: Embryonic stem, EPL: Primitive ectoderm-like, NSC: Neural stem cell. “Extra-neural” indicates cell types not normally resident in the brain.
Figure 1.8

Pluripotent stem cells

Extra-embryonic Endoderm

Visceral Endoderm

Mesoderm

Ectoderm

ES cell

Primitive Ectoderm (EPL cells)

Induction

Selection

Further differentiation

Teratoma

Endoderm

Mesoderm

Ectoderm

Terminally differentiated “Extra-neural” cells

Mature neurons and glial cells

Committed progenitors

NSC

Neurectoderm

Ectoderm

Surface Ectoderm

Mesoderm

Visceral Endoderm

Extra-embryonic Endoderm

Terminally differentiated “Extra-neural” cells
Conditioned media systems such as MEDII, produced by the hepatocarcinoma cell line HepG2 have also showed neural induction activity (Rathjen et al., 1999, Rathjen et al., 2002). The MEDII differentiation protocol involves induction of ES cells cultured either in monolayer, or in suspension as aggregates (EBs cultured in MEDII, or EBMs) to primitive ectoderm-like cells (Rathjen et al., 1999), and ultimately to neural progenitors and other terminally differentiated cells of a neuroectodermal lineage, grown in suspension (Rathjen et al., 2002). These early differentiation events are illustrated in Figure 1.8. The active compounds in MEDII and their mechanism of action have not yet been determined.

Interestingly, others have reported that neural differentiation may be the "default" pathway for ES cells deprived of other exogenous signals (Tropepe et al., 2001). Further investigation of this hypothesis has shown that rather than a default pathway, neural differentiation requires autocrine fibroblast growth factor (FGF) signalling (Ying et al., 2003). Although the exact pathways and signals involved in neural differentiation of ES cells are yet to be elucidated, various studies are beginning to implicate certain factors as being of critical importance to these pathways, such as Wnt antagonism (Aubert et al., 2002), the Hedgehog signalling cascade (Maye et al., 2004), FGF signalling (Ying et al., 2003), and the activity of protein kinases in the regulation of neural differentiation (Schumacher et al., 2003, Ding et al., 2003) and maintenance of pluripotency (Sato et al., 2004). The isolation of specific factors displaying neural induction activity, and ultimately a more comprehensive understanding of the signals involved in this important pathway, should allow the development of more specific protocols for neural induction and differentiation. Although some studies have suggested that exogenous signals, whether provided in the form of added factors, conditioned media or co-culture are not necessary for neural differentiation (Tropepe et al., 2001, Ying et al., 2003), it has also been suggested that there may exist a stochastic element or community effect amongst differentiating cells (Ying et al., 2003), contributing to the heterogeneity observed in differentiated cell populations. Taking this view, the use of specific agents in directed differentiation protocols might serve to enhance the induction or effect of endogenous signalling, not necessarily resulting in homogeneous induction, but significantly increasing the efficiency of neural differentiation (as represented by the blue arrow in Figure 1.8). In combination with further protocols for cell preparation and selection, this could
ultimately contribute to greater purity of in vitro differentiated cell populations, particularly for the generation of mature neural phenotypes.

1.7.3.1.b Selection

Selection stages of neural differentiation protocols generally involve the selective expansion of neural phenotypes, (such as multipotent neural stem cells, or NSCs) at the exclusion of non-neural and pluripotent phenotypes. This has been achieved using a variety of methods, the most common of these being the transfer of cells following induction into a serum-free medium containing mitogens such as epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF, also called FGF-2), shown to promote the growth of NSCs (Okabe et al., 1996). Additionally, the withdrawal of serum from the medium is thought to inhibit the growth of other phenotypes, such as those of a mesodermal lineage (Guan et al., 2001). Recently, it has been shown that culture in serum-free medium alone is not sufficient to exclude the proliferation or survival of extra-neural cell lineages (Ying et al., 2003), highlighting the need for further selection strategies. However, these culture conditions may provide for the selective proliferation of NSCs or progenitors, effectively enriching for these phenotypes in a cell population.

Studies have suggested that NSCs and other immature neural cells may have distinctly different adherence properties to other extra-neural cells in culture (Zhang et al., 2001). This property has also been exploited as a means of further purifying or enriching for these cells, in conjunction with serum-free culture conditions (Zhang et al., 2001).

Other strategies that have been employed for the specific selection of NSCs or precursors have included genetic modification to facilitate expression of either green fluorescent protein (GFP) under control of the neural-specific promoter Sox1 (Ying et al., 2003) for specific fluorescence-activated cell sorting (FACS) purification, or of neomycin phosphotransferase under control of the neural-specific promoter Sox2 to facilitate antibiotic selection of neural lineages (Li et al., 1998). Although these approaches can potentially result in the efficient elimination of non-neural cell types, they are inherently limited by the requirement of genetic modification in the source population of ES cells, potentially limiting their greater application in various differentiation protocols.
As studies reveal more about the fundamental properties of adult stem cells such as NSCs, further opportunities for their selection and isolation from mixed ES cell-derived populations arise. Numerous studies have focused on FACS conditions for the isolation of NSCs, based on their endogenous properties (Rietze et al., 2001). Recently, the discovery of the relevance of orphan nuclear receptor TLX to NSC maintenance has introduced further possibilities for FACS purification of NSCs (Shi et al., 2004). With future studies contributing to greater understanding of the fundamental properties of NSCs, the range of targets for selection approaches of this cell type will likely expand further.

The selection stage of differentiation protocols is likely to be of increasing importance with the increase in in vivo cell transplantation studies, and hopefully, ultimate application to human patients. Without efficient and thorough protocols for the elimination of extra-neural lineages and particularly pluripotent cells, the significant risk of teratoma formation following transplantation will limit the potential application of ES cell-based therapeutic approaches. This is illustrated in Figure 1.8; with efficient neural induction and selection protocols prior to transplantation, homogeneous differentiation to mature neural phenotypes (such as mature neurons and glial cells) could theoretically be achieved. ES cells that either differentiate to an “extra-neural” lineage or remain in a pluripotent state following in vitro differentiation protocols will ultimately form mature cells of non-neural lineages, or teratomas following transplantation.

1.7.3.1.e Further differentiation of ES cells to mature neural phenotypes

This final stage of ES cell differentiation is generally only employed in therapeutic approaches where there is a requirement for a specific phenotype of mature neural cell, such as in the case of Parkinson’s disease (Rolletschek et al., 2001). This further differentiation is generally induced by the withdrawal of mitogens used to induce NSC proliferation in suspension (bFGF and EGF), and the plating of cells in an adherent culture (Zhang et al., 2001). Differentiation to specific neural subtypes (such as astroglia, oligodendrocytes, dopaminergic neurons, serotonergic neurons, GABAergic neurons and motor neurons) can also be induced by the addition of specific growth factor combinations to the culture medium (Barberi et al., 2003). Generation and long term in vitro survival of dopaminergic neurons has also been shown to be
improved by the addition of various neurotrophic factors and cytokines promoting survival to the culture medium (Rolletschek et al., 2001).

Presently work is being carried out in order to understand the biochemical pathways involved in the generation of specific cell phenotypes and the culture conditions required to reliably generate these cells. These studies should ultimately expand the potential of ES cells, particularly for the generation of cell populations for various stem cell therapies. The fundamental challenge in the development of differentiation protocols for transplantation studies is obtaining a therapeutically useful population of sufficient purity to minimise (or ideally) eliminate the risk of tumour formation. Tumours have not been reported following the transplantation of NSCs derived from adult or fetal primary cultures, but have been reported following numerous ES cell-based transplantation studies (Björklund et al., 2002; Choi et al., 2002; Wakitani et al., 2003). Despite many studies reporting successful derivation of NSCs from both human and mouse ES cells (Rathjen et al., 2002; Stavridis and Smith 2003; Barberi et al., 2003), differentiation protocols need to be able to efficiently and consistently exclude extra-neural, and particularly, pluripotent cells (see Figure 1.8). This may well represent quite a challenge, since the presence of even a single residual pluripotent cell could theoretically result in the formation of a teratoma following transplantation.

1.7.4 Ethical and political issues associated with stem cell research

In addition to the technical issues associated with ES cell research, this field has been the subject of extensive debate in many institutions globally, concerning the ethics associated with the use of ES cells derived from human embryos, human ES cell research generally, and human cloning (both reproductive and therapeutic). Much of this debate has involved discussion of human cloning, which has on occasion unfortunately obscured the debates regarding the derivation and use of human ES cells, at least in public discourse. Human cloning (in all its potential forms) involves an entirely different set of ethical issues. Since therapeutic cloning (for example, somatic cell nuclear transfer) is not necessarily required for the development of effective stem cell therapies in humans, discussion of the issues relating to human cloning are beyond the scope of this thesis.
Although the studies described in this thesis have involved the use of mouse ES cells, the proximate aim of these studies is to be able to apply techniques and lessons learned using mouse ES cells to human ES cells, with the ultimate goal of developing effective therapies in humans. The primary ethical issues relating to the use of human ES cells for research concern the source from which they were derived; pre-implantation embryos normally generated during ART protocols.

These ethical issues concern both the moral status of the embryo, that is, at which stage is a fertilised embryo to be considered an individualised human entity with the inherent potential to become a person, and whether the use of cells or tissues from an embryo beyond this point (obtained with informed consent of the donors due to being surplus to ART procedures) is justified by being for the "greater good"; that is, to improve the understanding of basic human biology, or to help better treat human disease. (Outka, 2002; Sitko, 2002; Oduncu, 2003; Lauritzen, 2003). Due to the wide range of moral positions (both religious and secular) in our society, achieving consensus on all of these issues is difficult, if not impossible.

In such a rapidly changing ethical field, with new scientific discoveries providing fresh perspective on these issues on a regular basis, a detailed discussion of all the ethical, philosophical and political issues pertaining to stem cell research is beyond the scope of this thesis. However, legislation has now been passed in many countries to provide a regulatory framework in which stem cell research can proceed.

In Australia, two Acts were recently passed through Federal parliament- the Prohibition of Human Cloning Act 2002, and the Research Involving Human Embryos Act 2002 (see http://www.health.gov.au/ahmrc/embryo/index.htm). The first of these prohibits human cloning outright in Australia (either reproductive or therapeutic cloning), while the second introduces strict guidelines within which ES cell research can proceed. The Research Involving Human Embryos Act 2002 allows the use of embryos for research (including the derivation of new human ES cell lines) as long as the embryos were not created specifically for research, the informed consent of the donor couple is obtained, and the embryos are surplus to the requirements of the ART procedure for which they were generated. Additionally, the Federal Government is also able to provide funding for research of this kind.

The United Kingdom has, in effect, arguably the most progressive legislation for the regulation of stem cell research. What became the Human Fertilisation and
Embryology (Research Purposes) Regulations (2001SI 2001 No. 188) were debated and passed by the House of Commons on 19 December 2000 and by the House of Lords on 22 January 2001. These regulations allow research on pre-implantation embryos (either as surplus to ART procedures, or created specifically for research, and obtained with the informed consent of the donors), including the derivation of ES cells.

In the United States of America, a statement was made by President George W. Bush on August 9, 2001 (Office of the Press Secretary, 2001), which authorised funding of stem cell research using existing pluripotent stem cell lines that were derived from human embryos before August 9, 2001, provided that the informed consent of the donors was obtained, the embryos were created for reproductive purposes only and were in excess of clinical need, there were no financial inducements to the donors, and the embryos were not created specifically for research purposes. The National Institutes of Health (NIH) has since been providing grants to conduct human ES cell research starting in the fiscal year 2002.

Many other countries throughout the world have additionally passed legislation regarding stem cell research, or are in the process of debating such legislation. In a practical sense, the existence of a similar regulatory framework (between Australia, the United Kingdom and the United States of America) ensures that for now at least, there are no political barriers impeding development of allogeneic stem cell therapies in humans using human ES cells.

1.7.5 Stem cell therapy

Stem cell therapy involves the use of normal pluripotent or multipotent cells to repopulate a particular tissue in a disease involving cellular death or dysfunction (Smith, 1998). Stem cell therapies have shown promise for a range of somatic diseases including diabetes (Soria et al., 2000) and cardiac ischemia (Min et al., 2002), along with various brain diseases (discussed below). Genetically modified stem cells can also be used for ex vivo gene therapy as vectors for delivery of a therapeutic protein (Rathjen et al., 1998; Smith, 1998; Park et al., 2002).

Due to the self renewing nature of stem cells (particularly ES cells), precise genetic changes could potentially be carried out in the cell population, as even a very small proportion of modified cells could be selected and expanded without reaching senescence (Snyder and Vescovi, 2000). When implanted, all progenitors of the stem
cell population would thus contain this genetic modification. Additionally, as stem cells have been shown to differentiate based on local developmental cues to become other terminally differentiated cell types following transplantation (Fricker et al., 1999), they could possibly persist and express foreign genes on a much longer term basis than other terminally differentiated cell types used for *ex vivo* gene therapy, such as fibroblasts (Taylor and Wolfe, 1997). Stem cell therapy thus represents a promising strategy for long term treatment of genetic disease such as LSD.

Stem cell therapy for a range of diseases may eventually involve the use of either adult (tissue) stem cells isolated prior to use, or cell lines established from ES or EG cells, differentiated to a therapeutically useful phenotype. Genetic modifications could be undertaken (if required) when the cells are in their most undifferentiated state (and thus self-renewing and potentially most proliferative), before differentiating the cells to an appropriate phenotype prior to implantation (Park et al., 2002; Daley et al., 2003). The ideal source of stem cells for a particular therapy would depend on numerous factors, both practical and political.

Adult stem cells (or tissue stem cells) are presently thought of as restricted in their differentiation potential, relative to other pluripotent cells. Although this could ultimately limit the range of cell types that could be obtained from these cells, this represents an advantage, however, when considering issues of safety for transplantation, compared to the use of pluripotent cells such as ES cells (discussed below). However, because adult stem cells have not displayed the same proliferative capacity as ES cells in culture, any therapeutic strategy would most likely require continual derivation of these cells in order to treat a wide range of patients (Daley et al., 2003). This could result in “batch inconsistencies”, depending on the source material used to obtain these cells. Due to the presently poorly understood plasticity of these cells, this could complicate the reproducibility of pre-implantation protocols.

Many of the studies carried out to date have involved conditional immortalisation (involving transduction with a retrovirus containing the *v-myc* gene) of neural progenitors to facilitate expansion of these cells in culture, such as described in Flax et al. (1998). This immortalisation approach has facilitated the generation of cell lines amenable to genetic modification and clonal isolation *in vitro*, which are found to retain their multipotency and downregulate the *v-myc* gene following transplantation (Flax et al., 1998). Further genetic modification in these cells (to facilitate the
expression of various therapeutic proteins) has, however, resulted in select clones losing their ability to successfully engraft following transplantation (Flax et al., 1998). This observation suggests caution in the genetic modification of stem cells due to the possibility of disrupting mechanisms that are at present poorly understood yet essential for their therapeutic utility. Modified stem cell clones should thus be thoroughly evaluated to ensure that they retain similar potential to unmodified cells. Minimal or, ideally, absence of genetic modification in stem cells would however reduce or avoid this potential problem altogether.

Embryonic stem cells have the advantage of theoretically being able to divide indefinitely in culture (without immortalisation), resulting in a virtually limitless supply for therapies, along with being a practical cell source for carrying out precise genetic modifications. The derivation of human ES cells representing all major MHC classes could potentially allow development of a “cell bank” for treatment of patients all over the world with a collection of relatively few well characterised cell lines. At present, protocols for the in vitro differentiation of ES cells are relatively primitive, and thus these cells still have greater safety issues associated with them than adult stem cells. These cells have been shown in numerous studies to have the potential to form teratomas when transplanted without differentiation through appropriate in vitro differentiation protocols (Choi et al., 2002; Wakitani et al., 2003). However, as our understanding of the basic biology underlying differentiation of these cells improves, so too will the protocols for deriving defined and well characterised cell phenotypes.

1.8 Stem cell therapy for CNS pathology in LSD

Much research into stem cell therapies in the last few years has focused on the investigation of these approaches for treatment of neurodegenerative diseases such as Parkinson’s disease. Research into a variety of animal models of neurological disease (including LSD) has shown stem cells to have a number of unique properties that suggest promise for the treatment of these diseases (reviewed in Daley et al., 2003). Many of these studies have involved the use of NSCs or conditionally immortalised neural progenitors derived from primary cultures of adult (or fetal) brain (reviewed in Park et al., 2002; Daley et al., 2003). Due to the fact that these cell types (neural progenitors and NSCs) can also be derived through differentiation of ES cells, the
outcomes of studies utilising these adult stem cells also indicates promise for the use of similar cell phenotypes derived from ES cells.

One of the most useful characteristics of stem cells for the treatment of brain disease in LSD is their ability to migrate following transplantation, since pathology in LSD is present throughout the CNS (Park et al., 2002). Implanted conditionally immortalised NSCs have been found to proliferate and propagate, migrating throughout the brain following implantation (Flax et al., 1998). More recently, implantation of clonal but genetically unmodified mouse NSCs was shown to be effective in decreasing the symptomatic tremor of the shiverer mouse by differentiating into functional oligodendrocytes, found to migrate throughout the brain (Yandava et al., 1999). Implanted NSCs were also found to migrate throughout the striatum following striatal implants (Svendsen et al., 1997). Other studies have further found that NSCs have the capacity to migrate towards sites of pathology in the brain which when combined with expression of an anti-tumour gene, have indicated the potential for this cell type to be used as an adjunct to other therapies in treating brain tumours (Aboody et al., 2000, Yip et al., 2003).

Another interesting property of stem cells such as NSCs found to be of potential clinical relevance relates to their apparent ability to support and repair host cells following transplantation. Implantation of NSCs in a mouse model of Parkinson’s disease was found to result not only in differentiation of implanted cells into dopaminergic neurons, but clinical recovery largely due to the “rescue” of dysfunctional endogenous neurons (Ourednik et al., 2002). Transplantation of NSCs into rats with spinal cord lesions additionally showed improvement following treatment, partially due to a reduction in glial scarring and tissue loss from secondary injury processes (Teng et al., 2002). Further studies found that NSCs support axonal regeneration following spinal cord injury and constitutively express various neurotrophic factors (Lu et al., 2003), underlying their regenerative potential following transplantation. The constitutive expression of these neurotrophic factors by stem cells might also prevent or reduce neurodegenerative processes involved in CNS pathology in LSD.

Approximately two-thirds of all LSD will develop CNS pathology (John Hopwood, personal communication), that usually results from a deficiency of a single enzyme. As a group, LSD therefore represent excellent candidate diseases for the use of
stem cells to provide deficient enzyme to the brain. The first study to pursue this approach investigated treatment of brain pathology in the MPS VII mouse (Snyder et al., 1995). This involved implantation of conditionally immortalised mouse NSCs retrovirally transduced to express β-glucuronidase (the deficient enzyme in MPS VII) into the cerebral ventricles of newborn mice. In implanted mice analysed up to eight months of age, donor-derived cells were found to be present throughout the neuraxis, and β-glucuronidase activity was observed throughout the brain, along with widespread correction of lysosomal storage in neurons and glial cells in affected mice. However, this study did not investigate the effect on other aspects of pathology. Similar results were subsequently obtained using a similar approach to facilitate expression of the β-hexosaminidase α-subunit (the deficient enzyme in Tay-Sachs disease, another LSD with CNS pathology), where implanted mice were found to have a significant reduction in lysosomal storage, examined out to eight weeks following transplantation (Lacorazza et al., 1996). These studies demonstrated proof of principle for the concept of implanting stem cells to engraft and secrete enzyme in LSD, mediating a reduction in storage. Recently, studies using conditionally immortalised human NSCs expressing β-glucuronidase implanted into MPS VII mice have also shown a similar reduction in lysosomal storage, although these animals were only analysed out to 25 days post-transplantation (Meng et al., 2003).

Further preliminary studies have investigated this approach for treatment of other LSD with CNS pathology such as Krabbe disease (Torchiana et al., 1998) using conditionally immortalised rat NSCs and Tay-Sachs disease (Flax et al., 1998) comparing conditionally immortalised human and mouse NSCs. These studies both demonstrated significant secretion of recombinant enzyme by NSCs in vitro, and showed that this enzyme was able to mediate a reduction in storage in deficient fibroblasts (Torchiana et al., 1998) or various neural cell types (Flax et al., 1998) in culture, supporting the potential use of these cells for stem cell therapy approaches.

To date, few studies have investigated the longer-term effects of stem cell implantation into the brain for treatment of CNS pathology in LSD. One such study involved implantation of marrow stromal cells (MSCs) expressing acid sphingomyelinase (ASM) into the hippocampus and cerebellum of a knockout mouse model of Niemann-Pick disease (NPD), another LSD with CNS pathology (Jin et al., 2002). Cells implanted into the hippocampus and cerebellum at three weeks of age
were found to survive for at least six months following transplantation (although only in low numbers) and migrate away from the injection sites. Recombinant ASM was not detectable at six months in total brain homogenates, but was found in very low amounts in Purkinje neurons by immunodetection (Jin et al., 2002). Despite low cell survival and recombinant enzyme expression, Purkinje cell loss in treated mice was found to be delayed, and these mice had a significantly improved lifespan compared to untreated controls, along with improved bodyweights and performance in a rotarod test of coordination (Jin et al., 2002). This study suggested that even low cell survival and thus enzyme distribution could possibly have a positive effect on brain pathology in LSD.

1.8.1 Prospects for α-mannosidosis therapy

Embryonic stem cell-based stem cell therapy represents a promising strategy for long-term treatment of CNS pathology in α-mannosidosis. The capacity of cells within the brain to take up lysosomal enzymes via M6P-mediated endocytosis (Jones et al., 1998), combined with the possibility of producing functional α-mannosidase (secreted into the extracellular environment) using genetically modified producer cells (Sun et al., 1999) suggests great promise for the therapeutic effect of local α-mannosidase production within the brain of α-mannosidosis individuals. The ability of implanted stem cells to migrate throughout the brain (reviewed in Park et al., 2002) and the observation that lysosomal enzymes can be delivered to other sites of the brain by retrograde axonal transport (Passini et al., 2002), additionally suggests the utility of stem cell therapy for widespread enzyme distribution and thus treatment of global brain pathology such as is present in α-mannosidosis. We hypothesise that following implantation of ES cell-derived neural cells (genetically modified to produce and secrete rhαM) into the α-mannosidosis guinea pig brain, endogenous cells would take up α-mannosidase via M6P-mediated endocytosis, resulting in pathological and behavioural normalisation.

The critical challenge in pursuing a stem cell therapy approach for LSD is thus achieving successful and widespread engraftment of implanted cells in order to provide a cellular substrate for therapeutic gene expression in the brain. The conditions required (in terms of cell phenotype, implantation site and cell dosage) to achieve successful engraftment in the α-mannosidosis guinea pig are presently unknown.
1.9.0 Specific aims

1. To establish and further characterise behavioural and neurological tests to measure the effect of pathology in the α-mannosidosis guinea pig.

2. To evaluate mouse ES cells and their differentiated derivatives as potential delivery vectors for α-mannosidase by constructing α-mannosidase overexpressing mouse ES cell lines.

3. To evaluate the potential of ES cell derived cells for treatment of brain pathology in α-mannosidosis by:
   a. Implanting these cells into the brains of newborn guinea pigs and evaluating survival, engraftment, and migration.
   b. Evaluating the effectiveness of this therapy in reducing CNS pathology in α-mannosidosis guinea pigs through assessment of behavioural and neuropathological outcomes.
Chapter 2:
Materials and Methods
2.1 Materials

2.1.1 Molecular biology
Primers (MUTR and 4AF):
10x PCR buffer:
Taq DNA polymerase:
Deoxyribonucleoside triphosphates (dNTPs):
Sterile water:
Restriction enzymes (and buffers):
Bovine serum albumin:
Proteinase K:
T4 DNA ligase:
LR Clonase:
LR Reaction buffer (5x):
Agarose (DNA grade):
Agarose (Hi-Res):
Ethidium bromide:
SPP-1 EcoR1, pUC19 HpaII, 1kb+ size markers:
TOP 10 electrocompetent E.coli:
Bacto agar:
Yeast extract:
Tryptone/peptone:
Ampicillin:
Kanamycin sulphate:

2.1.2 Buffers, solutions and substrates
10 x Loading buffer:

Acetate buffer:
TAE:
TE:
Phosphate buffered saline (PBS):
HEPES buffered saline (HeBS):
Gelatin solution:
LB medium:

Invitrogen, Australia
Boehringer Mannheim, Germany
Roche Diagnostics, USA
Boehringer Mannheim, Germany
Pharmacia and Upjohn, Bentley, WA, Australia.
New England Biolabs Inc., USA
Sigma Chemical Co., USA
Sigma Chemical Co., USA
Boehringer Mannheim
GIBCO-BRL, Invitrogen, Australia
GIBCO-BRL, Invitrogen, Australia
Boehringer Mannheim, Germany
Boehringer Mannheim, Germany
Boehringer Mannheim, Germany
Geneworks (Adelaide, South Australia)
Invitrogen, Australia
Becton Dickinson, Franklin Lakes, NJ, USA
Becton Dickinson, Franklin Lakes, NJ, USA
Becton Dickinson, Franklin Lakes, NJ, USA
Sigma Chemical Co., USA
Boehringer Mannheim, Germany

50% (v/v) glycerol, 1% (w/v) SDS, 100mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue.
82ml acetic acid (0.1M), 18ml sodium acetate (0.1M), pH 4.5.
40mM Tris-acetate, 2mM EDTA (pH 8.3).
10mM Tris-HCl, pH 7.5, 0.1mM EDTA.
137mM NaCl, 2.7mM KCl, 4.3 mM Na2HPO4 (pH 7.2).
1.64% NaCl (w/v), 1.19% HEPES (w/v), 0.021% Na2HPO4, pH 7.05.
0.2% gelatin in PBS, autoclaved.
1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl,
PH to 7.5 with NaOH.
Epoxy resin: 22ml Dodeceny1 Succinic Anhydride (DDSA), 10ml Procure 812, 6ml Araldite 502, and 560μl DMP-30 (2,4,6(tri(Dimethylaminoethyl)phenol)).

20x SSC:

Hybridisation buffer (for mouse microsatellite in situ hybridisations):

450μl 100% Formamide, 250μl 20x SSC, 250μl 10% (w/v) Chondroitin Sulphate, 25μl ssDNA (herring sperm single stranded DNA), 25μl sterile water.

PBS lysis buffer:

1% Sodium Deoxycholate (w/v), 0.1% SDS, 0.5% Nonidet P40, in PBS.

Glycine stop buffer:

200mM glycine, 157mM Na₂CO₃, 125mM NaOH, pH 10.7.

LB agar:

1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl, pH to 7.5 with NaOH, 1.5% (w/v) Bacto-Agar.

4MU substrate:

13.53mg of 4-methylumbelliferyl-α-D-mannopyranoside, dissolved in 10ml 0.1M acetate buffer, pH 4.5

4MU standard:

25mg 4-methylumbelliferone dissolved in 1ml ethanol, made up to 1L with distilled water

2.1.3 Cell lines and bacteria:

CHO-K1 (Chinese Hamster Ovary cells):

ATCC, CRL-9618

D3 (Mouse Embryonic Stem cells):

ATCC, CRL-1934

HepG2 cells:

ATCC, HB-8065

SF3949 (Human α-mannosidosis skin fibroblasts):

National Referral Laboratory, WCH

SF4858 (Human normal skin fibroblasts):

National Referral Laboratory, WCH

SF4976 (Human normal skin fibroblasts):

National Referral Laboratory, WCH

E.coli strain: TOP10

F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 mupG

2.1.4 Surgical equipment:

Hand drill, 1mm diameter: P&N, Australia

Stereotaxic frame (Model 900, with guinea pig adaptors): David Kopf Instruments, Tujunga, CA, USA

SP200iz syringe pump: KD Scientific, USA

Olympus SZ40 dissecting microscope: Olympus, New York, USA
Boyle Apparatus anaesthesia machine; Microlight 150; CIG, Australia

2.1.5 Pharmaceuticals and surgical supplies:
Xylocaine spray (lignocaine hydrochloride):
Atropine (0.6mg/ml):
Ketamine (100mg/ml):
Lium Xylazil (Xylazine 20mg/ml):
Fortane (Isoflurane):
Finadyne (Flunixin meglumine 50mg/ml):
Bactrim (Sulphamethoxazole/Trimethoprim 40/8mg/ml):
Neoral (Cyclosporin A, 100mg/ml):
Lethabarb (pentobarbitone sodium 325mg/ml):
Lacrilube (liquid paraffin 42.5% w/w, soft white paraffin 57.3% w/w):
Sutures (Dysilk 4.0s, reverse cutting):
Sterile saline (0.9% w/v):
Sterile water:
Oxygen:
Dextrose:
Saline:
Syringes:
Needles:
Scalpel blades:
Gauze:
Ethanol:
Gammex sterile surgical gloves:
Astra Zeneca Pty Ltd, North Ryde NSW, Australia
Apex Laboratories Pty. Ltd. NSW, Australia
Parnell Laboratories Pty. Ltd., Australia
Troy Laboratories, Australia
Abbott Kurnell Australasia Pty. Ltd., Australia
Schering-Plough Animal Health, NSW, Australia
Roche, NSW Australia.
Novartis Australia Pty. Ltd.
Virbac Australia Pty. Ltd., Peakhurst, NSW, Australia
Allergan Gordon, NSW, Australia
Dynek (Hendon, Australia)
Pharmacia and Upjohn, Australia
Pharmacia and Upjohn, Australia
CIG, Australia
Baxter Healthcare Pty. Ltd., NSW, Australia
Baxter Healthcare Pty. Ltd., NSW, Australia
Becton Dickinson
Becton Dickinson (Franklin Lakes, NJ, USA)
Swann-Morton (Sheffield, England)
Smith and Nephew Pty. Ltd. Australia
Ajax Chemicals, Australia
Ansell Medical, Glen Waverley, Australia

2.1.6 Tissue culture:
Disposable tissue culture flasks, plates and pipettes:
Dulbecco’s Phosphate Buffered Saline (PBS) without calcium and magnesium:
Fetal Calf Serum (FCS):
Trypsin/Versene solution (0.1% w/v):
Dulbecco’s Modified Eagle Medium (DMEM):
Corning, USA
CSL Limited, Australia
CSL Limited, Australia
CSL Limited, Australia
GIBCO-BRL, Invitrogen, Australia
Ham's F12 Medium (F12):
G418 sulphate (Neomycin):
Penicillin/Streptomycin sulphate:
LIF:
β-Mercaptoethanol:
MEDII:
L-glutamine:
ITSS supplement:
FGF-2:

2.1.7 Histology:
Parafomaldehyde:
Glutaraldehyde:
Normal Donkey Serum:
Triton X-100:
Mouse microsatellite probe (DIG labelled):
Pepsin:

2.1.8 Antibodies:
Rat α-mouse Nestin IgG1
(Rat 401 monoclonal):
Rabbit α-NF200 IgG1
(N4142 polyclonal):
Mouse α-TH (LNC1 monoclonal):
Rabbit α-GFAP (G9269 polyclonal):
Goat α-Oct4 IgG
(sc-8628 (N-19) polyclonal):
Mouse α-SMA IgG
(A2547 Clone 1A4 monoclonal):
Mouse α-MAP2 IgG1
(MAB3418 monoclonal):
Donkey α-mouse CY3 (715-165-151):
Sheep α-mouse IgG FITC (AQ325F):
Donkey α-rabbit FITC (711-095-152):
Streptavidin-CY3:
Streptavidin-CY5:
Donkey α-Guinea Pig (biotinylated):
Monoclonal α-rhαM (clone 4C5):
Sheep α-mouse IgG:

2.1.9 Miscellaneous materials, chemicals and kits:
Isoton II:

GIBCO-BRL, Invitrogen, Australia
GIBCO-BRL, Invitrogen, Australia
CSL Ltd, Australia
Chemicon (Temecula, California, USA)
Sigma Chemical Co., USA
Bresagen, Ltd, Australia
CSL Limited, Australia
Boehringer Mannheim, Germany
Peprotech, USA
Sigma Chemical Co., USA
Sigma Chemical Co., USA
Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA)
Sigma Chemical Co., USA
Geneworks (Adelaide, South Australia)
Sigma Chemical Co., USA
Yale, USA
Sigma Chemical Co., USA
DiaSorin, Stillwater, USA
Sigma Chemical Co., USA
Santa Cruz Biotechnology, Inc., USA.
Sigma Chemical Co., USA
Chemicon, Temecula, California, USA
Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA)
Chemicon, Temecula, California, USA
Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA)
Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA)
Jackson ImmunoResearch Laboratories, Inc. (Pennsylvania, USA)
Barbara King, WCH
Silenus, Chemicon Australia

Beckman Coulter, USA
Mannose-6-phosphate: Sigma Chemical Co., USA
5-Azadeoxycytidine: Sigma Chemical Co., USA
4-Methylumbelliferyl-α-D-Mannopyranoside: Sigma Chemical Co., USA
4-Methylumbelliferone: Sigma Chemical Co., USA
Dimethylsulphoxide (DMSO): Sigma Chemical Co., USA
EDTA (Ethylenediaminotetraacetic acid): Sigma Chemical Co., USA
Tris Base: Ajax Chemicals, Australia
Sodium Chloride: Boehringer Mannheim
Sodium Azide (NaAz): BDH Chemicals Australia, Pty. Ltd., Melbourne, Australia
Procure Araldite Embedding Kit: Sigma Chemical Co., USA
(for epoxy resin): Sigma Chemical Co., USA
Polyvinylchloride plates (96 well): ProSciTech (Queensland, Australia)
Thermanox coverslips: Costar (Cambridge, MA, USA)
(for seeding EBMs): Nunc (Rochester, NY, USA)
C18 (EC) ISOLUTE cartridges (25mg): Adelab, Australia
GENECLEAN™ KIT: BIO 101 Inc., USA
Herring sperm DNA: Sigma Chemical Co., USA
Miniprep kits: Qiagen Pty. Ltd., Clifton Hill, Victoria, Australia
Midiprep kits: Qiagen Pty. Ltd., Clifton Hill, Victoria, Australia
Instagene extraction kit: BioRad, USA
Bio-Rad Protein Assay Dye reagent: BioRad, USA
White water based paint: Wattyl, Australia.

All other chemicals used in this study were analytical grade and purchased from
Ajax Chemicals Pty. Ltd. Auburn, Australia; BDH Chemicals Australia, Pty. Ltd., Melbourne, Australia, Boehringer Mannheim, Mannheim, Germany; and Sigma Chemical Co. St. Louis, USA.
2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Preparation of DNA

2.2.1.1a Minipreps

Minipreps were performed as per protocols supplied with the QIAprep Spin Miniprep Kit (Qiagen). All buffers described were supplied with the kit. Briefly, following plating of transformed bacteria, colonies were picked and inoculated into 1ml LB medium containing 100μg/ml Ampicillin, and incubated overnight at 37°C with shaking. Bacteria were then pelleted by microcentrifugation, and resuspended in 250μl Buffer P1. Buffer P2 (250μl) was then added and cells were mixed by gentle inversion 4-6 times before incubation for 5 minutes at room temperature. Buffer N3 (350μl) was then added prior to gentle inversion 4-6 times and the solution was centrifuged at approximately 17,900g for 10 minutes. The remaining supernatant was then applied to the QIAprep Spin Column, which was then microcentrifuged for 60 seconds. The flow-through was discarded and the column was then washed with 750μl Buffer PE (again discarding the flow through following centrifugation), before further microcentrifugation for 60 seconds to remove any residual wash buffer. DNA was then eluted by adding 50μl of sterile water (Pharmacia and Upjohn) to the column, letting it stand for 60 seconds and then centrifuging for a further 60 seconds into a clean tube.

2.2.1.1b Midipreps

Midipreps were performed as per protocols supplied with the HiSpeed Plasmid Midi Kit (Qiagen). All buffers described were supplied with the kit. Briefly, following plating of transformed bacteria, colonies were picked and inoculated into 1ml LB medium containing 100μg/ml Ampicillin and incubated overnight at 37°C with shaking. The following day, 200μl of culture was used to inoculate each of two 75ml flasks containing LB medium with 100μg/ml Ampicillin, which were again incubated overnight at 37°C with shaking. Bacterial cells were then pelleted by centrifugation at 6,000g for 10 minutes at 4°C, before removing all supernatant and resuspending the pellets in 6ml buffer P1. Buffer P2 (6ml) was then added before gentle mixing by inversion and incubation of the mixture at room temperature for five minutes. Buffer P3 (6ml) was then added before mixing by inversion 4-6 times, after which the lysate was poured into the QIAfilter Cartridge and incubated for 10 minutes at room
temperature. A HiSpeed Midi column (Qiagen) was then equilibrated with 4ml of Buffer QBT and allowed to empty by gravity flow, before applying the filtered lysate to the column. The column was then again allowed to empty by gravity flow, before washing with 20ml Buffer QC. DNA was then eluted with 5ml of Buffer QF into a glass tube, before precipitation by addition of 3.5ml isopropanol. DNA was then centrifuged at 10,000g for 60 minutes at 4°C, before gentle aspiration of the supernatant, followed by washing with 5ml 70% ethanol. DNA was then centrifuged again at 10,000g for 60 minutes at 4°C, before removing the supernatant, allowing the DNA to air dry in a laminar flow hood, and resuspending in 200μl sterile water (Pharmacia and Upjohn). DNA was then quantitated as described in Section 2.2.1.1c.

2.2.1.1c Quantitation

DNA was quantitated by dilution 1:200 in sterile water, followed by measurement of optical density (O.D.) at 260nm using a UV-1201 spectrophotometer (Shimadzu Corporation, Japan). The equation:

\[ \text{O.D.}_{260nm} \times \text{dilution factor (200)} \times 50 = [\text{DNA}] \mu g / ml \]

was then used to calculate the concentration of DNA in the original solution.

2.2.1.2 Agarose gel electrophoresis

Analysis of DNA by electrophoresis was done by combining DNA with 0.25 volumes of dye loading buffer and using gel containing 1-5% (w/v) agarose in 1x TAE buffer. Following electrophoresis, the gel was stained in a solution of 10µg/ml ethidium bromide before visualisation of DNA under UV light using a UviPro Gel documentation system with UviPhoto software for digital image acquisition (UviTec, Cambridge, UK).

2.2.1.3 Restriction digests

All restriction enzyme digestions were carried out utilising the conditions specified for each individual enzyme as recommended by the manufacturer.
2.2.1.4 Gel purification of DNA fragments

DNA was purified from 1x TAE agarose gels using a GENECLEAN™ kit (BIO 101 Inc., USA). After staining in ethidium bromide the gel was visualised under UV light and the appropriate band excised. The slice of gel containing the DNA of interest was combined with three volumes of Geneclean sodium iodide solution in the supplied tube and heated at 55°C for three to five minutes to dissolve the agarose. Geneclean Glassmilk (500µl) was then added to the tube, mixed by inversion and incubated at room temperature for five minutes. The Glassmilk/DNA was then pelleted by a 15 second microcentrifugation and washed with 500µl of Geneclean “New Wash” solution by microcentrifugation/resuspension, before drying the pellet for two minutes. DNA was eluted with sequential elutions of 15µl and 10µl sterile water (Pharmacia and Upjohn) into the catch tube supplied.

2.2.1.5 Ligation

Ligations were performed in the presence of 1x ligation buffer (Boehringer Mannheim) and 1µl of T4 DNA ligase, in a total reaction volume of 20µl. All reactions were prepared on ice before incubation. Linearised plasmid DNA and insert DNA preparations were semi-quantitated via agarose gel electrophoresis with 500ng of molecular size markers in an adjacent lane. For each plasmid/insert ligation, several reactions were done. A control ligation reaction contained the linearised, dephosphorylated plasmid, T4 DNA ligase and no insert. An additional control reaction was sometimes included in which only the linearised plasmid and ligation buffer were present. Several DNA ligation reactions containing various molar ratios of plasmid:insert were usually done. Three such ratios routinely used were 1:1, 1:2 and 1:5. Ligation reactions were performed at 4°C overnight and then stored at -20°C prior to transformation.

2.2.1.6 Recombination reactions

Recombination reactions were performed using the Gateway system (Invitrogen). Reactions were performed in the presence of 1x LR reaction buffer (Invitrogen), 4µl of LR Clonase (Invitrogen), in a total volume of 20µl. All reactions were prepared on ice before incubation. Linearised entry clone DNA and destination vector DNA preparations were semi-quantitated via agarose gel electrophoresis with
500ng of molecular size markers in an adjacent lane. For each entry clone/destination vector recombination, several reactions were done. A control ligation reaction contained the linearised, destination vector, LR Clonase and no entry clone. Several DNA recombination reactions containing various molar ratios of entry clone:destination vector were usually done. Three such ratios routinely used were 2:1, 1:1 and 1:2. Following addition of the LR Clonase mix, reaction mixes were mixed by vortexing briefly before incubation at 25°C for 60 minutes. Proteinase K (2µl) was then added to each reaction before incubation at 37°C for 10 minutes. Recombination reactions were then stored at -20°C prior to transformation.

2.2.1.7 Transformation of electrocompetent bacteria

Electrocompetent TOP10 E.coli (Invitrogen) were used for all transformations. Cells (25µl) were thawed on ice before mixing with 1µl of DNA (ligation mix, recombination mix, or plasmid). This mixture was then transferred to a pre-chilled electroporation cuvette and encouraged to sink to the bottom of the cuvette by gently tapping on the bench. The cuvette was then placed in the electroporator (BTX Electro Cell Manipulator ECM 395) before a voltage of 1.5kV was applied. Room temperature L-broth (250µl) was then immediately added before this mixture was transferred to a 10ml tube, incubated at 37°C for 1 hour with shaking and subsequently plated on L-agar plates (containing ampicillin) and incubated overnight at 37°C.

2.2.2 Protein analysis

2.2.2.1 Fluorogenic assay for α-mannosidase

α-Mannosidase activity was measured using the fluorogenic substrate 4-methylumbelliferyl-α-D-mannopyranoside (Avila and Convit, 1973). Samples to be analysed (generally 10µl for a cell lysate, or 20µl for a conditioned media sample) were added to 100µl 4MU substrate (PBS was used as a blank) in plastic fluorimeter tubes, and incubated at 37°C in a water bath for 30 minutes. Following incubation, the reaction was stopped by addition of 1.5ml glycine stop buffer and mixed by vortexing. Samples were then analysed on a fluorometer (Perkin Elmer Luminescence Spectrophotometer, LS 50B). Enzyme activities were calculated according to the following formula:
“Standard” samples were 1.6ml of glycine stop buffer, with 20μl of 4MU standard added. Specific activity was calculated by dividing activity by the amount of total cell protein, as quantitated in Section 2.2.2.3, below.

2.2.2.2 Immunocapture assay for recombinant human α-mannosidase (rhαM)

Sheep α-mouse antibody was diluted in 0.1M NaHCO₃, pH 8.5, to a concentration of 20μg/ml. This solution was then added to each well of a 96 well plate to be used for this assay (100μl/well), and incubated overnight at 4°C. Plates were then washed three times with Tris/NaCl (0.25M NaCl, 0.02M Tris pH 7.2), using an automatic plate washer (ADIL Instruments, France) before applying 4C5 mouse α-rhαM supernatant (Barbara King), 100μl/well. Plates were then incubated at room temperature for four hours, before washing three times with Tris/NaCl, and blocking for one to two hours in Tris/NaCl/1% (w/v) milk powder (Diploma). Following aspiration, samples were added (50μl/well) and incubated overnight at 4°C. Plates were then washed three times with Tris/NaCl before addition of 100μl 4MU substrate, incubation at 37°C for five hours and subsequent transfer of solutions to plastic fluorometer tubes containing 1.5ml glycine stop buffer followed by analysis as described in Section 2.2.2.1 above. Specific activities of rhαM in original samples were calculated by including rhαM standards of known concentration in the immunocapture protocol and equating calculated activities of measured samples to these standards.

2.2.2.3 Protein quantitation

Protein concentration of all samples was assayed using the Bio-Rad Protein Assay Dye reagent concentrate, according to the manufacturer’s specifications (Bio-Rad).
2.2.3 Cell culture

2.2.3.1 CHO cell

2.2.3.1a Culture

Chinese hamster ovary (CHO) cells (ATCC CRL-9618) were grown in Ham's F12 medium, supplemented with 10% (v/v) FCS. CHO cells were grown under 5% CO₂ in a humidified incubator. CHO cells were fed twice weekly. For splitting, CHO cells were washed twice with PBS before adding 5ml of 10% trypsin (trypsin versene, diluted 1:10 in PBS) and incubating for 5-10 minutes at 37°C. Cells were then encouraged to detach by gentle trituration, before seeding new flasks. CHO cells were generally split 1:10 once confluent.

2.2.3.1b Calcium phosphate transfection

The day prior to transfection, CHO cells were split 1:10 into 10cm plates. Cells were fed 4 hours before the transfection with 9ml fresh media. 10μg DNA in sterile water (prepared as in Section 2.2.1.1b) was made up to 450μl in sterile water, before adding 50μl 2.5M CaCl₂. This solution was then added dropwise to 500μl 2x HeBS, whilst vortexing in a laminar flow hood to form a fine precipitate. This mixture was then allowed to sit for 20 minutes at room temperature before adding dropwise to the CHO cells in 10cm plates, swirling to ensure even distribution. Cells were then incubated for 16 hours before washing twice with PBS and feeding with fresh media; 48 hours later, cells were split 1:25 into fresh medium containing 10μg/ml puromycin (for pEFiresα-mann transfections), or 750μg/ml G418 (for pCAGα-mann transfections). Cells were fed in this selective media every 48 hours until they attained confluence, after which time they were split 1:10 into fresh selective media and fed as before. Once cells again grew to confluence, they were trypsinised as before and harvested into PBS lysis buffer (as described in Section 2.2.3.4f) prior to analysis.

2.2.3.2 ES cell

2.2.3.2a Culture

D3 mouse ES cells (Doetschman et al., 1985) were used in this study. ES cells were grown under 10% CO₂ in a humidified incubator. ES cells were grown in DMEM supplemented with 10% (v/v) FCS, 1mM L-glutamine, 0.1mM β-mercaptoethanol (βME), and 1000 units of LIF, and generally fed daily. ES cells were split by washing
twice in PBS before adding 2ml of 50% (v/v) trypsin (trypsin/versene diluted 1:2 in PBS) and incubating at room temperature for approximately two minutes. Cells were then encouraged to detach by trituration, and harvested into 10ml tubes before 1ml of medium was added to neutralise the digestion. Cells were then counted (as per Section 2.2.3.4g below) and reseeded at a density of either 5x10^4 or 1x10^5 cells/ml into fresh plates (ie. 5x10^5 or 1x10^6 cells per 10cm dish).

2.2.3.2b Freezing/thawing ES cells

When required, ES cells were frozen by trypsinising cells and resuspending in FCS/10% (v/v) dimethyl sulphoxide (DMSO). Cells were then frozen to -70°C gradually (approximately 1 degree per minute) using an isopropanol-containing freezing container. Following overnight freezing at -70°C, cells were transferred to liquid N₂ for storage.

ES cells were revived by thawing in a water bath until just melted, followed by dropwise addition of fresh prewarmed ES cell media. Cells were then pelleted, washed again in ES cell media to remove any residual DMSO and seeded into fresh media at a density of 2x10^5 cells/ml.

2.2.3.3 Transfection of ES cells

2.2.3.3a Electroporation

ES cells were harvested by trypsinisation as described in Section 2.2.3.2a, prior to being counted, as described in Section 2.2.3.4g. 5x10^7 ES cells were transfected with 5µg DNA (either pEFIRESa-mann or pCAGα-mann, prepared as in Section 2.2.1.1b) by electroporation using a Bio-Rad Gene Pulsar at 210V and a capacitance of 960 µD.

2.2.3.3b Selection

Following transfection, ES cells were plated at a density of 5x10^4 cells/cm² (in 15cm dishes) in culture media as described in Section 2.2.3.2a. The following day, cells were fed with fresh media supplemented with 1.5µg/ml puromycin (for pEFIRESa-mann transfected ES cells) or 250µg/ml G418 (for pCAGα-mann transfected ES cells). Cells were then fed daily with selective media until well defined antibiotic-resistant colonies were visible and there were no surviving cells on the control plates (no DNA transfected). This generally took approximately 7-10 days.
2.2.3.3c Picking transfected clones

Following selection, ES cell clones were isolated using sterile glass cloning rings. These rings were placed over each colony, allowing addition of 100μl 50% (v/v) trypsin, incubation for 2 minutes, then trituration to isolate cells from each clone. Isolated clones were seeded into 24 well plates and fed daily (with selective media as described in Section 2.2.3.3b), before expansion up to six well plates and finally 10cm plates, before freezing (as described in Section 2.2.3.2b) or harvesting for further analysis.

2.2.3.4 ES cell differentiation

2.2.3.4a MEDII production

All MEDII used in this study was produced and supplied by BresaGen Pty. Ltd., as described in Rathjen et al. (1999). Briefly, HepG2 cells (Knowles et al., 1980) were trypsinised to a single cell suspension and seeded at 5x10^4 cells/cm² in DMEM supplemented with 10% FCS and 1mM L-glutamine to give a ratio of 1.75x10^5 cells/ml medium. Conditioned medium was collected after four days culture, sterilised by filtration through a 0.22μm membrane and supplemented with 0.1mM β-mercaptoethanol (β-ME) before use. MEDII was stored at 4°C for one to two weeks, or frozen at −70°C prior to use.

2.2.3.4b Generation of EBMs

All cell aggregates (embryoid bodies grown in MEDII, or EBMs) were formed by seeding 1x10^6 ES cells (as a single cell suspension) into 10ml of 50% (v/v) MEDII (DMEM with 10% FCS, 1mM L-glutamine and 0.1mM β-ME, supplemented with 50% (v/v) MEDII produced as described in Section 2.2.3.4a) in sterile bacterial Petri dishes (to prevent attachment). EBMs were formed and grown under 5% CO₂ in a humidified incubator. EBMs were divided 1:2 (into two new dishes with fresh media, per plate) on days two and four of the protocol. EBM-4 cells were harvested on day four, for analysis or transplantation.

For the generation of EBM-9 and EBM-12 cells, fresh medium was added on days five and six of the protocol. On day seven, EBMs were transferred to serum-free medium (50% (v/v) DMEM, 50% (v/v) Ham’s F12; supplemented with 1x insulin-
transferrin-sodium selenite (ITSS) supplement and 10ng/ml FGF-2). EBM-9 cells were harvested two days later for analysis or transplantation. For generation of EBM-12 cells, fresh serum-free medium was added on day nine and day 11 of the protocol, before harvesting of EBM-12s the following day.

2.2.3.4c Serum starvation protocol/seeding of EBM

Later stage EBM populations (such as EBM-21) were generated by seeding EBM onto an adherent growth substrate on day nine of the protocol. This was done by transferring EBM to gelatin-treated tissue culture-grade plasticware (Falcon), and feeding with serum-free medium as described above. EBM were again fed on days nine, 11, 13, 15 and 18 of the protocol. On day 21, media was harvested by aspiration, and EBM/cell outgrowths were harvested by trypsinisation (involving incubation in 50% trypsin for five minutes, followed by triturating to detach and harvest cells) prior to harvesting in PBS lysis buffer as described in Section 2.2.3.4d.

2.2.3.4d Seeding of EBM on coated Thermanox coverslips

Thermanox coverslips were first treated by overnight incubation (in 24 well plates) in a 20μg/ml solution of polyornithine at 37°C. After rinsing three times with sterile water, coverslips were then incubated overnight in a 1μg/ml solution of laminin at 37°C, before rinsing three times in sterile water and storing in PBS prior to use.

At the time of seeding (EBM-9), EBM (approximately two to four bodies per well) were transferred into serum-free media as described above, in 24 well dishes containing Thermanox coverslips. EBM were then cultured as described above until EBM-21. At this point, media was gently aspirated (except for the last 200μl, such that processes were not dislodged) before gently washing twice in PBS and incubating in 4% paraformaldehyde in PBS for 10 minutes. Coverslips were again washed twice in PBS before storing at 4°C in PBS/0.1% (w/v) NaAz prior to processing with immunofluorescent protocols. Note: for Oct4 immunofluorescence, fixation was carried out in 4% paraformaldehyde in 70% (v/v) ethanol for 10 minutes, prior to washing and storage in 70% ethanol.

2.2.3.4e Preparation of single cell suspension for implants

EBM-4 embryoid bodies were prepared as described in 2.2.3.4b. On day four,
EBMs were collected in a 50ml Falcon tube and allowed to settle by gravity. The supernatant was then gently aspirated, before suspending EBMs in 8ml PBS (in a 10ml tube) and centrifuging for two minutes at 800g. The supernatant was then removed and EBMs were resuspended in 2ml trypsin and incubated for five minutes at 37°C. EBMs were then triturated through three flame-drawn glass pipettes of progressively finer diameter, 10 times through each. Four millilitres of 50% (v/v) MEDII (as described in 2.2.3.4b) was then added to block trypsin digestion before centrifuging cells at 800g. The supernatant was again removed, cells were resuspended in 2ml DMEM (with no supplements) and allowed to stand for five minutes, such that any clumps of cells would settle to the bottom of the tube. 550µl of cell suspension was then drawn from approximately 1ml below the surface of the medium, before performing a cell count using a trypan blue exclusion assay (using 50µl for this cell count). Additional DMEM was then added to make up cells to a concentration of 1.5x10⁶ cells/ml. If a more concentrated cell suspension was required (such as 10⁸/ml), or cells were too dilute, cells were pelleted at 800g before resuspension in the appropriate volume. Cells were stored on ice prior to implantation or analysis.

2.2.3.4f Harvesting/cell lysis for enzyme assays

CHO cells, ES cells, seeded EBMs or skin fibroblasts were trypsinised as described in Sections 2.2.3.1a, 2.2.3.2a, 2.2.3.4c, and 2.2.3.5, respectively. EBMs were harvested by collection into a 10ml centrifuge tube. Following harvest, cells were centrifuged at 800g, washed once in PBS and centrifuged again at 800g. Pellets were then resuspended in 500µl PBS lysis buffer (per 10cm plate or flask) and triturated to mix. Lysed cells were incubated at 4°C overnight before microcentrifugation and storage at -20°C prior to analysis.

2.2.3.4g Trypan blue exclusion assay

Trypan blue was added to the cell suspension to be counted at a ratio of 1:1. Ten microlitres of this mixture was then applied to a haemocytometer (Sigma), before counting cell numbers.

2.2.3.5a Characterisation- cross correction experiment

Both α-mannosidosis (SF3949) and normal (SF4858 and SF4976) human skin
fibroblasts were used for cross correction studies. Fibroblasts were maintained and expanded in DMEM/10% FCS until confluent, grown under 5% CO₂ in a humidified incubator. Cells were then trypsinised by addition of 5ml 50% trypsin, before being split 1:2 into fresh media. The following day, conditioned media samples were applied.

Conditioned media was obtained from populations of seeded EBMs (as described in Section 2.2.3.4c), harvested at day 21 of the differentiation protocol (such that media had been conditioned for 72 hours). Conditioned media was filtered using a 0.22μm filter prior to further use. α-Mannosidase activity was measured in media samples, and further diluted in unconditioned serum-free media if required. Mannose-6-phosphate was added to conditioned media at a concentration of 5mM, where indicated. The legend for Figure 4.15 provides details of the cells used, α-mannosidase activity and M₆P added for various samples in this experiment. Skin fibroblasts were washed twice with PBS before application of conditioned media samples. Fibroblasts were left for 48 or 96 hours before harvesting by trypsinisation (as described above) into PBS lysis buffer (as per Section 2.2.3.4f).

2.2.3.5b Quantitation of oligosaccharides using mass spectrometry

Following application of conditioned media and harvest of fibroblasts, oligosaccharides Man₂NAcGluc and Man₃NAcGluc were quantitated. Total cell protein was first quantitated in harvested samples, in triplicate. Derivatisation of oligosaccharides was carried out as per the method of Pitt and Gorman (1997), with modifications.

Briefly, cell lysates containing 20μg of total cell protein were lyophilised, prior to the addition of 50μl derivatising solution per sample (250mM 1-phenyl-3-methyl-5-pyrazolone (PMP) in 0.4M ammonia) and incubation at 70°C for 1 hour. The solution was then acidified with 50μl 0.8M formic acid before adding 400μl milli-Q water to each sample. Excess PMP was then removed by the addition of 500μl chloroform (CHCl₃), prior to vigorous shaking. The solution was then centrifuged, the aqueous layer removed, and chloroform extractions repeated twice more. The final aqueous layer was applied to a 25mg C18 ISOLUTE solid-phase extraction cartridge, (equilibrated by sequential addition of 1ml acetonitrile, 1ml 50% (v/v) acetonitrile/0.025% (v/v) formic acid, and 1ml milli-Q H₂O). After the samples had completely entered the column beds, the columns were washed by addition of 500μl
milli-Q H₂O, followed by two washes of 1ml milli-Q H₂O, after which the columns were dried under vacuum for 15 minutes. Chloroform (1ml) was then added twice to ensure that all remaining PMP had been extracted, followed by drying the columns under vacuum for 15 minutes. Elution was carried out with three 200μl elutions of 50% (v/v) acetonitrile/0.025% (v/v) formic acid.

Methyl lactose (as an internal standard) was added to each sample prior to derivatisation and was present in the final preparations at a concentration of 1μM. Derivatised samples were stored at −20°C prior to analysis.

All mass spectrometry was carried out by Dr. Maria Fuller (WCH) using electrospray ionisation-tandem mass spectrometry on a PE SCiEX API 365 triple quadrupole mass spectrometer (Foster City, CA, USA). Oligosaccharides Man₂NAcGluc and Man₃NAcGluc were analysed by molecular ion monitoring of the M+1 ion and the m/z ion 175 resulting from the fragmentation of the PMP moiety. The relative amount of each oligosaccharide was calculated based on the signal response relative to the signal from the methyl lactose internal standard.

2.2.3.6 Demethylation experiment

Seeded EBMs were generated as described in Section 2.2.3.4c, with the addition of the methyltransferase inhibitor 5-azadeoxycytidine (5-azadC), 0.15μM on days 13 and 15 of the differentiation protocol (cells were fed with normal serum-free medium on day 18 to allow them to recover). On day 21, seeded cell populations were harvested into PBS lysis buffer as described in Section 2.2.3.4c and 2.2.3.4f, before analysis.

2.2.4 Animal work

All animal studies in this thesis were reviewed and approved by the Women’s and Children’s Hospital and University of Adelaide Animal Ethics Committees.

2.2.4.1 Genotyping of guinea pigs

α-Mannosidosis in the guinea pig is caused by a naturally occurring missense mutation leading to a substitution of arginine by tryptophan at amino acid position 227 (R227W, as shown in Figure 2.1, Berg and Hopwood, 2001). As such, a PCR-based approach was established to genotype the guinea pigs from blood spots (Berg and Hopwood, 2001).
**Figure 2.1: Genotyping of α-mannosidosis guinea pigs**

A: Gel photo of PCR results showing heterozygous (carrier), affected (α-mannosidosis) and normal guinea pigs. B: Diagram showing the mutation in the α-mannosidosis gene, resulting in α-mannosidosis. Gel photo and diagram provided by Dr. Thomas Berg.
**Figure 2.1**

**A**

Undigested PCR product  
Digested PCR products

<table>
<thead>
<tr>
<th>Carriers</th>
<th>Affected</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5</td>
<td>6 7 8 9 10</td>
<td>11 12 13</td>
</tr>
</tbody>
</table>

| 23 45 67 | 8 9 10 |

| GAAGAGAC | ( | K | AAGAGAC | ( |

**B**

Normal: AACTGGTGTTGAGAGAGAGAC  
Affected: AACTGGTGCGGAAGAGAGAC

<table>
<thead>
<tr>
<th>L</th>
<th>V</th>
<th>R</th>
<th>K</th>
<th>K</th>
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<tbody>
<tr>
<td>W</td>
<td>K</td>
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</table>

| 400 | 300 | 200 | 100 | 165 | 226 | 241 | 61 |
Following swabbing with ethanol, blood was collected on a Guthrie card by pricking the ear of newborn guinea pigs with a sharp needle. The ear was then gently squeezed to provide drops of blood for application to the card. Guthrie cards were allowed to dry for at least one day prior to inclusion in the PCR-based genotyping protocol. A blood smear was also prepared and stained with May-Grunwald Giemsa stain (WCH Core Laboratory performed this staining). All blood smears were visualised by Dr. Dyane Arrol to identify α-mannosidosis guinea pigs by the presence of abnormal clear vacuolation within the lymphocytes. All suspected α-mannosidosis guinea pigs had their genotype confirmed using the PCR-based protocol detailed below.

A 3mm disc was punched from the Guthrie card using a hole puncher and blood extracted from the Guthrie card using a Bio-Rad extraction kit, as per the manufacturer’s recommendations. All buffers were supplied with the kit. Briefly, this protocol involved incubation of the blood spot in 1ml sterile water at room temperature for 10 minutes twice, followed by incubation at 65°C in 100µl extraction reagent twice, before incubation in 100µl wash buffer twice at room temperature. Blood spots were then washed in 200µl of 70% (v/v) ethanol before air drying prior to the reaction.

Polymerase chain reaction was set up using primers as follows, from a previously established method (Berg and Hopwood, 2001):

mutR: AGTGAAGAGGTCCGCGCAG
4AF: ACTTTCTACCTGTGCTCTGAC

Primers were added from a stock of 150µM (1µl per reaction, in 100µl reactions) to provide a final concentration of 0.15µM. dNTPs were added to a final concentration of 200µM, along with 5U of Taq polymerase and PCR buffer at 1x concentration (Boehringer Mannheim). PCR was carried out for 42 cycles, each consisting of 94°C for 30 seconds, 52°C for 45 seconds and 72°C for 40 seconds.

Confirmation of amplification was first carried out by resolution of an aliquot of reaction product (mixed with 10x loading buffer) using agarose gel electrophoresis with a 2% (w/v) agarose gel. Reaction products were then restricted with AcI (17.5µl reaction product, 2µl NE Buffer 3, and 0.5µl AcI per digest) before resolution of products with a 2.5% (w/v) agarose gel (2% (w/v) DNA grade agarose, 0.5% (w/v) HiRes agarose). Figure 2.1A shows a photograph of a gel containing products from
normal, heterozygous and α-mannosidosis guinea pigs (Berg and Hopwood, 2001).

Heterozygous guinea pigs have not been shown to display pathology (Dr. Allison Crawley, personal communication) and thus data obtained for these animals was collapsed with normal animals for analyses. “Normal” phenotype in this thesis henceforth refers to either normal or heterozygous genotype.

2.2.4.2 Enriched environment

All guinea pigs were housed in pens of approximately five to eight animals, in an enriched environment consisting of housing boxes, PVC pipes, and a varied diet of pellets, lettuce, carrot and apple.

2.2.4.3 Stereotaxic surgery/recovery

Guinea pigs were pretreated with 0.05mg/kg atropine (subcutaneous injection) approximately 30 minutes before surgery. A moderate level of anaesthesia was induced with isoflurane, before shaving the top of the head and clipping the hair around the ears. Deeper anaesthesia was then induced (confirmed by non-responsiveness to toe pinch) and maintained with isoflurane, to effect. The animal was then fitted into the stereotaxic frame, such that both ear bars were secure and the surface of the skull was horizontal. Anaesthesia was maintained by the use of a face mask constructed from the finger of a latex glove (enclosing the tooth bars of the stereotaxic frame), with a tube delivering isoflurane/oxygen to the animal. The animal was kept warm during surgery by placing a heated wheat bag under the body. Both eyes of the animals were treated with Lacrilube to ensure they remained moist during the procedure. An Olympus SZ40 dissecting microscope and a Fibreoptic Lightguides Microlight 150 was used to improve visibility of all surgical procedures.

The surface of the animal’s head was swabbed with ethanol, and one dose (105μl) of lignocaine was applied to the site using the applicator pump provided and left for two minutes. An incision approximately 3cm long was then made along the surface of the head along the midline, starting approximately 1cm from the nose, to expose the skull and cranial suture lines lambda and bregma. Further incisions were made through the membranes on the skull surface, allowing them to be cleared away with a sterile cotton bud. Throughout the procedure, the site was flushed regularly with sterile saline to ensure that the skull remained moist. The head position of the animal
was then adjusted until lambda and bregma were both level, before positioning the injection arm above the appropriate injection site. The injection coordinates used for these studies (with respect to bregma and the midline) were as follows:

**Striatum:**
- P: 0.9mm
- L: ±2.7mm
- V: 5.0mm

**Dentate gyrus:**
- P: 2.8mm
- L: ±1.0mm
- V: 4.0mm

Marks were made on the surface of the skull using a black marker and 1mm holes were drilled using a sterile hand drill. Drilling proceeded slowly, such that the dura underlying the skull were not penetrated during the procedure. Fine forceps were used to clear the dura of any bone fragments, before flushing with sterile saline and piercing the dura with a 27G needle. The injection needles (connected to the syringe pump with plastic tubing) were lowered to the surface of the dura, before slowly advancing the needles to the required depth, over the course of approximately three minutes. The syringe pump was then used to infuse 1µl of cells (or vehicle) per side over the next three minutes. Needles were left stationary for a further three minutes, before being raised over the following 3-4 minutes.

The animal was removed from the frame, and the incision was sutured. Flunixin (2.5mg/kg, intramuscular injection) was administered, along with 3ml/100g warmed dextrose/saline (4% (w/v) dextrose, 0.18% (w/v) saline, subcutaneous injection). Animals were then placed in a dark box on top of a hot water bottle until they had regained consciousness and were able to walk, before returning them to their pen. Animals were closely monitored following surgery and then daily thereafter. Skin infections of the surgery site were treated by irrigation with a dilute solution of Betadine antiseptic, and twice daily with Bactrim (0.625ml/kg, administered orally). Sutures were removed after one week.

**2.2.4.4 Cyclosporin treatment/monitoring**

Animals were treated with Neoral (Cyclosporin A (CSA), 50mg/ml), administered orally. Neoral solution was diluted 1 in 5 with apple juice (to provide a
solution of 10mg/ml CSA), before mixing thoroughly to create an emulsion. Guinea pigs were treated with 20mg/kg/day CSA (commencing the day before surgery) for 14 days, then 10mg/kg/day orally until sacrifice. Whole blood CSA trough levels were measured by collection of >100µl of blood from ear prick, into EDTA tubes, then measured by the WCH Core Laboratory, using the Abbott monoclonal FPIA method on an AXSYM analyser. CSA levels were measured one week following onset of treatment. All animals were weighed daily and blood samples were collected from animals observed to have a notable reduced growth rate (relative to other CSA treated animals in the cohort). Of these animals, those with whole blood trough CSA levels above 200µg/L had their administered dose halved to 5mg/kg daily, because of the negative effect on growth observed when CSA was above this level. These animals were then retested approximately seven days following the dosage change.

2.2.4.5 Morris water maze
Specific methods for the Morris water maze test are described in Section 3.1.1.

2.2.4.6 Gait analysis
Specific methods for Gait analysis are described in Section 3.2.1.

2.2.4.7 Neurological examination
Specific methods for the neurological examinations are described in Section 3.3.1.

2.2.4.8 Euthanasia
Animals to be euthanased were pre-anaesthetised with 12mg/kg Xylazine and 30mg/kg Ketamine (subcutaneous injection) 30 minutes before the procedure began. Isofluorane was then used to ensure a very deep level of anaesthesia (confirmed by non-responsiveness to a toe pinch). An incision was then made along the midline of the abdomen and the skin was separated away from the ribcage by blunt dissection. The end of the sternum was then lifted, such that an incision could be made under the ribcage, exposing the diaphragm. The diaphragm was cut from the midline laterally, before cutting the ribs (on both sides, away from the sternum) up to the shoulders, after which the separated ribcage was clamped, exposing the heart. The descending vena
cava was then clamped before perfusion began. Perfusion was carried out into the left ventricle, and a 19G needle was inserted into the right ventricle to provide an exit. Blood was collected from this needle at this point, when required. Chilled PBS was perfused until almost clear (approximately 200ml), followed by 200ml of 4% (w/v) paraformaldehyde in PBS (chilled).

Euthanasia in animals not required for histology was carried out with a similar injectable anaesthesia protocol as described above, followed by injection of 60mg/kg lethabarb (pentobarbitone sodium) directly into the heart.

2.2.4.9 Analysis of mouse-specific antibodies in guinea pig serum

EBM-4 cells were collected and prepared as a single cell suspension as described in 2.2.3.4c. Cells were resuspended in PBS/0.5% (v/v) heat inactivated FCS (provided by Chris Boulter, WCH) at a concentration of 1x10⁶ cells in 50µl (2x10⁷ cells/ml). Fifty microlitres of guinea pig serum (collected at euthanasia by cardiac puncture and diluted 1:8 in IsotonII) from each animal to be analysed was added to each cell sample, swirled gently, and incubated at room temperature for 30 minutes. Cells were then centrifuged at 560g and washed twice in IsotonII, before resuspension in biotinylated donkey α-guinea pig IgG, (diluted 1:500 in IsotonII) and incubated for 30 minutes at room temperature. Cells were then again centrifuged at 560g and washed twice in IsotonII, before resuspension in Streptavidin conjugated Cy5 (diluted 1:500 In IsotonII), and incubated for 30 minutes at room temperature. Following a final two washes in IsotonII, cells were fixed by resuspension in a solution of 0.5% formaldehyde in PBS, prior to flow cytometry.

Gating conditions were set using forward and side scatter characteristics to identify viable cells, excluding debris and non-viable cells. Gating conditions were set by Dr. Greg Hodge, WCH. Data was collected on 1x10⁴ cells on a Becton Dickinson FACScalibur and analysis of fluorescence in this cell population was performed using CellQuest 3.1. Gated cells were analysed for median fluorescence intensity (MFI), against a background set such that control cells (no added guinea pig serum) had a MFI of 2%.
2.2.5 Histology

2.2.5.1 Embedding of brains

2.2.5.1a Paraffin method

All paraffin embedding was carried out by staff in the WCH Department of Histopathology according to standard protocols. Sections were cut at a thickness of 5μm and stained with haematoxylin/eosin, also according to standard protocols (Department of Histopathology, WCH).

2.2.5.1b Polyethylene glycol (PEG) embedding

Following euthanasia and perfusion with paraformaldehyde as described in 2.2.4.8, brains were removed and post-fixed in 4% (w/v) paraformaldehyde/0.1% (w/v) sodium azide (NaAz) overnight at 4°C. Whole brains were then washed twice in PBS/0.1% (w/v) NaAz, before storage in the same solution at 4°C, prior to embedding.

Brains were cut using a large scalpel blade into 5mm sagittal slices as shown in Figure 2.2, by first removing the cerebellum (cut 1), before cutting approximately 5mm either side of the midline (cuts 2 and 3) and then finally cutting along the midline (cut 4).

Brain slices were dehydrated by sequential incubation in ethanol solutions (50%, 70% and 100%) for 45 minutes each, at room temperature, before incubation in DMSO for approximately 60 minutes at room temperature (with occasional gentle swirling). Slices were then again incubated in 100% ethanol for five minute and 30 minute sequential incubations, before overnight incubation in PEG 400 at room temperature. Slices were then transferred to a mixture of PEG 1450/PEG 1000 (3:1 ratio) at 50°C under vacuum for two to three hours (until they had sunk) before embedding in the same PEG mixture. Both left and right slices were processed and embedded midline side down (such that sectioning could proceed laterally out from the midline).

Blocks were stored in airtight containers containing desiccant (silica beads) prior to sectioning. Sections were cut at 20μm and stored free floating in PBS/0.1% (w/v) NaAz in 24 well trays at 4°C prior to analysis.

2.2.5.1c Epoxy resin embedding

Immediately following euthanasia and perfusion as described in 2.2.4.8,
samples of hippocampus to be embedded in resin were further dissected from whole brains. Hippocampus samples were taken from the left side of the brain, such that the right side could be embedded in PEG as described above.

Sagittal sections were cut approximately 1mm thick (taken next to midline) using a scalpel blade, before further dissecting out samples of hippocampus, such that they were approximately 1.5-2mm square by approximately 1mm deep. These tissue samples were then incubated overnight in 4% (w/v) paraformaldehyde/ 1.25% (v/v) glutaraldehyde/ 4% (w/v) sucrose in PBS, pH 7.2, at 4°C. Tissue samples were then washed twice for 10 minutes in PBS/4% (w/v) sucrose, before post-fixing in 2% (v/v) osmium tetroxide (OsO₄) for 1 hour. Tissue was then dehydrated by sequential incubation in 70% (v/v), 90%, 95% and then 100% ethanol; each ethanol concentration involved three 20 minute incubations. Tissue samples were then incubated in propylene oxide for 60 minutes. All incubations were carried out at room temperature, with gentle agitation.
Figure 2.2A: Diagram showing sectioning of guinea pig brains prior to PEG embedding
Lines indicated by numbers 1, 2, 3 and 4 show the sequential cuts that were made to perfused guinea pig brains *in toto* prior to embedding.

Figure 2.2B: Diagram of a sagittal section of guinea pig brain
Injection sites (dentate gyrus) are indicated, along with surrounding structures.
Figure 2.2

A

Lateral Olfactory
NOSE

Injection sites

Cortex

Hippocampus

Cerebellum

5mm 5mm
L R

B

Cortex

Dentate gyrus (injection site)

Lateral ventricle
Olfactory bulb

NOSE

Hippocampus

Cerebellum

TAIL
Resin infiltration of tissue samples then commenced by incubation in a 1:1 mixture of propylene oxide and epoxy resin overnight at room temperature (with gentle agitation), before three sequential incubations in 100% epoxy resin, for approximately eight hours each. Samples were then embedded in freshly made epoxy resin, which was polymerised at 70°C under vacuum for at least 24 hours to harden.

All sections were cut at a thickness of 0.5μm by Lyn Waterhouse (Adelaide Microscopy, University of Adelaide) and stained with toluidine blue prior to analysis.

2.2.5.2 Mouse microsatellite in situ hybridisation

Mouse cells implanted into guinea pig brains were detectable using a biotinylated oligonucleotide specific for the mouse microsatellite sequence (Brüstle et al. 1995). This sequence is as follows:

GGACCTGGAATATGGCGAGAAAACTGAAAAATCACGGAAAATGAGAAATACACA
CTTTAGGACGTGAAATATGGCGAGAAAACTG

Brain sections cut in 2.2.5.1b above were washed in sterile milli-Q water in 48 well trays (two free-floating sections per well) for at least 10 minutes prior to commencing the protocol. Sections were then incubated at 37°C in a 200μl solution of 0.05mg/ml pepsin/0.012M HCl, for 30 minutes in Eppendorf tubes, before stopping the digestion by immediately placing the tubes on ice and adding 500μl of 5xSSC. Sections were then returned to 48 well trays, and washed in 5xSSC for at least 10 minutes.

Oligonucleotide was added to the hybridisation buffer (made fresh before each hybridisation) at a concentration of 5μg/ml (from a stock oligonucleotide solution of 0.5μg/μl) to make the hybridisation solution. Sterile flat-bottomed glass tubes were used for the hybridisation procedure, with 200μl of hybridisation solution used per tube. Following transfer of the brain sections to the hybridisation solution, tubes were sealed and placed at 95°C for five minutes, before allowing them to cool at room temperature for three minutes. Hybridisation was then carried out at 45°C for 90 minutes. Sections were then removed and washed three times in 2x SSC containing Brij 35 (2.5μl/ml) for at least 10 minutes, before washing in 0.1x SSC (with 2.5μl/ml Brij 35) for 10 minutes. Sections were then equilibrated in PBS/0.3% (v/v) Triton X-100 for five minutes, then incubated overnight in Streptavidin-Cy3 diluted 1:600 in
PBS/0.3% (v/v) Triton X-100 at room temperature. Plates were wrapped in aluminium foil for this incubation, to avoid photo-bleaching of the chromophore (Cy3). Sections were then washed three times in PBS/0.3% (v/v) Triton X-100 for at least 10 minutes each time, before either mounting for visualisation or processing with further immunofluorescence protocols.

2.2.5.3 Immunofluorescence protocols for lineage analysis

Following in situ hybridisation protocols described in Section 2.2.5.2, tissue sections were further processed using immunofluorescence protocols for various phenotypic markers. Sections were processed in 48 well trays, using 200µl of solution per well.

Samples were first washed three times in PBS/0.3% Triton X-100 (v/v) for 10 minutes, before blocking in PBS/0.3% (v/v) Triton X-100/10% (v/v) normal donkey serum (NDS) for 30 minutes. Samples were then incubated in primary antisera (diluted in PBS/0.3% (v/v) Triton X-100 by a factor specified below) overnight at room temperature, before washing three times in PBS/0.3% (v/v) Triton X-100 for 10 minutes each. Samples were then incubated in secondary antisera (diluted in PBS/0.3% (v/v) Triton X-100 by a factor specified below) overnight at room temperature, wrapped in foil to prevent photobleaching. Samples were then washed three times in PBS/0.3% (v/v) Triton X-100 for 10 minutes before mounting in buffered glycerol (supplied by BresaGen Pty. Ltd.). All protocols for the use of primary and secondary antisera were optimised by Dr. Adrian Meedeniya of BresaGen Pty. Ltd., providing the working concentrations for antibodies listed below.

EBMs seeded on coverslips (as detailed in Section 2.2.3.4d) were processed in a similar fashion, with the addition of 10 minute incubation in DMSO following the first three washes in PBS/0.3% (v/v) Triton X-100. This was followed by three washes in PBS/0.3% (v/v) Triton X-100 before blocking with PBS/0.3% (v/v) Triton X-100/10% (v/v) NDS.
Primary antisera:

- Rat α-mouse *Nestin* 1:500
- Rabbit α-NF200 1:500
- Mouse α-TH 1:500
- Rabbit α-GFAP 1:200
- Goat α-Oct4 1:1000
- Mouse α-SMA 1:2000
- Mouse α-MAP2 1:1000

Secondary antisera:

- Donkey α-mouse CY3 1:300
- Sheep α-mouse FITC 1:300
- Donkey α-rabbit FITC 1:100

2.2.5.4 Light microscopy

Light microscopy was carried out using an Olympus BX51 with attached Olympus DP12 digital camera.

2.2.5.5 Fluorescence microscopy: epifluorescence

Fluorescence microscopy was carried out using an Olympus AX70 fitted with epifluorescence and a video camera connected to a PC computer. For Cy3, a Chroma 31002 filter block (beam splitter: 565nm, excitation filter: 515-50nm, barrier filter: 575-615nm); for FITC, a Chroma 31002 filter block (beam splitter: 505nm, excitation filter: 465-95nm, barrier filter: 515-55nm) and for DAPI, a Chroma 41008 filter block (beam splitter: 660nm, excitation filter: 590-650nm, barrier filter: 665-740nm) were used.

2.2.5.6 Fluorescence microscopy: confocal

All confocal image acquisition was carried out by Dr. Grant Hennig (Centre for Neuroscience, Flinders University of South Australia) or Dr. Meredith Wallwork (Adelaide Microscopy, University of Adelaide). The microscope used was an Olympus AX70 with a Biorad 1024 (Cambridge, UK) confocal head. Excitation and emission wavelengths for Cy3 and FITC were as described in Section 2.2.5.5 (no filter was available for DAPI on this microscope). Confocal digital image processing was carried out by Dr. Grant Hennig (Centre for Neuroscience, Flinders University of South Australia).
Australia) using a modified (incorporating macros written by Dr. Grant Hennig) version of NIH image (Bethesda, MD, USA).

2.2.6 Statistical analysis

All statistical analysis was performed by Craig Hirte and Janine Jones, Public Health Research Unit, WCH.

Morris water maze:

The time taken for each animal to locate the platform was considered as the dependent variable in these analyses. A repeated measures analysis was used to assess differences in time taken over days, between groups (ie. normal and α-mannosidosis) and trials (1-4). Each assessment period (acquisition and reversal) was considered separately. The analysis was performed with SPSS v10.

Analyses based on the area under the curve-method (Matthews et al., 1990) were used in all comparisons of whether animals exhibited each particular behaviour for each day (the four trials of each day were combined for these analyses). Each assessment period (acquisition and reversal) was considered separately. The analysis was performed with SPSS v10.

Probe phase data was analysed using a two-tailed student t-test, comparing the time spent in a particular quadrant for each group, and the time spent in individual quadrants, within groups. The analysis was performed with SPSS v10.

Other statistical analyses:

Gait analysis data was analysed using a two-tailed student t-test for comparisons between treatment groups for each variable at each time. MFI observed for serum-incubated mouse cells (described in Section 2.2.4.10) was analysed using a two-tailed student t-test in a similar fashion. These analyses were also performed with SPSS v10.
Chapter 3:

Behavioural characterisation of the α-mannosidosis guinea pig
3.0 Introduction

Neurological pathology in LSD (including α-mannosidosis) manifests in a range of different clinical symptoms in both patients and animal models. Some of the more complex aspects of this pathology include cognitive deficits (including memory and learning difficulties), ataxia, and a range of other symptoms. Accordingly, this thesis has involved behavioural characterisation of the α-mannosidosis guinea pig model by establishing the Morris water maze test, as well as further characterising pathology using gait analysis and a multiple component neurological examination.

3.1 Morris water maze

A number of maze procedures have been used to assess cognitive function in animals, including radial arm mazes, T and Y mazes, and the Morris water maze. The Morris water maze was originally established for use in rodents as a behavioural test to assess spatial learning ability (Morris, 1984). The maze consists of a circular pool filled with water, containing a hidden platform submerged just beneath the surface. In the Morris water maze, the relatively low temperature of the water (21°C to 24°C), along with the unfamiliar environment provide an incentive to escape the pool. This escape takes the form of mounting the hidden platform, allowing the animal to raise itself out of the water and stop swimming. The lack of local cues in water, for example scent cues used in some other tests (Hodges, 1996) provides a sufficiently difficult test that can be used to sensitively detect cognitive differences between different groups of animals.

The Morris water maze has been used extensively with mice, but has only recently been shown to be of use for testing guinea pigs (Dringenberg et al., 2001). This test has been used successfully to quantitate memory and learning impairments in LSD mouse models such as MPS VII (Chang et al., 1993) and MPS IIIA (Gliddon and Hopwood, 2004), two other LSD with CNS pathology. Due to the similarities in the pathogenesis of neurological disease in MPS VII, MPS IIIA and α-mannosidosis, we hypothesise that there may be deficits in memory and learning in α-mannosidosis guinea pigs which could also be quantitated using the Morris water maze test. The protocol used in the present study has been modified from the study published by Dringenberg et al. (2001), to minimise the level of stress in guinea pigs during testing.
3.1.1 Specific methods:
3.1.1.1 Equipment:

3.1.1.1a The pool

An inflatable wading pool with an internal diameter of 180cm, 45cm high, divided into four quadrants (See Figure 3.1) was used for all tests. The pool was filled 30cm deep with water, made opaque through the addition of 250ml of non-toxic, water-based white paint (Wattyl), such that the platform could not be seen by the animals to be tested (see Figure 3.2). The water was cold enough to provide a negative stimulus (along with the unfamiliar environment of the water itself) such that the animals would search for an escape, but not so cold as to influence the animal’s physical ability to perform the test. For this reason, a water temperature between 21°C and 24°C was used for guinea pigs, similar to that reported for previously published studies (23-24°C was used by Dringenberg and others in 2001). The water temperature was regulated by addition of either ice or hot water following the filling of the pool. For this reason, a wider temperature range resulted than in the previous study.

3.1.1.1b The platform

The platform was constructed from clear Perspex by Women’s and Children’s Hospital Building Services. The top of the platform was 20cm in diameter, while the base was 30cm in diameter. The platform was 29cm high. This platform was smaller than the 30cm diameter platform used for previously published studies (Dringenberg et al., 2001). Coarse white nylon mesh was glued to the top of the platform, to provide grip for the animals while climbing onto and standing on the platform (see Figure 3.3). The platform was placed 10cm from the wall of the pool, and was fully submerged (1cm below the surface of the water) such that it was not visible.

3.1.1.1c Cues

The animals navigate through the pool finding the platform based on cues outside the pool itself. These cues consist largely of visual cues within the room, which was set up as shown in Figure 3.5. The poster shown in Figure 3.4 serves as one of these visual cues, along with the operator, the basin and the door within the room (see Figures 3.4 and 3.1). Additionally, the inside wall of the pool was covered with a
Figure 3.1: The layout of the Morris water maze room
The layout of the Morris water maze room was consistent for all testing periods. The housing boxes were outside the room, not visible to the animals during the test. The poster, operator, basin and door were the primary visual cues within the room. Q1, Q2, Q3 and Q4 are the four quadrants of the pool, and 1, 2, 3 and 4 show the cardinal points at which consecutive trials were commenced. The platform was located in Quadrant 4 (as shown) during the acquisition phase of the test. This diagram is not to scale.
Figure 3.1

housing boxes

basin

door

poster

pool

platform

Q1

Q2

Q3

Q4

operator
Figure 3.2: The pool
The inflatable pool used for the Morris water maze is filled with water and 250ml of non-toxic white paint, to make the water opaque, obscuring the platform. The lines drawn on the top of the pool divide the pool into four quadrants. These lines were not visible to the animal during the test.

Figure 3.3: The platform
The platform used is shown submerged in the pool (without the addition of white paint). The platform was weighted at the base with bricks as shown. This platform was not visible to the animals during the test.
Figure 3.4: Visual cues- the poster and the operator
The brightly coloured poster shown and the operator were the two most prominent cues animals used to navigate through the maze.

Figure 3.5: Housing boxes for the Morris water maze
Animals were housed in these boxes immediately before and after the Morris water maze trials. While in the boxes, surgical drapes were used to cover the box as shown, to provide a dark environment. The box on the left also contained a heating pad, to allow the animal to dry itself following swimming.
pattern that represented an internal visual cue (see Figures 3.2 and 3.3). To ensure reproducibility between different testing periods, all cues were set up in an identical location for each test. The same operator carried out all testing in this study and wore the same blue gown for each testing period.

3.1.1.2 Handling and general housing protocol

Animals were housed in an enriched environment as described in Section 2.2.4.2. All animals in this study (except for the six month old naïve animals tested in the first "pilot study" experiment) were weighed and treated daily with CSA (as described in Section 2.2). Additionally, during the two week period in which the Morris water maze trial was run, animals were treated with Bactrim (as per Section 2.2) as a prophylactic measure against respiratory infection during the test. The animals became quite tame and familiar with handling, and they appeared relaxed during the testing period itself. All weighing and drug administration procedures were carried out prior to testing on a given day.

The Morris water maze test was always commenced on a Saturday, such that the weekly routine of guinea pig pen cleaning fell on consistent days throughout the different trial periods. Scheduled cage cleaning (and feeding) always took place after testing was completed for that day, to ensure that the animals began the test with a minimal level of stress.

3.1.1.3 Housing of animals for the test

Initial preliminary studies involving testing of guinea pigs in the Morris water maze suggested that stress was a potentially major confounding factor in these experiments. As such, every effort was taken to ensure the animals were as relaxed as possible prior to testing. One of the measures developed to facilitate this was the use of boxes to house the animals before and after the test (as shown in Figure 3.5). This system was established in collaboration with Dr. Dyane Auclair (WCH). Before testing, each animal was taken from its pen and placed in the "pre-testing box" shown on the right of Figure 3.5, and allowed to remain there for approximately five minutes. Covered with the surgical drape as shown, this box provided a quiet and dark environment for the animal prior to testing. Immediately after the four test trials, the animal was placed in the post-testing box, which was set up in a similar fashion to the
pre-testing box, with the addition of a heating pad. This allowed the animals to dry and
groom themselves in a warm, dark, quiet environment following the test, before being
returned to their pen. Additionally, each animal was handled with a clean towel on each
day, to avoid the possibility of scent cues from other animals confounding the test.

3.1.1.4 Trials

All Morris water maze trials were carried out first thing in the morning,
following drug administration. Animals were allowed to return to their pens and rest for
approximately one hour following weighing and drug administration, such that Morris
water maze trials usually commenced between 10am and 11am.

A trial commenced by gently lowering the animal (supported with a spread hand
under its thorax and abdomen) into the water at one of the cardinal points (see Figure
3.1) facing the wall of the pool. The trial commenced at the time the animal was
released. Each trial was carried out for a maximum of 45 seconds. The time taken for
the animal to find and mount the platform was recorded using a stopwatch. Each of
four consecutive trials was commenced at cardinal points 1, 2, 3 then 4, as shown in
Figure 3.1.

If the animal was observed to vocalise during the trial period, or attempted to
climb out the sides of the pool rather than search for the platform, this was noted. If the
animal found the platform and attempted to climb onto it but was unable to (for
example, due to the angle of approach), the animal was helped onto the platform and
the time at which it first tried to mount the platform was taken as the score for that trial.
If the animal was unable to find the platform during the 45 second test period, it was
gently guided to the platform, a score of 45 seconds was recorded and the test was
counted as a fail.

Once the animal either found or was guided to the platform, it was allowed to
remain there for 15 seconds before commencing the next trial or being removed from
the pool. If the animal walked off the platform back into the pool during this 15 second
period, this was noted and the animal was placed back onto the platform for a total of
15 seconds. If the animal attempted to jump off the platform to exit the pool entirely,
this was also noted. The operator remained completely stationary during this test period
such that leaving the platform represented a rejection of the platform as an escape,
rather than a predator escape response, which could occur as the operator went to
remove the animal from the pool.

3.1.1.5 Testing protocol

The Morris water maze testing protocol employed in this study consists of four test phases: Acquisition (days 1-7), Rest (days 8-12), Probe (day 13) and Reversal (days 13-14).

3.1.1.5a Acquisition phase

Days 1-7 (inclusive) of the Morris water maze test represented the acquisition phase. The platform was placed in Quadrant 4 for each trial during this period. Each day of this period consisted of four 45 second trials, as described above.

3.1.1.5b Rest

Animals were not tested at all on days 8 to 12, but did receive CSA and Bactrim as described above. This rest period was consistent with the protocol described by Dringenberg et al., 2001.

3.1.1.5c Probe phase

On day 13, animals underwent the probe test. This involved removing the platform from the pool, and (with all other factors set up as described previously), the animal was allowed to swim around the pool for 60 seconds, starting from cardinal point 1 (see Figure 3.1). The time spent in each quadrant was recorded by use of a Walkman and cell counter. The Walkman played a metronome tone recorded to sound every second, such that every time the operator heard the tone, the appropriate button on the cell counter was pushed, corresponding to the quadrant the animal was in at that time. The operator heard this tone through an earpiece, such that it was not audible to the animal being tested.

3.1.1.5d Reversal phase

The reversal phase was also commenced on day 13, after the probe test. The reversal phase was continued on day 14 (previously published studies only included a one day reversal phase, as detailed in Dringenberg et al., 2001). Following the probe test, animals were allowed to dry and return to their pens for at least 30 minutes before
commencing this part of the test. The platform was placed in Quadrant 2 (the opposite side of the pool to the previous location) and the animals were tested as described before, with four 45 second trials per day. The time taken to find the platform in this different location was recorded for each trial.

3.1.1.6 Visible platform test
Following testing in the reversal phase on day 14, some animals (tested in trial period five, as shown in Appendix 1) underwent the visible platform test. Animals were dried and allowed to return to their pens for one hour before this test commenced. The visible platform test involved draining the pool and refilling it with water, minus the paint previously used to make it opaque. The water level was filled to approximately 5mm below the level of the platform, such that the animals could clearly see the platform whilst swimming. The platform was placed in Quadrant 3 for these trials. Four trials were carried out as described previously, with the time taken to find the platform in each trial recorded.

3.1.1.7 Data analysis
Sex differences have previously been reported in Morris water maze studies involving rats and mice (Roof and Stein, 1999; livonen et al., 2003). However, previous studies using the Morris water maze for testing of memory and learning in guinea pigs reported no significant sex differences, and thus collapsed male and female data for analysis (Dringenberg et al., 2001). Due to this report from the previous study and the logistics of the present study, data from both male and female guinea pigs were thus analysed together. Additionally, the testing of three month old naïve guinea pigs (described in Section 3.1.2.5) was carried out in five trial periods, as shown in Appendix 1. Once again, due to the logistics of the present study, data from all five trial periods were analysed together. Statistical analysis of data was carried out as described in Section 2.2.6.

3.1.2 Results: Morris water maze
3.1.2.1 Pilot study: six months naïve
To ascertain whether α-mannosidosis guinea pigs experienced memory and learning impairments, a pilot study was first carried out using a small cohort of
normal/heterozygous (n=3) and α-mannosidosis (n=3) guinea pigs, at six months of age. These animals had not previously been tested in the Morris water maze (they were “naïve”). The water maze test was carried out as per the protocol detailed above. In contrast to subsequently tested animals, guinea pigs in this group did not receive CSA, and thus they only received daily handling during the testing period (when they received Bactrim). Statistical analysis was carried out as per Section 2.2.6.

3.1.2.1a Acquisition phase: six months naïve

At the beginning of this phase, both normal and α-mannosidosis animals were slow to find the hidden platform, taking an average of 34 and 45 seconds respectively, although this represented failure in half the trials for normal animals compared to all the trials for α-mannosidosis animals. Throughout the seven day acquisition phase, both groups improved in the time taken to find the platform, with normal animals reaching an average time of 17 seconds on day 7, compared to 27 seconds for α-mannosidosis animals. Although α-mannosidosis guinea pigs generally took longer to find the platform during most of the days of this test period (Figure 3.6.1a), repeated measures analysis (see Section 2.2.6) showed that this increase was not statistically significant (p=0.184). However, a significant increase in the rate of trial failure by α-mannosidosis guinea pigs compared to normal guinea pigs (failing to find the platform within the 45 second trial period) was noted during this period (p=0.05, Figure 3.6.1b).

Additionally, α-mannosidosis animals were observed to have a significantly increased incidence both of rejecting the platform (Figure 3.6.1c, p=0.046) and attempting to climb up the sides of the pool during the trial period (Figure 3.6.1d, p=0.046). No significant differences in either vocalising (Figure 3.6.1e) or jumping out of the pool (Figure 3.6.1f) were observed between normal and α-mannosidosis guinea pigs in this period.

3.1.2.1b Reversal phase: six months naïve

In six month old naïve animals, the reversal phase of the Morris water maze test did not reveal any significant differences between normal and α-mannosidosis guinea pigs. Although the day 13 time for α-mannosidosis animals appears to be elevated (see Figure 3.6.1a), statistical analysis considering the reversal phase as a whole did not
reveal any significant differences between these groups. Additionally, the other measures analysed in this stage of the test (Figures 3.6.1c, 3.6.1d, 3.6.1e and 3.6.1f) showed no significant differences between normal and α-mannosidosis animals.

3.1.2.1c Probe test: six months naïve

The probe test was conducted on day 13, following five days of rest (no swimming). Normal animals showed a very strong preference for the target quadrant (where the platform was located during the acquisition phase), spending an average of 26 seconds (see Figure 3.6.1g) in this quadrant (random searching would theoretically result in 15 seconds spent in this quadrant). This represented a statistically significant increase (p=0.045) compared to the time spent in the other quadrants (see Figure 3.6.1h), indicating that normal animals had strong retention of the task of finding the hidden platform. This increase was also highly significant (p=0.005) when compared to the time α-mannosidosis animals spent searching the target quadrant (11 seconds, see Figure 3.6.1g), indicating that α-mannosidosis animals were significantly impaired in the cognitive abilities required to successfully perform this test. Indeed, α-mannosidosis animals showed no significant differences in the time spent in the target quadrant (p=0.096) compared to the other quadrants (see Figure 3.6.1h). Interestingly, α-mannosidosis animals did appear to display a preference for the operator quadrant (Quadrant 1) compared to the other quadrants, although this did not represent a statistically significant increase (p=0.202).
Figure 3.6.1a: Morris water maze pilot study: six month old naïve animals. 

n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. Data shown is the mean of four trials performed per animal, for each phenotype group, on each day, ±1SEM. Maximum duration of each trial was 45 seconds, after which time the animal was guided to the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.1b: Proportion of trials failed: six month old naïve animals. 

n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. Failed trials were defined as when the animal did not find the platform during the 45 second trial period. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- □ α-mannosidosis
- ▲ normal
Figure 3.6.1a

Average time (seconds)

Day

ns p=0.184
ns p=0.905

Figure 3.6.1b

Proportion

Day

* p=0.05
ns p=0.099
Figure 3.6.1c: Proportion of trials subject rejected platform: six month old naïve animals.
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. “Subject rejecting platform” was defined as when the animal, having found or having been guided to the platform, swam back into the pool instead of waiting on the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.1d: Proportion of trials subject attempted to climb out of pool: six month old naïve animals.
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. “attempting to climb out of the pool” was defined as when the animal, during the course of the 45 second trial period, was observed scratching on the sides of the pool, thus attempting to climb out of the pool, as opposed to searching for the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- □ α-mannosidosis
- ■ normal
Figure 3.6.1c

* p=0.046

ns p=1

Figure 3.6.1d

* p=0.046

ns p=1

Day

Proportion

1 2 3 4 5 6 7 13 14

1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0

Day

Proportion

1 2 3 4 5 6 7 13 14

1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0
Figure 3.6.1e: Proportion of trials subject vocalising: six month old naïve animals. 
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. Vocalising during the trial period was an indication of panic in the subject animal. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.1f: Proportion of trials subject attempted to jump out of pool: six month old naïve animals. 
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. “Attempting to jump out of the pool” was defined as when the animal, during the 15 second rest period following the trial period, jumped off the platform and out of the bounds of the pool. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.1g: Probe test (overview): six month old naïve animals.
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. Quadrant 4 was the target quadrant (where platform was located in previous trials), Quadrant 1 was where the operator was located. Statistical analysis was a t-test performed between the time spent in the target quadrant (Quadrant 4) for normal animals compared to α-mannosidosis animals, and a t-test performed between the time spent in the operator quadrant (Quadrant 1) for normal animals compared to α-mannosidosis animals. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Quadrant 1
- Quadrant 2
- Quadrant 3
- Quadrant 4
Figure 3.6.1g

![Bar chart showing average time (seconds) for normal and α-mannosidosis groups.]

- * p=0.005
- ns p=0.444
Figure 3.6.1h: Probe test (target vs. non-target quadrants): six month old naïve animals.
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Target Quadrant” was the mean time spent searching for the platform in Quadrant 4, “Non-Target Quadrants” was the mean time spent in Quadrants 1, 2 and 3. Statistical analysis was a t-test performed between the time spent in target quadrant vs. time spent in non-target quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Target Quadrant
- Non-Target Quadrants

Figure 3.6.1i: Probe test (operator vs. non-operator quadrants): six month old naïve animals.
n=3 α-mannosidosis guinea pigs and n=3 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Operator Quadrant” was the mean time spent searching for the platform in Quadrant 1, the quadrant closest to the operator. “Non-Operator Quadrants” was the mean time spent in Quadrants 2, 3 and 4. Statistical analysis was a t-test performed between the time spent in the operator quadrant vs. time spent in non-operator quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Operator Quadrant
- Non-Operator Quadrants
Figure 3.6.1h

![Graph showing time (seconds) for normal and α-mannosidosis groups.](image)

- p = 0.045 for normal group.
- ns p = 0.096 for α-mannosidosis group.

Figure 3.6.1i

![Graph showing time (seconds) for normal and α-mannosidosis groups.](image)

- ns p = 0.900 for normal group.
- ns p = 0.202 for α-mannosidosis group.
Discussion: Pilot study, six month naïve animals

The combined data from animals tested at six months of age indicate that there are cognitive deficits that manifest in α-mannosidosis guinea pigs, and that these can be measured using the Morris water maze protocol. Although previous studies have shown that the Morris water maze test can be effectively performed by guinea pigs at approximately one month of age (Dringenberg et al., 2001), this test has not previously been published for use in older guinea pigs. The results of the preliminary experiments carried out in the present study suggested further investigation was warranted using this test to characterise cognitive deficits and determine the age at which they develop.

In these tests, a “fail” received a time of 45 seconds, despite the fact that the actual time to find the platform may have been greater had the animal been allowed to continue the trial. Thus, the observed increase in the rate of trial failure for α-mannosidosis animals compared to normal animals suggests that the mean times taken for α-mannosidosis animals may have been underestimated to a greater extent than for normal animals. Additionally, α-mannosidosis animals were observed to have a significantly increased incidence of rejecting the platform and attempting to climb up the sides of the pool during the testing period. This could suggest that the cognitive deficits present in α-mannosidosis guinea pigs may reduce their ability to remember the “goal” of the test, (to find the platform), and hence result in searching for an alternative escape from the water maze. These measurements have not previously been reported in Morris water maze studies. The lack of significant differences observed between normal and α-mannosidosis animals in the reversal phase may have been in part due to the relatively small sample size of animals tested at this age. Further testing with a larger cohort may reveal greater differences between these two groups.

The observation that α-mannosidosis animals appeared to have a preference for the operator quadrant during the probe phase of this test could suggest that these animals are able to learn certain aspects of this test, particularly that the operator will eventually remove them from the pool. This behaviour has previously been noted during testing of normal and MPS IIA mice using the Morris water maze with a similar quadrant configuration (Dr. Briony Gliddon, personal communication). The task of spotting and swimming towards the operator would certainly be less difficult than remembering the location of the hidden platform based on external cues. The lack of
any apparent preference for the operator quadrant amongst normal animals (see Figure 3.6.1i) could suggest that for these animals that were able to successfully perform the test, finding the platform was a favourable alternative to waiting for the operator to remove them from the pool.

3.1.2.2 Pilot study: two months naïve

In order to determine the age at which measurable cognitive deficits occur in α-mannosidosis, a small group of α-mannosidosis (n=4) and normal (n=9) guinea pigs were bred. All animals in this group were weighed and received daily treatments of CSA as described previously, along with Bactrim during the trial period. Additionally, all animals received a sham injection of vehicle at one week of age (DMEM, as described in Section 2.2.4.3). The Morris water maze test was commenced at two months of age.

3.1.2.2a Acquisition phase: two months naïve

There were no significant differences between the performance of α-mannosidosis (average 36 seconds to platform) and normal animals (average 33 seconds to platform) on day one of the acquisition phase (see Figure 3.6.2a). Throughout the acquisition phase, both normal and α-mannosidosis animals improved markedly in the time taken to find the platform, resulting in an average time of 23 seconds for α-mannosidosis animals to find the platform on day seven, and 10 seconds for normal animals to find the platform on this day. The separation between these groups became most apparent on days six and seven, at the end of the acquisition phase. However, repeated measures analysis on these data revealed that there was only a trend towards improved performance of normal animals (p=0.064). No significant differences were observed in the proportion of failed trials between these groups (p=0.439, see Figure 3.6.2b). No significant differences were observed between different normal and α-mannosidosis guinea pigs in the proportion of trials where the platform was rejected as an escape (p=0.089, Figure 3.6.2c), or the proportion of trials spent attempting to climb up the sides of the pool (p=0.699, Figure 3.6.2d), vocalising (p=0.876, Figure 3.6.2e), or jumping out of the pool (p=0.274, Figure 3.6.2f).
3.1.2.2b Reversal phase: two months naïve

Both normal and α-mannosidosis animals took similar times to find the platform during the reversal phase (days 13 and 14, see Figure 3.6.2a). No significant differences between normal and α-mannosidosis animals were observed in any of the measurements recorded for this stage of the test, except for the proportion of trials that α-mannosidosis animals rejected the platform (Figure 2c, p=0.004).

3.1.2.2c Probe test: two months naïve

Normal animals again showed a preference for the target quadrant (where the platform was located during the acquisition phase), spending an average of 20 seconds (see Figure 3.6.2g) in this quadrant. This represented a highly significant increase (p=0.002) compared to the time spent in the other quadrants (see Figure 3.6.2h), verifying that normal animals at this age had strong retention of the task of finding the hidden platform. This increase was also trending towards significance (p=0.054) when compared to the time α-mannosidosis animals spent searching the target quadrant (16 seconds, see Figure 2i). In contrast with normal animals, α-mannosidosis guinea pigs showed no significant increase in the time spent in the target quadrant (p=0.194) compared to the other quadrants (see Figure 3.6.2h). Both α-mannosidosis and normal animals once again showed an increase in the time spent in the operator quadrant, although this increase represented a trend toward an increase only for normal animals (Figure 3.6.2i, p=0.161 and p=0.056 for α-mannosidosis and normal animals, respectively).
Figure 3.6.2a: Morris water maze pilot study: two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Data shown is the mean of four trials performed per animal, for each phenotype group, on each day, ±1SEM. Maximum duration of each trial was 45 seconds, after which time the animal was guided to the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.2b: Proportion of trials failed: two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Failed trials were defined as when the animal did not find the platform during the 45 second trial period. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.2a

Average time (seconds)

Day

Figure 3.6.2b

Proportion

Day

ns p=0.064
ns p=0.439
ns p=0.260
ns p=0.754
Figure 3.6.2c: Proportion of trials subject rejected platform: two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Subject rejecting platform” was defined as when the animal, having found or having been guided to the platform, swam back into the pool instead of waiting on the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.2d: Proportion of trials subject attempted to climb out of pool: two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Attempting to climb out of the pool” was defined as when the animal, during the course of the 45 second trial period, was observed scratching on the sides of the pool, thus attempting to climb out of the pool, as opposed to searching for the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.2c

Figure 3.6.2d
Figure 3.6.2e: Proportion of trials subject vocalising: two month old naïve animals.
n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Vocalising during the trial period was an indication of panic in the subject animal. "*" indicates statistical significance, "ns" indicates no statistical significance.

Figure 3.6.2f: Proportion of trials subject attempted to jump out of pool: two month old naïve animals.
n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. "Attempting to jump out of the pool" was defined as when the animal, during the 15 second rest period following the trial period, jumped off the platform and out of the bounds of the pool. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

α-mannosidosis
normal
Figure 3.6.2e

ns p=0.876

Figure 3.6.2f

ns p=0.274

ns p=0.538
Figure 3.6.2g: Probe test (overview): two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. Quadrant 4 was the target quadrant (where platform was located in previous trials), Quadrant 1 was where the operator was located. Statistical analysis was a t-test performed between the time spent in the target quadrant (Quadrant 4) for normal animals compared to α-mannosidosis animals, and a t-test performed between the time spent in the operator quadrant (Quadrant 1) for normal animals compared to α-mannosidosis animals. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- Quadrant 1
- Quadrant 2
- Quadrant 3
- Quadrant 4
Figure 3.6.2g

The graph shows the average time (in seconds) for normal and α-mannosidosis groups. The y-axis represents the average time, ranging from 0 to 30 seconds. The x-axis represents different quartiles (Q1, Q2, Q3, Q4).

For normal:
- Q1: ns p=0.774
- Q2: ns p=0.054
- Q3: ns p=0.774
- Q4: ns p=0.054

For α-mannosidosis:
- Q1: ns p=0.774
- Q2: ns p=0.054
- Q3: ns p=0.774
- Q4: ns p=0.054
Figure 3.6.2h: Probe test (Target vs. Non-Target Quadrants): two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Target Quadrant” was the mean time spent searching for the platform in Quadrant 4, “Non-Target Quadrants” was the mean time spent in Quadrants 1, 2 and 3. Statistical analysis was a t-test performed between the time spent in target quadrant vs. time spent in non-target quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Target Quadrant
- Non-Target Quadrants

---

Figure 3.6.2i: Probe test (Operator vs. Non-Operator Quadrants): two month old naïve animals.

n=4 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Operator Quadrant” was the mean time spent searching for the platform in Quadrant 1, the quadrant closest to the operator. “Non-Operator Quadrants” was the mean time spent in Quadrants 2, 3 and 4. Statistical analysis was a t-test performed between the time spent in the operator quadrant vs. time spent in non-operator quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Operator Quadrant
- Non-Operator Quadrants

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3.1.2.3 Pilot study: three months non-naïve

The animals tested above at two months of age were tested again at three months of age. One α-mannosidosis animal had to be excluded from this test group because of a failure to thrive. This left a cohort of three α-mannosidosis guinea pigs and nine normal guinea pigs. All animals were weighed and treated with CSA daily, along with Bactrim during the test period.

3.1.2.3a Acquisition phase: three months non-naïve

On the first day of this testing phase, both α-mannosidosis and normal guinea pigs were able to find the platform much faster (11 seconds for normal animals, 23 seconds for α-mannosidosis guinea pigs) than on the first day of testing at two months, possibly because of previous exposure to the water maze test. However, normal animals demonstrated consistently faster times to the platform compared to α-mannosidosis animals throughout the whole acquisition phase (Figure 3.6.3a). Statistical analysis showed this difference to be statistically significant (p=0.013). Additionally, α-mannosidosis guinea pigs showed a significantly higher proportion of fails (Figure 3.6.3b, p=0.010) during this period, suggesting that the time differences between normal and α-mannosidosis animals may be understated. α-Mannosidosis animals also displayed a significant increase in the proportion of trials rejecting the platform (Figure 3.6.3c, p=0.023), despite all being previously exposed to the test. No significant increases were observed, however, in the proportion of trials the animals tried to climb out of the pool (Figure 3.6.3d, p=0.636), vocalise (Figure 3.6.3e, p=0.373), or attempted to jump out of the pool (Figure 3.6.3f, p=0.150).

3.1.2.3b Reversal phase: three months non-naïve

α-Mannosidosis animals once again took longer to find the platform in this phase than normal animals (Figure 3.6.3a), although this was not found to be statistically significant (p=0.874). The increase in the proportion of trials failed by α-mannosidosis guinea pigs was also not found to be significant (p=0.227). α-Mannosidosis animals again displayed a significant increase in the proportion of trials rejecting the platform (Figure 3.6.3c, p=0.011), although no significant differences were found between groups in the other measurements investigated.
3.1.2.3c Probe test: three months non-naïve

Normal animals again showed a preference for the target quadrant (where the platform was located during the acquisition phase), spending an average of 21 seconds (see Figure 3.6.3g) in this quadrant. This represented a highly significant increase (p=0.002) compared to the time spent in the other non-target quadrants (see Figure 3.6.3h). This increase was also significant (p=0.042) when compared to the time α-mannosidosis animals spent searching the target quadrant (18 seconds, see Figure 3.6.3g). α-Mannosidosis animals also showed only a trend towards an increase in the time spent in the target quadrant (p=0.057) compared to the other non-target quadrants (see Figure 3.6.3h), in contrast to normal animals described above. Neither the α-mannosidosis or the normal animals showed a significant increase in the time spent in the operator quadrant (Figure 3.6.3i, p=0.580 and p=0.116, respectively), compared to the time spent in the non-operator quadrants.
Figure 3.6.3a: Morris water maze pilot study: three month old non-naïve animals. n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Data shown is the mean of four trials performed per animal, for each phenotype group, on each day, ±1SEM. Maximum duration of each trial was 45 seconds, after which time the animal was guided to the platform. "*" indicates statistical significance, "ns" indicates no statistical significance.

Figure 3.6.3b: Proportion of trials failed: three month old non-naïve animals. n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Failed trials were defined as when the animal did not find the platform during the 45 second trial period. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- □ α-mannosidosis
- ▲ normal
Figure 3.6.3c: Proportion of trials subject rejected platform: three month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Subject rejecting platform” was defined as when the animal, having found or having been guided to the platform, swam back into the pool instead of waiting on the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.3d: Proportion of trials subject attempted to climb out of pool: three month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “attempting to climb out of the pool” was defined as when the animal, during the course of the 45 second trial period, was observed scratching on the sides of the pool, thus attempting to climb out of the pool, as opposed to searching for the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.3c

* p=0.023

* p=0.011

Figure 3.6.3d

ns p=0.636

ns p=0.629
Figure 3.6.3e: Proportion of trials subject vocalising: three month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Vocalising during the trial period was an indication of panic in the subject animal. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.3f: Proportion of trials subject attempted to jump out of pool: three month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Attempting to jump out of the pool” was defined as when the animal, during the 15 second rest period following the trial period, jumped off the platform and out of the bounds of the pool. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.3g: Probe test (overview): three month old non-naive animals.

\(n=3\) α-mannosidosis guinea pigs and \(n=9\) normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, \(\pm\) ISEM. Quadrant 4 was the target quadrant (where platform was located in previous trials), Quadrant 1 was where the operator was located. Statistical analysis was a t-test performed between the time spent in the target quadrant (Quadrant 4) for normal animals compared to α-mannosidosis animals, and a t-test performed between the time spent in the operator quadrant (Quadrant 1) for normal animals compared to α-mannosidosis animals. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

<table>
<thead>
<tr>
<th>Quadrant 1</th>
<th>Quadrant 2</th>
<th>Quadrant 3</th>
<th>Quadrant 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.6.3g

Average time (seconds)

ns p=0.532

* p=0.042

Q1 Q2 Q3 Q4 Q1 Q2 Q3 Q4
normal α-mannosidosis
Figure 3.6.3h: Probe test (Target vs. Non-Target Quadrants): three month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Target Quadrant” was the mean time spent searching for the platform in Quadrant 4, “Non-Target Quadrants” was the mean time spent in Quadrants 1, 2 and 3. Statistical analysis was a t-test performed between the time spent in target quadrant vs. time spent in non-target quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

☑ Target Quadrant
☑ Non-Target Quadrants

Figure 3.6.3i: Probe test (Operator vs. Non-Operator Quadrants): three month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Operator Quadrant” was the mean time spent searching for the platform in Quadrant 1, the quadrant closest to the operator. “Non-Operator Quadrants” was the mean time spent in Quadrants 2, 3 and 4. Statistical analysis was a t-test performed between the time spent in the operator quadrant vs. time spent in non-operator quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

☑ Operator Quadrant
☑ Non-Operator Quadrants
Figure 3.6.3h

Q4

* p=0.002

Time (seconds)

normal

α-mannosidosis

Figure 3.6.3i

Q1, Q2, Q3

ns p=0.057

ns p=0.116

Time (seconds)

normal

α-mannosidosis

Q1, Q2, Q3, Q4

ns p=0.580
3.1.2.4 Pilot study: four months non-naïve

α-Mannosidosis (n=3) and normal (n=9) animals tested above at two and three months of age were swum again for this four month testing period. All animals were weighed and treated with CSA daily, along with Bactrim during the test period.

3.1.2.4a Acquisition phase: four months non-naïve

Due to exposure to the test on two previous occasions, both α-mannosidosis and normal guinea pigs began this phase with low seek times (22 seconds for α-mannosidosis animals and just 10 seconds for normal guinea pigs, see Figure 3.6.4a). Throughout the acquisition phase, α-mannosidosis animals further improved to find the platform in an average of 13 seconds by day seven and normal animals improved to find the platform in an average of 5 seconds. However, perhaps due to the overall familiarity of the test to all the animals and the variation in seek times of α-mannosidosis animals, repeated measures analysis did not indicate significant differences between these groups (p=0.13). There was, however, a trend towards a significant increase in the proportion of trials failed by α-mannosidosis animals in this testing period (Figure 3.6.4b, p=0.08). No significant increases were observed amongst α-mannosidosis guinea pigs with regard to the other measurements investigated in the acquisition phase (Figures 3.6.4c, 3.6.4d, 3.6.4e and 3.6.4f).

3.1.2.4b Reversal phase: four months non-naïve

Changing the location of the platform for the reversal phase resulted in an increase of the seek time of normal animals to 13 seconds on day 13, compared to 30 seconds for α-mannosidosis guinea pigs (Figure 3.6.4a). Although the seek times for the reversal phase considered as a whole did not reveal a statistically significant increase between these groups, the proportion of trials failed was observed to be significantly greater in α-mannosidosis animals (Figure 3.6.4b, p=0.040). The proportion of trials in which α-mannosidosis animals rejected the platform (Figure 3.6.4c) was found to be greatly increased in α-mannosidosis animals (p=0.001), with this group displaying this behaviour in 50% of all trials on day 13, and normal animals not displaying this behaviour at all during this testing period. The other measurements analysed (Figures 3.6.4d, 3.6.4e and 3.6.4f) did not reveal any significant differences
between these groups.

### 3.1.2.4c Probe test: four months non-naïve

Normal animals once again showed a preference for the target quadrant, spending an average of 21 seconds (see Figure 3.6.4g) in this quadrant. This represented a highly statistically significant increase \((p=0.004)\) compared to the time spent in the other quadrants (see Figure 3.6.3h). This increase was not significant \((p=0.204)\) when compared to the time \(\alpha\)-mannosidosis animals spent searching the target quadrant (17 seconds, see Figure 3.6.4g). Additionally, as a group, \(\alpha\)-mannosidosis animals showed no significant increase in the time spent in the target quadrant compared to the other quadrants (see Figure 3.6.4h, \(p=0.188\)), in contrast to normal animals described above. Both \(\alpha\)-mannosidosis and normal animals once again showed an increase in the time spent in the operator quadrant, although this increase was only found to be significant for normal animals (Figure 3.6.4i, \(p=0.062\) and \(p=0.007\) for \(\alpha\)-mannosidosis and normal animals, respectively).

Table 3.1 summarises the results obtained for the different aspects of the Morris water maze test, for all ages tested in the pilot study.
Figure 3.6.4a: Morris water maze pilot study: four month old non-naïve animals. 
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Data shown is the mean of 
four trials performed per animal, for each phenotype group, on each day, ±1SEM. 
Maximum duration of each trial was 45 seconds, after which time the animal was guided 
to the platform. “*” indicates statistical significance, “ns” indicates no statistical 
significance.

Figure 3.6.4b: Proportion of trials failed: four month old non-naïve animals. 
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Failed trials were defined 
as when the animal did not find the platform during the 45 second trial period. “*” 
indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.4c: Proportion of trials subject rejected platform: four month old non-naïve animals.

n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Subject rejecting platform” was defined as when the animal, having found or having been guided to the platform, swam back into the pool instead of waiting on the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.4d: Proportion of trials subject attempted to climb out of pool: four month old non-naïve animals.

n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Attempting to climb out of the pool” was defined as when the animal, during the course of the 45 second trial period, was observed scratching on the sides of the pool, thus attempting to climb out of the pool, as opposed to searching for the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.4e: Proportion of trials subject vocalising: four month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Vocalising during the trial period was an indication of panic in the subject animal. "*" indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.4f: Proportion of trials subject attempted to jump out of pool: four month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. “Attempting to jump out of the pool” was defined as when the animal, during the 15 second rest period following the trial period, jumped off the platform and out of the bounds of the pool. "*" indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.4e

Figure 3.6.4f
Figure 3.6.4g: Probe test (overview): four month old non-naïve animals.

n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. Quadrant 4 was the target quadrant (where platform was located in previous trials), Quadrant 1 was where the operator was located. Statistical analysis was a t-test performed between the time spent in the target quadrant (Quadrant 4) for normal animals compared to α-mannosidosis animals, and a t-test performed between the time spent in the operator quadrant (Quadrant 1) for normal animals compared to α-mannosidosis animals. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- Quadrant 1
- Quadrant 2
- Quadrant 3
- Quadrant 4
Figure 3.6.4g

Average time (seconds)

<table>
<thead>
<tr>
<th>Normal</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
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<tr>
<td>Q1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>α-mannosidosis</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Q3</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ns p = 0.337
ns p = 0.204
Figure 3.6.4h: Probe test (Target vs. Non-Target Quadrants): four month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Target Quadrant” was the mean time spent searching for the platform in Quadrant 4, “Non-Target Quadrants” was the mean time spent in Quadrants 1, 2 and 3. Statistical analysis was a t-test performed between the time spent in target quadrant vs. time spent in non-target quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Target Quadrant
- Non-Target Quadrants

Figure 3.6.4i: Probe test (Operator vs. Non-Operator Quadrants): four month old non-naïve animals.
n=3 α-mannosidosis guinea pigs and n=9 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Operator Quadrant” was the mean time spent searching for the platform in Quadrant 1, the quadrant closest to the operator. “Non-Operator Quadrants” was the mean time spent in Quadrants 2, 3 and 4. Statistical analysis was a t-test performed between the time spent in the operator quadrant vs. time spent in non-operator quadrants, for each group. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Operator Quadrant
- Non-Operator Quadrants
Figure 3.6.4h

Time (seconds)

* p=0.004
ns p=0.188

Q4
Q1, Q2, Q3

Q4
Q1, Q2, Q3

normal
α-mannosidosis

Figure 3.6.4i

Time (seconds)

* p=0.007
ns p=0.062

Q1
Q2, Q3, Q4

Q1
Q2, Q3, Q4

normal
α-mannosidosis
Table 3.1: Morris water maze pilot study: Summary of differences observed between normal and α-mannosidosis guinea pigs

<table>
<thead>
<tr>
<th>Age</th>
<th>Acquisition Times</th>
<th>Fail</th>
<th>Probe</th>
<th>Reversal Times</th>
<th>Fails</th>
<th>Acq. Platform</th>
<th>Sides</th>
<th>Vocalise</th>
<th>Jumping</th>
<th>Reversal Platform</th>
<th>Sides</th>
<th>Vocalise</th>
<th>Jumping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Six months naïve</td>
<td>*</td>
<td></td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Two months naïve</td>
<td>trend</td>
<td></td>
<td>trend</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Three months</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Four months</td>
<td>-</td>
<td>trend</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All statistical analysis was performed as described in Section 2.2.6, with all comparisons made between α-mannosidosis and normal guinea pigs. “Naïve” indicates the animals have had no previous exposure to the Morris water maze test. “-” indicates no significant differences (p≥0.08), “trend” indicates a trend towards significance (p<0.08) and “*” indicates significant differences (p<0.05). “Times” and “Fails” refers to comparisons between the time taken to find the platform and the proportion of trials failed, respectively. The measurements “Platform”, “Sides”, “Vocalise” and “Jumping” are described in detail in Section 3.1.1.4.
Pilot study discussion:

α-Mannosidosis guinea pigs tested using the Morris water maze begin to show memory and learning impairments compared to normal animals from the earliest time point analysed, two months of age. However, some of these differences only represent a statistical trend at this age.

When tested again at three months of age, α-mannosidosis guinea pigs were found to display significant cognitive deficits compared to normal animals, particularly during the acquisition and probe phases of this test. Certain infrequent behaviours such as a tendency to swim off the platform following mounting (rejecting the platform) additionally seem to be characteristic of α-mannosidosis guinea pigs in this test. Caution must be taken when interpreting probe test data during this period (and indeed, any non-naïve testing period), due to the greater amount of previous exposure to the test. However, the results of these tests suggest that by three months of age, cognitive deficits in α-mannosidosis guinea pigs had progressed to such an extent that they could be effectively measured using the Morris water maze.

At four months of age, differences previously observed between α-mannosidosis and normal animals appeared to be reduced in the acquisition phase of this test. The fact that α-mannosidosis animals still showed cognitive deficits compared to normal animals in the probe phase of the test, coupled with the fact that neurological pathology in the α-mannosidosis guinea pig is progressive (and thus would be expected to be more pronounced at four months of age than at three months) suggests that this may be an artefact of the repeated testing protocol, rather than a reflection of the actual pathological state of this group of animals. Having performed the Morris water maze test twice previously, it may be that both normal and α-mannosidosis animals were so familiar with the test (and thus displayed such low seek times), that it became more difficult to detect statistically significant differences between groups.

As such, the three month time point was chosen for further testing in the Morris water maze. This age was chosen to provide an age at which memory and learning deficits in α-mannosidosis animals had developed to such an extent that significant separation between these and normal animals could be observed using the Morris water maze test. Additionally, this age was chosen in order to minimise the contribution of
other aspects of somatic pathology (such as skeletal and muscular pathology) to
differences observed between normal and α-mannosidosis guinea pigs.

3.1.2.5 Morris water maze: three months naïve

One of the aims in establishing the Morris water maze as a test of cognitive
function in the α-mannosidosis guinea pig is for use in evaluating potential therapies. For this purpose, a group of three month old naïve α-mannosidosis (n=8) and normal (n=12) animals were tested as described previously. This age was chosen following the pilot study detailed above. All animals were weighed and treated daily with CSA, along with Bactrim during the testing period. Additionally, all animals received a sham injection of vehicle at one week of age (DMEM, as described in Section 2.2.4.3). These animals served as the control groups for evaluating the therapy described in Chapter 6 of this thesis.

3.1.2.5a Acquisition phase: three months naïve

Both normal and α-mannosidosis animals began the acquisition phase with similar seek times, between approximately 30 and 35 seconds (Figure 3.6.5a). Following statistical analysis, significant differences were observed between groups, however, in their rate of improvement in these times.

Aside from days one and three, normal animals displayed seek times consistently at least one standard error of the mean lower than α-mannosidosis guinea pigs. Statistical analysis also showed this to be significant over the testing period (p=0.009). Additionally, α-mannosidosis animals were also found to fail a significantly greater proportion of trials (Figure 3.6.5b, p=0.005), suggesting once again that seek times reported may be underestimation to a greater extent for these α-mannosidosis animals than for normal animals. Surprisingly, none of the other measurements examined (proportion of trials rejecting the platform, climbing up the sides of the pool, vocalising, or jumping out of the pool) revealed any significant differences between normal and α-mannosidosis animals over these trials.

3.1.2.5b Reversal phase: three months naïve

Statistical analysis on the reversal phase of this test did not show any significant
differences in the seek times between normal and α-mannosidosis animals (Figure 3.6.5a). Fail rates (Figure 3.6.5b), and all other measures analysed (Figures 3.6.5c, 3.6.5d, 3.6.5e and 3.6.5f) were additionally not found to be significantly different between groups.

3.1.2.5c Probe test: three months naïve

Normal animals showed a strong preference for the target quadrant during the probe test, spending an average of 20 seconds in this quadrant (see Figure 3.6.5g). This represented a statistically significant increase (p=0.018) compared to the time spent in the other quadrants (see Figure 3.6.5h). Normal animals at this age thus demonstrate strong retention of the task of finding the hidden platform. In contrast to normal guinea pigs, α-mannosidosis animals showed no significant increase in the time spent searching the target quadrant (p=0.507) compared to the other quadrants (see Figure 3.6.5h). Both normal and α-mannosidosis animals once again showed increases in the time spent in the operator quadrant, although these were not found to be significant (Figure 3.6.5i, p=0.218 and p=0.155, respectively).

When comparisons were made between the time searching the target quadrant for normal and α-mannosidosis animals, normal animals were found to have a significantly increased search time in this quadrant (20 seconds compared to 14 seconds, p=0.026, Figure 3.6.5g).
Figure 3.6.5a: Morris water maze pilot study: three month old naïve animals.

$n=8$ α-mannosidosis guinea pigs, and $n=12$ normal guinea pigs. Data shown is the mean of four trials performed per animal, for each phenotype group, on each day, ±1SEM. Maximum duration of each trial was 45 seconds, after which time the animal was guided to the platform. "*" indicates statistical significance, "ns" indicates no statistical significance.

Figure 3.6.5b: Proportion of trials failed: three month old naïve animals.

$n=8$ α-mannosidosis guinea pigs and $n=12$ normal guinea pigs. Failed trials were defined as when the animal did not find the platform during the 45 second trial period. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.5c: Proportion of trials subject rejected platform: three month old naïve animals.
n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. “Subject rejecting platform” was defined as when the animal, having found or having been guided to the platform, swam back into the pool instead of waiting on the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.6.5d: Proportion of trials subject attempted to climb out of pool: three month old naïve animals.
n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. “Attempting to climb out of the pool” was defined as when the animal, during the course of the 45 second trial period, was observed scratching on the sides of the pool, thus attempting to climb out of the pool, as opposed to searching for the platform. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.5e: Proportion of trials subject vocalising: three month old naïve animals.

n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. Vocalising during the trial period was an indication of panic in the subject animal. "*" indicates statistical significance, "ns" indicates no statistical significance.

Figure 3.6.5f: Proportion of trials subject attempted to jump out of pool: three month old naïve animals.

n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. “Attempting to jump out of the pool” was defined as when the animal, during the 15 second rest period following the trial period, jumped off the platform and out of the bounds of the pool. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.6.5e

ns = 0.669

ns p = 0.722

Figure 3.6.5f

ns p = 1

ns p = 0.414
Figure 3.6.5g: Probe test (overview): three month old naïve animals.

n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. Quadrant 4 was the target quadrant (where platform was located in previous trials), Quadrant 1 was where the operator was located. Statistical analysis was a t-test performed between the time spent in the target quadrant (Quadrant 4) for normal animals compared to α-mannosidosis animals, and a t-test performed between the time spent in the operator quadrant (Quadrant 1) for normal animals compared to α-mannosidosis animals. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- Quadrant 1
- Quadrant 2
- Quadrant 3
- Quadrant 4
Figure 3.6.5g

Time (seconds)

<table>
<thead>
<tr>
<th>Q1</th>
<th>Q2</th>
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<th>Q4</th>
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<tbody>
<tr>
<td>15</td>
<td>10</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

normal

<table>
<thead>
<tr>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>10</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

α-mannosidosis

* p=0.026

ns p=0.703
Figure 3.6.5h: Probe test (Target vs. Non-Target Quadrants): three month old naïve animals.
n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. "Target Quadrant" was the mean time spent searching for the platform in Quadrant 4, "Non-Target Quadrants" was the mean time spent in Quadrants 1, 2 and 3. Statistical analysis was a t-test performed between the time spent in target quadrant vs. time spent in non-target quadrants, for each group. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- Target Quadrant
- Non-Target Quadrants

Figure 3.3.5i: Probe test (Operator vs. Non-Operator Quadrants): three month old naïve animals.
n=8 α-mannosidosis guinea pigs and n=12 normal guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. "Operator Quadrant" was the mean time spent searching for the platform in Quadrant 1, the quadrant closest to the operator. "Non-Operator Quadrants" was the mean time spent in Quadrants 2, 3 and 4. Statistical analysis was a t-test performed between the time spent in the operator quadrant vs. time spent in non-operator quadrants, for each group. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- Operator Quadrant
- Non-Operator Quadrants
3.1.2.6 Visible platform test

To verify that differences in seek times were indeed a result of cognitive deficits in α-mannosidosis guinea pigs and not physical aspects of pathology (such as vision, ability to swim or ability to physically mount the platform), a visible platform test was carried out on a group of normal (n=5) and α-mannosidosis (n=4) animals (all from trial period five, as described in Appendix 1). This test was carried out following the completion of the last trials of the reversal phase, on day 14.

Although α-mannosidosis animals began the first of four trials during this test with a significantly elevated seek time (41 seconds compared to 24 seconds, as shown in Figure 3.6.6a), in the last of the four consecutive trials no significant difference was observed between the seek times for normal and α-mannosidosis animals, and the proportion of trials failed by both groups fell to zero by the end of these trials (see Figure 3.6.6b). Additionally, the swimming ability of three month old α-mannosidosis guinea pigs was not found to be noticeably impaired compared to normal animals. This was subjectively assessed by the operator, however, and was not specifically tested in this study.

Table 3.2 summarises the results obtained following testing of three month old naïve guinea pigs in the Morris water maze, in comparison to pilot study results discussed previously.
**Figure 3.6.6a: Visible platform test: three month old naïve animals.**
n=4 α-mannosidosis guinea pigs and n=5 normal guinea pigs. Data shown is the mean time for each group of animals to find and mount the platform, in each of the four trials, ±1SEM. Maximum duration of each trial was 45 seconds, after which time the animal was guided to the platform. "*" indicates statistical significance, "ns" indicates no statistical significance.

**Figure 3.6.6b: Visible platform test: proportion of trials failed: three month old naïve animals.**
n=4 α-mannosidosis guinea pigs and n=5 normal guinea pigs. Failed trials were defined as when the animal did not find the platform during the 45 second trial period. "*" indicates statistical significance, "ns" indicates no statistical significance.

**Legend:**

- α-mannosidosis
- normal
Table 3.2: Summary of differences observed between normal and α-mannosidosis guinea pigs at all ages tested

<table>
<thead>
<tr>
<th>Age</th>
<th>Acquisition</th>
<th>Probe</th>
<th>Reversal</th>
<th>Acquisition</th>
<th>Reversal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Times</td>
<td>Fails</td>
<td>Times</td>
<td>Fails</td>
<td>Platform</td>
</tr>
<tr>
<td>Six months naïve</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Two months naïve</td>
<td>trend</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three months</td>
<td>*</td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Four months</td>
<td></td>
<td></td>
<td>trend</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Three months naïve</td>
<td>*</td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

Table summarising the results obtained following testing of three month old naïve guinea pigs in the Morris water maze, in comparison to pilot study results discussed previously. All statistical analysis was performed as described in Section 2.2.6, with all comparisons made between α-mannosidosis and normal guinea pigs. “Naïve” indicates the animals have had no previous exposure to the Morris water maze test. “-” indicates no significant differences (p>0.08), “trend” indicates a trend towards significance (p<0.08) and “*” indicates significant differences (p<0.05). “Times” and “Fails” refers to comparisons between the time taken to find the platform and the proportion of trials failed, respectively. The measurements “Platform”, “Sides”, “Vocalise” and “Jumping” are described in detail in Section 3.1.1.4.
3.1.3 Discussion: Morris water maze

The pilot study investigated the utility of the Morris water maze to detect changes in cognitive ability between normal and α-mannosidosis animals at six months of age. Following the observation of significant differences in these tests, further investigation was carried out to determine the age at which these cognitive deficits could be readily observed using the Morris water maze test. These studies involved testing a group of animals at two, three and four months consecutively.

α-Mannosidosis guinea pigs were found to display changes in their memory and learning ability at two months, observed as a reduced rate of learning in the acquisition phase, and a decrease in the time spent searching in the target quadrant (compared to normal animals) during the probe phase (see Table 3.2). However, these differences only represented a statistical trend at this age. Additionally, α-mannosidosis animals were observed to reject the platform more frequently than normal animals. By three months of age these differences were more pronounced and were found to be statistically significant. The lack of significant differences between normal and α-mannosidosis animals in the acquisition phase at four months probably did not reflect the lack of pathology at this age; indeed, α-mannosidosis guinea pigs were found to display many notable aspects of pathology at four months, when tested with the neurological examination detailed later in this thesis. Rather, the level of familiarity these animals developed with the water maze test (as a result of being tested twice previously) may have resulted in both groups performing so well that any differences fell below the level of sensitivity of the test as performed. This result contrasts with data obtained in a similar pilot study for the testing of MPS IIIA mice using the Morris water maze, where MPS IIIA mice were observed to perform progressively worse when tested at seven weeks, 15 weeks, 20 weeks and 28 weeks of age (Gliddon, 2002).

This test should thus ideally be carried out on naïve animals, in order to most sensitively examine cognitive differences between normal and α-mannosidosis guinea pigs. Additionally, the small sample size for α-mannosidosis animals (n=3) would have reduced the power of statistical comparisons in these studies. The ideal experimental design for this pilot study would have involved testing larger groups of naïve animals at two, three and four months of age (such that each group of animals was tested only once). Unfortunately, this was not logistically possible within the scope of this thesis.
Following the pilot study, the testing of three month old naïve animals in this study verified that significant memory and learning deficits are present in α-mannosidosis animals at three months of age, (compared to normal animals), and that these deficits can be effectively measured using the Morris water maze test.

When a visible platform test was carried out, both normal and α-mannosidosis animals began these trials with higher seek times than the last trial completed during the second day of the reversal phase (day 14). This may reflect that although the platform was visible in these trials, there was still a learning component involved-animals had to learn to recognise the visually unfamiliar platform as the escape. Previous studies involving visible platform Morris water maze tests in guinea pigs have also showed a similar learning component, with animals improving progressively over the course of all trials (Dringenberg, 2001). The observation that all animals improved in their seek times throughout the four trials suggests that both normal and α-mannosidosis animals were able to find the platform, swim towards it and mount it as an escape.

This study has observed and reported numerous novel observations in the Morris water maze, namely, the different types of behaviour exhibited by guinea pigs during testing, expressed as a proportion of trials in which they occurred. To our knowledge, these observations have not previously been published in Morris water maze studies. Some of these measurements were not found to be particularly useful as a point of difference between normal and α-mannosidosis animals (for example, vocalising and jumping off the platform out of the pool, see Table 3.2). However, others that represented a rejection of the task by the animal (attempting to climb out the sides of the pool, and in particular, rejecting the platform once mounted) were found to be significantly different between normal and α-mannosidosis guinea pigs in some of the periods tested (as shown in Table 3.2). Indeed, one behaviour that seems to be characteristic of α-mannosidosis guinea pigs in the Morris water maze is an erratic performance. Where normal animals tend to consistently and predictably improve throughout the testing period, α-mannosidosis animals were often found to perform well one day (or even one trial), then in the next day (or trial) fail to find the platform and/or swim off the platform once guided to it. These behaviours quite often resulted in wider variation in the seek times of α-mannosidosis animals (see Figures 3.6.1a, 3.6.2a,
than those observed for normal animals. Similarly erratic performance has been reported following Morris water maze studies using MPS IIIA mice, compared to normal animals (Gliddon, 2002).

A maximum trial time of 45 seconds was chosen for the Morris water maze protocol in this thesis, based on previous Morris water maze studies using the guinea pig (Dringenberg et al., 2001). This relatively short trial time (compared to 90 seconds routinely used for trials involving mice by others in our laboratory; Gliddon and Hopwood, 2004) was chosen in order to minimise exhaustion in the guinea pigs, and thus the potential for stress confounding the task of finding the platform. This short trial time potentially reduced the test sensitivity, as the actual time the animal may have taken to find the platform if allowed to continue swimming may have been greater than 45 seconds. As guinea pigs in this study (both normal and α-mannosidosis) all demonstrated the ability to swim strongly for the full length of all four trials on a given day, future testing protocols could possibly involve a longer trial time. This could contribute to even more significant differences between normal and α-mannosidosis animals (perhaps even detectable at two months of age), and thus an even more sensitive test for evaluating memory and learning ability in α-mannosidosis guinea pigs following therapy.

The probe phase of this test was found to be highly effective at demonstrating significant differences in memory between normal and α-mannosidosis guinea pigs at most ages tested (summarised in Table 3.2), particularly following a five day rest period after the acquisition phase (as per the protocol described in Dringenberg et al., 2001). This rest period was included to increase the difficulty of the animals remembering the location of the hidden platform, compared to conducting the probe phase the day after the last day of the acquisition phase. The data obtained in the pilot study suggests that both normal and α-mannosidosis animals are able to remember the location of the platform not only following a five day rest period, but also month to month. This longer-term retention was also seen in a similar pilot study involving MPS IIIA mice (Gliddon, 2002). The strength of memory retention could vary, however, between normal and α-mannosidosis guinea pigs. In order to extend the useful aspects of the Morris water maze test, future protocols could possibly involve a second probe test after an additional and longer rest period following the first probe test, to further
increase the difficulty of the animals remembering the location of the hidden platform. This could once again further contribute to the sensitivity of this test.

The reversal phase of this testing protocol was found to be poor at revealing significant differences between normal and α-mannosidosis animals in any of the testing periods reported, in contrast to what has been observed previously between MPS IIIA and normal mice (Gliddon, 2002; Gliddon and Hopwood, 2004). This observation could be due to the fact that guinea pigs take up a much greater proportion of the pool relative to mice, and swim in much wider circles in their search for the platform. Thus, where mice can spend the majority of time in a trial searching one quadrant of the pool (and thus miss the platform entirely), guinea pigs are much more likely to search other quadrants by default, finding the platform more quickly in a new location. The use of a larger pool in future testing protocols could thus further contribute to the sensitivity of this test, resulting in greater observed differences between groups in the reversal phase of this test.

3.2 Gait analysis

Gait abnormalities have been previously reported in the guinea pig model of α-mannosidosis (Crawley et al., 1999) and the mouse model of MPS IIIA (Hemsley and Hopwood, submitted). Preliminary studies carried out with α-mannosidosis guinea pigs have shown significant quantitative differences in gait length (Kim Hemsley, unpublished data). As a result of these studies it was decided that further characterisation of gait changes in this animal model would be valuable.

Normal/heterozygous and α-mannosidosis guinea pigs were tested at both two and three months of age. All animals were treated daily with CSA and received sham surgery at one week of age, as described above.

3.2.1 Specific methods

Hind-limb gait was determined by dipping the rear paws into ink and allowing the guinea pigs to walk along a piece of paper (Steinberg et al., 1989). The box used for performing the gait analysis is shown in Figure 3.7. Animals were placed at the rear end and allowed to walk into the enclosed space at the other end of the box, where they found a reward (pieces of apple). The first run was conducted with no ink, such that the animals could become familiar with the environment and the task. After the first run,
the hind paws of the animal being tested were dipped in non-toxic food colouring before being placed in the box and then being allowed to walk across the paper again. This was repeated until two clean runs (ie. animal walking all the way along the paper) were obtained for each animal at each time.

A cross was then drawn at the front of each paw print (ie. the front of the centre pad, not including marks made by the claws or toes), before gait length and width were measured as shown (see Figure 3.8). Left and right gait lengths were averaged to provide a mean gait length, and width was calculated as shown in Figure 3.8. All gait lengths and widths were normalised to the weight of each animal, to ensure that observed differences reflected actual gait changes as a result of α-mannosidosis pathology, and not just the slower growth rate of α-mannosidosis animals. Previous studies have reported no significant sex-related gait differences in mice (Fernagut et al., 2002). As a result (and to provide a sufficient sample size for analysis), male and female animals were analysed together in this study. Statistical analysis was performed as described in Section 2.2.6.
Figure 3.7: Box used for gait analysis
The guinea pig was placed in the closest end of the box (as shown) and allowed to walk along the paper to the enclosed area at the far end of the box. The enclosed area was dark and contained pieces of chopped apple, representing a reward for the animal.
Figure 3.7
Figure 3.8: Schematic example of gait analysis
Crosses represent guinea pig footprints. To calculate stride length, lines were drawn between all left footprints and all right footprints as shown. The distance between footprints (L1, L2, L3, etc.) was then measured. Stride width was calculated by drawing the perpendicular from each “length” line across to the corresponding footprint, as shown. The stride widths (W1, W2, W3, etc.) were then measured.
Figure 3.8
3.2.2 Results

Normal/heterozygous and α-mannosidosis guinea pigs were tested at both two and three months of age (see Section 2.2.4.6): at two months, n=5 α-mannosidosis guinea pigs, n=11 normal/heterozygous guinea pigs, and at three months, n=8 α-mannosidosis guinea pigs and n=12 normal/heterozygous guinea pigs.

Measurement of stride length (Figure 3.9a) revealed highly significant increases in gait length in α-mannosidosis animals at three months of age (p=0.008). No significant differences were observed at two months of age in this measure (p=0.151), however.

Measurement of stride width (Figure 3.9b) revealed more striking differences between normal and α-mannosidosis animals, with affected guinea pigs displaying highly significant increases in gait width at two and three months (p<0.001 for both time points).

When comparisons were made between the length/weight ratio of tested animals (Figure 3.9c), normal animals were found to have a significantly higher ratio at both two and three months (p=0.001 and p=0.003, respectively). Thus, although gait length was found to be increased in α-mannosidosis animals at three months of age (relative to the body weight of each animal), width was found to increase much more dramatically (relative to body weight once again) at both time periods tested.
Figure 3.9a: Stride length of guinea pigs at two and three months of age.

α-Mannosidosis guinea pigs n=5 (two months), n=8 (three months)
Normal guinea pigs n=11 (two months), n=12 (three months)

Data shown is the mean of stride length divided by bodyweight for each animal, ±1SEM. “*” indicates statistical significance, “ns” indicates no statistical significance.

Figure 3.9b: Stride width of guinea pigs at two and three months of age.

α-Mannosidosis guinea pigs n=5 (two months), n=8 (three months)
Normal guinea pigs n=11 (two months), n=12 (three months)

Data shown is the mean stride width divided by bodyweight for each animal, ±1SEM. “*” indicates statistical significance, “ns” indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.9a

Distance/weight (mm/g)

ns p=0.151

2 months

3 months

Figure 3.9b

Distance/weight (mm/g)

* p<0.001

2 months

3 months
Figure 3.9c: Stride length/width ratio of guinea pigs at two and three months of age.

α-Mannosidosis guinea pigs n=5 (two months), n=8 (three months)
Normal guinea pigs n=11 (two months), n=12 (three months)

Data shown is mean stride length divided by mean stride width, ±1SEM. "*" indicates statistical significance, "ns" indicates no statistical significance.

Legend:

- α-mannosidosis
- normal
Figure 3.9c

* p=0.001

* p=0.003
3.2.3 Discussion: gait analysis

These results demonstrate striking gait changes in α-mannosidosis as a characteristic feature of cerebellar dysfunction in this disease. These gait analyses did not take into account speed of travel of the animals during measurement, which has been shown to be an important consideration for measuring stride length (Clarke and Still, 1999). This potentially resulted in a greater level of variation in the data than may have otherwise been observed, particularly in stride length. However, the fact that such highly significant differences were observed between normal and α-mannosidosis animals suggests the potential utility of this measurement for future investigations into α-mannosidosis pathology. Gait changes have also been observed as a feature of pathology in the MPS IIIA mouse (Hemsley and Hopwood, in preparation).

3.3 Multiple component neurological examinations

Due to the wide range of symptoms presenting in the α-mannosidosis guinea pigs and the progressive nature of this pathology, a generalised neurological examination test was developed to observe changes occurring over time in this disease. This test was originally developed by Dr. Allison Crawley, and then further modified for the purposes of this study in collaboration with Dr. Allison Crawley, based on a small animal neurological examination suggested in De Lahunta (1983). One of the advantages of this test is that, like gait analysis, it can be employed on a repeated basis, allowing observation of individual animals over time. The testing of cell-implanted animals with this test (described in Chapter 6) also allowed regular detailed observation of these animals. This was important due to the risk of teratoma formation following cell implantation; any rapid changes in the neurological presentation of these animals that might indicate the presence of a teratoma could be observed fairly early, allowing early euthanasia.

3.3.1 Specific methods

Neurological examinations were performed approximately weekly on the bench on a surgical drape, with up to six guinea pigs tested at a time. Testing groups included both normal and α-mannosidosis animals, to enable accurate assessment of relative differences at a given age. Animals tested together were usually all from the same pen, to minimise stress. This also encouraged interaction and thus enabled better assessment
of behaviours. Where this was not possible (ie. when a pen contained only affected animals), normal females from other pens were used for comparison, to avoid fighting. Test components were given scores, some graded according to increasing severity. Scores were then summed and each animal was given a total score (expressed as a percentage of the total possible score). The observer did not refer to previous scores when testing; all data was collated and analysed after the testing period. Figure 3.10 shows the sheet used for these tests.

3.3.1.1 Physical features:

Test item 1: Facial features

Affected animals were examined (sitting on the bench) in comparison to unaffected animals for their degree of facial dysmorphia (broad face and short rounded nose). Animals were given a score out of three for this test item.

Test item 2: Foreleg position

The position of the animal’s forelegs while stationary was examined. Animals with forelegs tucked under their body (not clearly visible) were given one point for this test item.

Test item 3a: Body condition (muscular)

Animals were examined for their general muscular condition. Animals that felt weak during handling and had relatively reduced muscle mass (compared to age-matched unaffected animals) were given one point for this test item.

Test item 3b: Body condition (hair)

Animals with noticeably coarse hair (compared to age-matched unaffected animals) were given one point for this test item.

Test item 3c: Body condition (grooming)

Animals with dirt and food in their coat (from inadequate grooming) were given one point for this test item.
GUINEA-PIG NEUROLOGICAL EXAMINATION

ID:  
DOB:  
Age:  
Bodyweight:  

***Circle appropriate score***; normal will have a low score;

**Physical Appearance:**
1. *facial features - broad face & short rounded nose normal/mild/moderate/severe  
2. position forelegs - tucked under body/unable to see easily? - no/yes  
3. body condition normal/moderate or poor  
   - muscle  
   - hair (coarse)  
   - grooming

**Behaviour/Gait:**
On benchtop
4. *bright, alert & inquisitive /moderately lethargic/ dull & unresponsive  
5. smooth & coordinated movement /slightly abnormal movement/ slow, laboured & jerky mvmt  
6. main posture hunched no/yes

During handling
7. struggles when caught - no/mild/a lot/freaks out

On benchtop
8. *can lift head up - yes/no  
9. whole body sway when walks/ataxia - no/yes  
10. hindlimb gait  
    - normal / mild abnormalities / obvious bunny hop or shuffling  
11. stretches body and extends head to walk - no/yes  
12. hyperaesthesia (noise) - no/moderate/excessive

**Postural Reactions:**
    - normal/ slow correction/ slow and falls to other side  
14. wide head excursions following righting - no/yes  
15. nystagmus following righting - no/mild/severe  
16. wheelbarrow - normal/ stumble or nose on ground

**Cranial Nerves: (pg 372-377)**
17. medial canthus touch (sensory V; motor VII) - normal/abnormal  
18. lateral canthus touch (sensory V; motor VII) - normal/abnormal  
19. *nose, nostril & whisker movement (motor VII)  
    - normal/ coarse mvmt only/ no mvmt  
20. - in response to touch: normal/reduced  
21. facial symmetry incl. eye position - normal/abnormal  
22. head tilt (VIII) - no/yes  
23. nystagmus resting - no/yes  
24. vertical - no/yes  
25. horizontal - no/yes

**General Comments/Summary**
3.3.1.2 Behaviour/Gait:

Test item 4: Alertness

Animals were observed walking around the bench, investigating the environment and interacting with other guinea pigs for this test. Animals that were observed to be less bright, alert and inquisitive (i.e. moderately lethargic, compared to age-matched unaffected animals) were given two points for this test item; animals that were dull and unresponsive, and generally not moving around much, were given four points for this test item.

Test item 5: Movement

Animals were examined while active on the bench for this test, with regard to the ease at which they were able to walk and move around. Animals with slightly abnormal movement (compared to age-matched unaffected animals) were given one point for this test item; animals with noticeably slow, laboured and jerky movement were given two points for this test item.

Test item 6: Posture

The posture of the animals tested was examined, both walking and sitting. Animals that displayed a noticeably hunched posture (compared to age-matched unaffected animals) were given one point for this test item.

Test item 7: Struggling during handling

Animals were picked up off the bench, held with one hand around the back of their shoulders and their front legs, such that their hind legs could hang freely. Unaffected animals tended to be fairly relaxed in this position, whereas affected animals often struggled noticeably. Animals were given a score out of three for their degree of struggling in this position.

Test item 8: Head lifting ability

While sitting on the bench, animals were allowed to chew on the end of a plastic ball point pen. The pen was then raised slowly, such that they could follow it with their mouth. Unaffected animals were generally able to raise their head to follow the pen,
whereas affected animals were less able to raise their head to this angle and often placed a paw on the pen for support. Animals that were not easily able to raise their head in this test were given one point for this test item.

Test item 9: Whole body sway while walking

Animals were observed walking once again for this test. An observation of the whole body swaying while walking (indicative of ataxia) led to the animal being given two points for this test item.

Test item 10: Hind limb gait

The hind limb gait of animals was observed whilst they were walking around on the bench. Mild abnormalities in hind limb gait (compared to age-matched unaffected animals) were given two points, while obvious “bunny hopping” or shuffling by the animal led to it being given four points for this test item.

Test item 11: Body stretch while walking

Animals were observed walking around on the bench. Animals that noticeably stretched their body and extended their head while walking (compared to age-matched unaffected animals) were given one point for this test item.

Test item 12: Hyperaesthesia

Animals were observed as a group on the bench, for their reaction to a moderate volume auditory stimulus (hand clap). Animals that were observed to have a slightly pronounced flinching reaction (compared to age-matched unaffected animals) were given one point for this test item. Animals with a very pronounced flinching reaction (compared to age-matched unaffected animals) were given two points for this test item.

3.3.1.3 Postural reactions:

Test item 13: Righting reflex

This test was performed in clear space on a surgical drape on the bench (ie. no other animals in the immediate vicinity). The animal being tested was held with one hand around the back of its shoulders and gently flipped onto its back. The righting
reflex observed was the animal’s ability to right itself back into a normal posture. Animals that demonstrated slow correction to normal posture (compared to age-matched unaffected animals) were given two points for this test item, while animals that both showed slow correction and over-correction (falling to the other side following correction) were given four points for this test item.

Test item 14: Head excursions following righting

Animals were observed immediately following the righting test procedure described in test item 13. Animals that displayed wide head excursions following righting (compared to age-matched unaffected animals) were given one point for this test item.

Test item 15: Nystagmus following righting

Animals were observed immediately following the righting test procedure detailed in test item 13. Animals that displayed noticeable nystagmus (involuntary rhythmic eyeball oscillations) following righting were given one or two points for this test item, depending on the severity of the nystagmus.

Test item 16: “Wheelbarrow”

This test consisted of holding the animal by its hind legs/hips, with its front legs still on the bench top, moving the animal forward gently, such that it could move along the bench top using its front legs. Animals that stumbled during this procedure, or could not support their head such that their nose touched the bench top, were given one point for this test item.

3.3.1.4 Cranial nerves:

Test items 17 and 18: Medial canthus touch and lateral canthus touch, respectively

These tests were performed with the animal sitting on the bench top. The tip of a ball point pen was gently touched to either the medial or lateral canthus and the resultant response of the animal was noted. Unaffected animals usually responded by blinking and sometimes flinching. Animals with a reduced response (compared to age-matched unaffected animals) were given one point for each of these test items.
Test item 19: Nose, nostril and whisker movement

Animals were examined while being held for this test item. Nose, nostril and whisker movements were observed. Animals that displayed only coarse movement of the nose, nostrils and whiskers (compared to age-matched unaffected animals) were given two points for this test item. Animals that displayed virtually no movement of the nose, nostrils and whiskers were given four points for this test item.

Test item 20: Nose, nostril and whisker movement in response to touch

Animals were examined in a similar fashion to test item 19, with gentle stimulation of the whiskers with the tip of a ball point pen. Animals that displayed reduced movement (compared to age-matched unaffected animals) were given one point for this test item.

Test item 21: Facial symmetry

Animals were examined for facial symmetry, including eye position. Animals that displayed greater asymmetry (compared to age-matched unaffected animals) were given one point for this test item.

Test item 22: Head tilt

Animals were examined to determine their head position. Animals that displayed a noticeable head tilt to one side were given one point for this test item.

Test item 23: Nystagmus (resting)

Animals were observed for this test while resting stationary on the bench top. Animals that displayed noticeable nystagmus were given one point for this test item.

Test item 24: Nystagmus (vertical)

This test was performed by holding the animal up and moving its head gently up and down. Animals that displayed noticeable nystagmus during this procedure were given one point for this test item.
Test item 25: Nystagmus (horizontal)

This test was performed as for test item 24, except by moving the animal’s head side to side. Animals that displayed noticeable nystagmus during this procedure were given one point for this test item.

3.3.2 Results: Neurological examinations

The total score for each animal was graphed over time, providing an overview of disease progression in α-mannosidosis guinea pigs compared with normal animals (see Figure 3.11a). Normal animals had maximal scores of approximately 8%, which was consistent throughout the period analysed (up to approximately 100 days old). α-Mannosidosis guinea pigs were found to score higher than normal animals in this test as early as 20 days old, which was seen to progressively increase with age throughout the period analysed, up to as high as 58% in some animals. α-Mannosidosis animals were clearly distinguishable from normal animals by approximately 30 to 40 days of age using this neurological examination test. Graphical representation of the neurological components of this test (Figure 3.11b) shows a similar progression of pathology in α-mannosidosis guinea pigs, indicating that the majority of the score attributed to α-mannosidosis animals in this examination was due to observations of neurological pathology. Significant variation was found in test scores for α-mannosidosis guinea pigs with regard to both the onset of observable pathology and rate of progression of pathology.

Individual graphs of the four test categories: “Physical Appearance” (Figure 3.11c), “Behaviour/Gait” (Figure 3.11d), “Postural Reactions” (Figure 3.11e) and “Cranial Nerves” (Figure 3.11f) additionally show a similar progression of pathology in α-mannosidosis animals, and indicate that the majority of score attributed to normal animals is due to the “Postural Reactions” category of this test. This score was generally obtained in the “righting reflex” component of the neurological examination (test item 13) where normal guinea pigs were found to occasionally have difficulty righting in this test.

Individual test items were also graphed (data not shown), providing an indication of the onset of pathology represented by each test item. These graphs enabled the construction of a timeline of progression of pathology.
Figure 3.11a: Neurological examination: total score (% affected)
Test items 1-25, inclusive (see Figure 3.10). Data shown is the score for each animal expressed as a percentage of the total possible score.

Legend:

- - α-mannosidosis

- - normal
Figure 3.11a
Figure 3.11b: Neurological examination: neurological score (% affected)
Test items 4-25, inclusive (see Figure 3.10). Data shown is the score for each animal expressed as a percentage of the total possible score.

Legend:

- [ ] α-mannosidosis
- [ ] normal
Figure 3.11c: Neurological examination: physical appearance summary
Test items 1-3c, inclusive (see Figure 3.10). Data shown is the score for each animal expressed out of a total maximum of 7 points.

Legend:

- [ ] ++ α-mannosidosis
- [ ] normal
Figure 3.11d: Neurological examination: behaviour/gait summary. Test items 4-12, inclusive (see Figure 3.10). Data shown is the score for each animal expressed out of a total maximum of 21 points.

Legend:

- [-] α-mannosidosis
- [ ] normal
Figure 3.11e: Neurological examination: postural reactions summary
Test items 13-16, inclusive (see Figure 3.10). Data shown is the score for each animal expressed out of a total maximum of 8 points.

Legend:

- α-mannosidosis
- normal
Figure 3.11f: Neurological examination: cranial nerves summary
Test items 17-25, inclusive (see Figure 3.10). Data shown is the score for each animal expressed out of a total maximum of 12 points.

Legend:

- ⬤ α-mannosidosis
- ⬤ normal
Timeline of α-mannosidosis pathology as observed through neurological examinations

<table>
<thead>
<tr>
<th>Age</th>
<th>Physical/neurological change observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 days</td>
<td>None; α-mannosidosis guinea pigs appear identical to normal/heterozygous guinea pigs.</td>
</tr>
<tr>
<td>20 days</td>
<td>Very mild facial dysmorphia first observed (test item 1); animals first begin to struggle more during handling (test item 7); first signs of mild hyperaesthesia (test item 12); slow correction first observed in righting reflex (test item 13).</td>
</tr>
<tr>
<td>30 days</td>
<td>Hunched posture while sitting first noticeable (test item 6); body posture (stretched) during walking first begins to change (test item 11); first signs of coarse movement in nose, nostrils and whiskers noticeable (test item 19).</td>
</tr>
<tr>
<td>40 days</td>
<td>Muscular condition first noticeably deteriorated (test item 3a); animals begin to appear less alert (test item 4); movement begins to appear slightly abnormal (test item 5); animals struggle to an even greater extent during handling (test item 7); mild abnormalities noticeable in hind-limb gait (test item 10); hyperaesthesia now more pronounced (test item 12); abnormal medial and lateral canthus reaction in response to touch noticeable (test items 17 and 18).</td>
</tr>
<tr>
<td>50 days</td>
<td>Moderate facial dysmorphia now observed (test item 1); nose, nostril and whisker movement in response to touch now noticeably reduced (test item 20).</td>
</tr>
<tr>
<td>60 days</td>
<td>Forelegs now observed to be tuck under body while animal is sitting (test item 2); hair appears coarse (test item 3b); some animals display reduced grooming (test item 3c); ability to lift head up is noticeably reduced (test item 8); “wheelbarrow” test in some animals shows noticeable stumbling (test item 16); Gait length and width (normalised to weight) are increased (relative to normal animals); gait length/width ratio is decreased (relative to normal animals); trends towards statistically significant memory and learning deficits are detectable using the Morris water maze test.</td>
</tr>
<tr>
<td>70 days</td>
<td>Movement of animals now appears slow, laboured and jerky (test item 5); animals now struggle excessively during handling (test item 7); righting reflex is further impaired- animal may be observed to fall to other side following righting (test item 13).</td>
</tr>
<tr>
<td>80 days</td>
<td>Animals now observed to be fairly dull and unresponsive (test item 4); hind-limb gait noticeably different with frequent “bunny hopping” or shuffling (test item 10); facial symmetry (including eye position) first noticeably different (test item 21); head tilt first noticed (test item 22);</td>
</tr>
</tbody>
</table>
horizontal nystagmus (moving head side to side) first noticed in some animals (test item 25).

90 days

More severe facial dysmorphia now noticeable (test item 1); gait length and width (normalised to weight) are further increased (relative to normal animals); gait length/width ratio is further decreased (relative to normal animals); significant memory and learning deficits are now detectable using the Morris water maze test.

>100 days

Noticeable ataxia (whole body swaying when walking, test item 9); wide head excursions evident following righting reflex test (test item 14); nystagmus observed following righting reflex test (test item 15); nose, nostril and whisker movement minimal (test item 19); resting and vertical nystagmus observed (test items 23 and 24).

3.3.3 Discussion: neurological examinations

Graphing of all measures observed in the neurological examinations showed a clear progression of pathology in α-mannosidosis animals. Normal animals did not demonstrate scores beyond four points out of a total of 48 (approximately 8%) for this test, demonstrating the effectiveness of this test for observing α-mannosidosis pathology specifically. Significant variation was observed in scores obtained for α-mannosidosis animals throughout the course of the period analysed. While this may be due in part to the subjective nature of the test, it also suggests that significant variation exists between individuals in the rate of progression and severity of pathology.

Due to the subjective nature of this test, it is not as rigorously quantitative as the Morris water maze and gait analysis. However, the convenience and possibility of repeated testing suggests promise for inclusion as a component of a battery of tests to chart the development of pathology, and thus as an additional tool for evaluating potential therapies. Additionally, the broad observations in this test provide an effective means of observing the neurological characteristics of all animals in a study, and thus any rapid changes that may occur in their condition. This is important where safety concerns exist, such as in evaluating the cell-based therapy described later in this thesis.
Chapter 4:
Construction and evaluation of a mouse embryonic stem cell line for the sustained expression of recombinant human \(\alpha\)-mannosidase
4.1 Introduction

Previous studies investigating stem cell-based approaches have shown promise for treatment of neurological pathology in LSD (Snyder et al., 1995; Lacorazza et al., 1996; Jin et al., 2002; Meng et al., 2003). These studies achieved therapeutic gene expression in the brain in mouse models of LSD by using genetically modified adult stem cells as a production and delivery vector for the deficient enzyme.

Two of the critical issues remaining with the use of adult stem cells such as NSCs are the requirement for ongoing derivation (with the potential lack of consistency between source populations that may arise as a result) and availability of source material. The latter is of particular concern, as the requirement for cadavers and abortuses is likely to ultimately preclude these approaches from widespread clinical application. Although other more readily obtainable adult stem cells have shown potential for treatment of neurodegenerative diseases (such as mesenchymal stem cells from bone marrow and cells from umbilical cord blood), issues concerning observations of plasticity and amenability to expansion and manipulation in culture still need to be resolved (Daley et al., 2003).

At present, ES cells represent one of the most promising source populations of cells for widespread therapeutic application; theoretically, they are infinitely expandable in culture, highly amenable to genetic modification and able to produce a wide variety of clinically relevant phenotypes, including NSCs (Tropepe et al., 2001; Rathjen et al., 2002; Stavridis and Smith 2003; Barberi et al., 2003). Additionally, strategies for the induction of tolerance to allogeneic ES cells may eventually facilitate ES cell-based cell therapies without the requirement for immunosuppression (Fändrich et al., 2003). With the further development of reproducible and efficient *in vitro* differentiation protocols for the generation of homogeneous cell populations for transplantation, ES cell-based therapies show great promise for treatment of neurological disease in LSD.

This thesis aims to pursue a stem cell-based approach for therapy in α-mannosidosis, by creating an α-mannosidase over-expressing mouse ES cell line. For this cell line to be therapeutically useful it must be able to produce and secrete α-mannosidase that is able to be taken up by host cells and effectively targeted to the lysosome. Additionally, high levels of expression should ideally be maintained throughout differentiation, such that terminally differentiated progeny of implanted
stem cells will continue to produce α-mannosidase following implantation and in vivo differentiation.

Mouse D3 ES cells (Doetschman et al., 1985) have been used for construction of these lines, rather than conditionally immortalised neural progenitors described in many of the previous approaches (Snyder et al., 1995; Lacorazza et al., 1996; Meng et al., 2003). Transgenic mouse D3 ES cells have been used previously for other studies such as the generation of Nurr1 expressing cell lines for generation of dopaminergic neurons (Chung et al., 2002). ES cells are less restricted in their differentiation potential than neural progenitors, are highly amenable to genetic modification (including clonal isolation of transformed cells), and demonstrate a high proliferative capacity (without genetic modification), theoretically being able to divide indefinitely in culture (Rathjen et al., 1998). Thus, ES cell lines represent one of the most flexible source populations for stem cell therapies, particularly when combined with various specific differentiation protocols for generation of diverse and potentially therapeutically useful cell populations, such as the MEDII differentiation protocol (Rathjen et al., 1999). This protocol has been developed for directed differentiation of ES cells to primitive ectoderm-like cells, and ultimately neural progenitors and other terminally differentiated cells of a neur ectodermal lineage (Rathjen et al., 1999; 2002). Cells generated using this protocol have been shown to exhibit similar differentiation potential to the conditionally immortalised neural progenitors described above (Rathjen et al, 2002).

In the present study, vectors constructed to facilitate expression of recombinant human α-mannosidase (rαM) were used to transfect ES cell lines. These cell lines were then screened to find over-expressing clones that were further characterised through differentiation using a modified version of the MEDII differentiation protocol, to confirm that this high expression was sustained throughout differentiation. The highest expressing clone was evaluated to ensure that genetic modification did not qualitatively change their differentiation potential. Finally, a cross-correction experiment was carried out to confirm that enzyme produced and secreted by these cells was able to be taken up and targeted to the lysosome of deficient cells, to mediate a reduction in levels of stored oligosaccharides.
4.2 Specific methods

The aim of this part of the study was to generate α-mannosidase over-expressing ES cell lines that retained the differentiation potential and cell culture characteristics of untransfected ES cells. The differentiation protocol employed involved the use of the conditioned media MEDII, as described by Rathjen and others in 1999. This protocol generated aggregates of cells referred to as EBMs (embryoid bodies conditioned in MEDII), with the following number indicating the number of days of differentiation post ES cell, i.e. EBM-4 refers to embryoid bodies conditioned in MEDII for four days (see Section 2.2.3.4).

One of the methods used to evaluate the cell lines generated was to record the subjective appearance of embryoid bodies during differentiation using the MEDII differentiation protocol. This was done by scoring the overall appearance of embryoid bodies based on a number of criteria, described below. These criteria and scoring system were developed in consultation with Dr. Joy Rathjen (Department of Molecular Biosciences, University of Adelaide). All other methods for molecular biology, cell culture and enzyme analysis utilised in this chapter are described in Chapter 2.

4.2.1 Scoring of embryoid bodies at day 4 (EBM-4) and day 12 (EBM-12) of the MEDII differentiation protocol

s: Smoothness of embryoid bodies

The MEDII differentiation protocol was employed in this study to induce the formation of neurectoderm in cultured embryoid bodies. Embryoid bodies that responded well to this induction often displayed a smooth surface, characteristic of neurectoderm formation (in contrast to nodules and "budding" on the surface of the bodies, indicative of endoderm formation; Rathjen et al., 2002). Bodies were scored with "+", "++", or "+++" based on the degree of smoothness they exhibited. Embryoid bodies observed to have numerous regions of non-neurectoderm tissue outgrowths, nodules or "budding" were scored with "-", "--", or "---" based on the frequency and size of these outgrowths. No "++" or "-" indicates that this cell population lay in the middle of the spectrum in this respect. This was defined as the majority of bodies displaying a smooth surface, with approximately 15-20% of bodies showing some evidence of endoderm formation.
n: Cavitation/neurectoderm of embryoid bodies

Neurectoderm induction in embryoid bodies also resulted in the characteristic cavitation in the centre of the bodies, indicative of neurectoderm formation (Rathjen et al., 2002). This involved encapsulation by a stratified epithelial sheet, in contrast to the formation of large cysts indicative of endoderm formation. The degree of cavitation was scored with “+”, “++”, “+++”, while the formation of cystic bodies was scored with “-”, “--”, or “---”. No “+” or “-” indicates that this cell population lay in the middle of the spectrum in this respect. This was defined as the majority of bodies displaying a relatively uniform appearance (without endodermal cysts), with stratified neurectoderm observed in approximately 60% of bodies.

x: Necrosis within embryoid bodies

Embryoid bodies that reliably formed neurectoderm following induction with MEDII were observed to have a single, internal area of cell death (black area) within the centre of the bodies at day four, in contrast to multiple areas of necrosis observed in embryoid bodies generated without MEDII induction (Rathjen et al., 2002). Embryoid bodies at day 12 were scored generally for necrosis (bodies that exhibited thick neurectoderm rarely displayed notably necrotic areas). The lack of widespread, multiple areas of necrosis was scored with “+”, “++”, or “+++”, and necrotic areas in the bodies scored with “-”, “--”, or “---” as described above. No “+” or “-” indicates that this cell population lay in the middle of the spectrum in this respect, which was defined as necrotic areas being observed in approximately 10% of bodies.

b: Beating areas within embryoid bodies

Beating muscle is formed by cells of a non-neurectodermal lineage. Thus, the presence of this cell type was an indication of ineffective or incomplete neural induction by the MEDII differentiation protocol. The absence of areas of beating muscle was scored with “+”, “++”, or “+++”, while the presence of beating muscle in the embryoid bodies was scored with “-”, “--”, or “---” as described above. No “+” or “-” indicates that this cell population lay in the middle of the spectrum in this respect, which was defined as only 1-2% of bodies displaying areas of beating muscle.
m: Maturity of embryoid bodies

This subjective observation was a reflection of how rapidly differentiation proceeded in the given population of embryoid bodies, relative to what was usually observed using this differentiation protocol with untransfected D3 ES cells; “+++” indicated a very rapid formation of morphologically distinct tissue types in the embryoid bodies, while “---” indicated a very slowly progressing cell population, with very little differentiation. Other scores indicated levels of maturity between these two extremes, with no “+” or “-” indicating “average” maturity at each time point for an embryoid body population.

4.2.2 Scoring of embryoid bodies seeded at day nine of the MEDII differentiation protocol and grown in an adherent culture for a further 12 days (EBM-21)

n: Neurites extending out from embryoid body

Embryoid bodies grown in adherent culture following seeding onto tissue culture plates displayed cell outgrowths of numerous types, including neurites (long fibrous projections). The level of neurite outgrowth was indicated by “+”, “++”, or “+++”, with the latter representing highly prevalent neurite formation; “-” indicated relatively poor neurite formation.

f: Fibroblastic cells growing around embryoid body

Following seeding of embryoid bodies, outgrowths of cells of a fibroblastic morphology were also observed. Prevalence of outgrowths of this appearance were indicated with “+”, “++”, or “+++”, with the latter representing observation of the most numerous outgrowths.

d: Density of outgrowths from embryoid body

Outgrowths from embryoid bodies varied considerably in density. Density of outgrowths was scored with “+”, “++”, or “+++”, with the latter representing highly dense outgrowths. Occasionally, few outgrowths were seen from seeded embryoid bodies; this was indicated with the score “-“.
4.3 Results

4.3.1a Construction of the vector *pEFIRESpuro-α-mann*

The vector *pEFIRESpuro-6* was provided by Dr. Dan Peet (University of Adelaide, see Appendix 2 for details of this vector). The vector *pcDNA3.1ATG2*, containing the human α-mannosidase cDNA, was provided by Dr. Thomas Berg and originally cloned by Nilssen et al., 1997 (see Appendix 2 for further details of this vector). *pcDNA3.1ATG2* was digested with XbaI to provide the 3.2kb human α-mannosidase cDNA fragment before subcloning into the XbaI site of *pEFIRESpuro-6*, to create the vector *pEFIRESpuro-α-mann* (see Figure 4.1 for details of the expression cassette of this vector). Insert orientation was confirmed by digestion with XbaI and NotI.

4.3.1b Construction of the vector *pCAGα-mann*

The vectors *pENTR11* and *pCAG1NeoGW* were provided by Dr. Stephen Wood (Child Health Research Unit, Adelaide, see Appendix 2 for details of these vectors). *pCAGα-mann* was created by first subcloning the 3.2kb fragment of the KpnI/Sall digested *pcDNA3.1ATG2* into the KpnI/XhoI linearised *pENTR11* (Gateway entry vector). The resultant *pENTRα-mann* was then combined with *pCAG1NeoGW* (Gateway destination vector) in a recombination reaction (see Section 2.2.1.6) to create *pCAGα-mann* (see Figure 4.1 for details of the expression cassette of this vector). The insert orientation was once again confirmed by separate KpnI and ScaI digests.
Figure 4.1: Schematic diagram of vectors constructed in this study
The expression cassettes of these two vectors pCAGα-mann (A) and pEFIREα-mann (B) were as shown. "CMV": Human CMV immediate early enhancer “chβA”: Chicken β-actin promoter. “rhαM”: Human α-mannosidase cDNA. “IRES”: Internal ribosomal entry site. “neo”: Neomycin resistance gene. “puro”: Puromycin resistance gene.
Figure 4.1

A: pCAG\(\alpha\)-mann

\[
\text{CMV} \quad \text{ch\(\beta\)A} \quad \text{rh}\(\alpha\)M \quad \text{IRES} \quad \text{neo}
\]

B: pEF\text{IFRES}\(\alpha\)-mann

\[
\text{EF}\text{1}\(\alpha\) \quad \text{rh}\(\alpha\)M \quad \text{IRES} \quad \text{puro}
\]
4.3.2 Testing of $\alpha$-mannosidase expression by $pCAG\alpha$-mann and $pEFIRE\alpha$-mann after transfection into CHO cells

Both vectors were linearised by digestion with the restriction enzyme PvuI prior to transfection. Calcium phosphate-mediated transfection was used for these transformations, as described in Section 2.2.3.1c. Briefly, CHO cells were grown to approximately 70% confluence before transfection. Calcium phosphate/DNA precipitate (containing 5µg of vector DNA) was incubated with cells for 24 hours; cells were then fed and allowed to recover for three more days before splitting and placing under selection with either 10µg/ml puromycin or 750µg/ml G418. Cells were maintained under these conditions for three passages (approximately two weeks) before harvesting by trypsinization and lysis in PBS lysis buffer. $\alpha$-Mannosidase activity was measured using the substrate 4-methylumbelliferyl-$\alpha$-D-mannopyranoside and protein concentrations were quantitated as described in Section 2.2.2. All activities and proteins were measured in triplicate, and activity was normalised to total cell protein.

Cell populations transfected with either $pEFIRE\alpha$-mann or $pCAG\alpha$-mann were found to have significantly elevated levels of $\alpha$-mannosidase activity (see Figure 4.2, 2.44±0.10nmol/min/mg for $pEFIRE\alpha$-mann transfectants, 4.20±0.12nmol/min/mg for $pCAG\alpha$-mann transfectants) compared to untransfected CHO cells (0.33±0.01nmol/min/mg), suggesting effective expression of functional $\alpha$-mannosidase with both of these vectors.

4.3.3 Transfection of $pCAG\alpha$-mann and $pEFIRE\alpha$-mann into D3 ES cells

Electroporation of vectors into D3 ES cells, selection of stably transformed clones and expansion of these clones was carried out as described in Section 2.2.3.3. Cells were placed under antibiotic selection 24 hours following transfection and fed daily with fresh medium. Selection was carried out in parallel with untransfected D3 ES cells to verify that the selection protocol allowed only the survival of successfully transfected clones. Clones were picked 8-11 days post-transfection, once all cells on the control plate (untransfected D3 ES cells) had died under selection and individual colonies were large enough to be accurately picked.
Figure 4.2: Testing of vectors in Chinese hamster ovary (CHO) cells
Expression of recombinant human α-mannosidase by pCAGα-mann and pEFIREα-mann was verified by transfection into CHO cells. Total α-mannosidase activities were quantitated as described in Section 2.2.2.1. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were ±1SD. Activities and proteins were measured in triplicate.
Figure 4.2

Specific Activity (nmol/min/mg)

- untransfected control
- pEFIREsa-mann transfected
- pCAGa-mann transfected
Sixteen \textit{pCAG\alpha-mann} transfected clones and fifteen \textit{pEFIRES\alpha-mann} transfected clones were isolated, expanded, frozen down and catalogued for future use. Embryoid bodies were also generated from each clone, as described below. All clones described were found to generate colonies within the selection period described. Untransfected cells did not generate resistant colonies in this protocol.

### 4.3.4 Testing of \(\alpha\)-mannosidase activity in clones throughout differentiation: experiment 1

To determine whether cell lines generated by transfection with these vectors were able to express high levels of recombinant human \(\alpha\)-mannosidase and sustain this expression throughout differentiation, embryoid bodies from each clone were set up using the MEDII differentiation system (see Section 2.2.3.4). Untransfected D3 ES cells were also used to generate embryoid bodies, as controls.

Samples from each clone were harvested as undifferentiated ES cells (prior to commencement of the differentiation protocol), EBM-4 (embryoid bodies harvested four days after commencement of the differentiation protocol), and EBM-12 (embryoid bodies harvested 12 days after commencement of the differentiation protocol). Each sample was collected by harvesting a 10cm plate of ES cells or embryoid bodies, in a volume of 500\(\mu\)L PBS lysis buffer. Total activities were measured (as per Section 2.2.2.1); human specific activities were measured in a similar fashion, except incorporating an immunocapture stage into the protocol using a mouse monoclonal specific for human \(\alpha\)-mannosidase (as per Section 2.2.2.2). Proteins were measured as described in Section 2.2.2.3. All activities and proteins were measured in triplicate.

ES cell clones transfected with \textit{pCAG\alpha-mann} were found to have only marginally elevated levels of total \(\alpha\)-mannosidase activity relative to levels observed in untransfected ES cells, in select clones (see Figure 4.3). Analysed at the EBM-12 stage of differentiation, \textit{pCAG\alpha-mann} transfected clones did not show a significant increase in total \(\alpha\)-mannosidase expression relative to endogenous \(\alpha\)-mannosidase expression levels measured in untransfected D3 ES cells (see Figure 4.3). Further analysis of human \(\alpha\)-mannosidase activity (see Figure 4.4) indicated that \(\alpha\)-mannosidase activity resulting from transgene expression (rhoM) was below endogenous \(\alpha\)-mannosidase expression levels. Thus, \textit{pCAG\alpha-mann} transfection was not found to facilitate over-
Figure 4.3: Total α-mannosidase activity in pCAGα-mann transfected ES cell clones: differentiation experiment 1

Total α-mannosidase activities were quantitated as described in Section 2.2.2.1. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with pCAGα-mann. “EBM-4” describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol.

Legend:
- ES cell
- EBM-4
- EBM-12

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Figure 4.4: Human α-mannosidase activity in \( pCAG\alpha\text{-mann} \) transfected ES cell clones: differentiation experiment 1

ραM activities were quantitated as described in Section 2.2.2.2. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with \( pCAG\alpha\text{-mann} \). "EBM-4" describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. "EBM-12" describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol.

Legend:

- ES cell
- EBM-4
- EBM-12
expression of rhαM in differentiated ES cell clones.

In contrast, ES cell clones transfected with *pEFIREScα-mann* displayed greatly increased total α-mannosidase activity at the undifferentiated ES cell stage (see Figure 4.5). These activities were observed to decrease notably throughout the course of differentiation. These results were verified by analysis of human α-mannosidase activity, which showed similar patterns throughout differentiation (see Figure 4.6). Despite the reduction in rhαM activity throughout differentiation, over-expression (total α-mannosidase activities significantly above endogenous levels measured in untransfected cells) was still observed in select *pEFIREScα-mann* transfected clones at the EBM-12 stage, of up to 325% of endogenous levels (measured in clone EF3; see Figure 4.6).

4.3.5 Testing of α-mannosidase activity in high expressing clones throughout further differentiation: experiment 2

Following the analysis of transfected clones differentiated to EBM-12 described above, differentiation was repeated with five clones transfected with each of *pCAGα-mann* and *pEFIREScα-mann*. Clones were chosen based on observed high expression levels at EBM-12 and EBM-4 stages (as described above), as well as growth characteristics and appearance in culture (data not shown). Clones G5, G8, G9, G12, G15, EF1, EF3, EF7, EF8 and EF15 were differentiated as described previously, with samples harvested at the undifferentiated ES cell stage (before commencing the differentiation protocol), EBM-4 and EBM-12 as before. Additionally, embryoid bodies from each clone were seeded onto 10cm tissue culture dishes (as per Section 2.2.3.4c) nine days following commencement of the differentiation protocol and grown until day 21 when numerous outgrowths were observed. Cells were then trypsinised and harvested as before. This stage was evaluated because it represented a heterogenous mixture of many terminally differentiated cell phenotypes, similar to what might be expected in a graft following implantation. Although *pCAGα-mann* transfected clones were not found to express high levels of human α-mannosidase in the analysis described above (relative to levels seen in *pEFIREScα-mann* transfected clones), they were included in this experiment to confirm their lack of potential utility for this approach.
Figure 4.5: Total α-mannosidase activity in pEFIREα-mann transfected ES cell clones: differentiation experiment 1

Total α-mannosidase activities were quantitated as described in Section 2.2.2.1. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with pEFIREα-mann. “EBM-4” describes embryoid bodies formed using the MEDII differentiation, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol.

Legend:

- ES cell
- EBM-4
- EBM-12
Figure 4.5

![Bar Chart]

**Specific Activity (nmol/min/mg)**

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</table>

The chart shows the specific activity for various clones, with clone EF12 having the highest activity.
Figure 4.6: Human α-mannosidase activity in \( pEFIRESa-mann \) transfected ES cell clones: differentiation experiment 1
rhoαM activities were quantitated as described in Section 2.2.2.2. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with \( pEFIRESa-mann \). “EBM-4” describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol.

Legend:

- ES cell
- EBM-4
- EBM-12
pCAGα-mann transfected clones provided similar results as described in Section 4.3.4 (see Figure 4.7), with clone G8 the only cell line demonstrated to maintain notable rhαM expression out to day 21 of the differentiation protocol (see Figure 4.8). This activity, however, represented less than a two-fold increase above endogenous expression levels, once again confirming that pCAGα-mann transfection does not facilitate over-expression of α-mannosidase in this system. pEFIREα-mann transfected clones again demonstrated a dramatic increase above expression levels observed in untransfected cells (see Figure 4.9), which was verified by analysis of rhαM activities (see Figure 4.10). Clone EF3 was found to produce the highest levels of rhαM at the EBM-21 stage, resulting in total α-mannosidase activity observed to be approximately 400% of endogenous levels. This clone also demonstrated embryoid body formation similar in morphology to that observed for untransfected D3 ES cells (see Tables 4.1, 4.2 and 4.3) in the MEDII differentiation protocol. For these reasons, this clone was chosen for further characterisation.

Subjective analysis of embryoid bodies generated from different clones was undertaken in both differentiation experiments to confirm that transfected clones were all able to generate bodies not morphologically dissimilar to those generated by untransfected cells. Clones were observed to all generate morphologically similar embryoid bodies (see Tables 1 and 2) and similar outgrowths following seeding (see Table 3), with the notable exception of clones G5 and G9. These two clones appeared to respond poorly to the neur ectoderm induction of the MEDII protocol in both differentiation experiments, and appeared to have fewer neural outgrowths following seeding. Although not a definitive method of analysing differentiation potential, this observation suggests that careful evaluation of these clones would be required if they were to be used for further studies, to ensure that they retained their ability to respond to extrinsic differentiation factors in a similar fashion to untransfected ES cells.
Figure 4.7: Total α-mannosidase activity in pCAGα-mann transfected clones: differentiation experiment 2

Total α-mannosidase activities were quantitated as described in Section 2.2.2.1. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with pCAGα-mann. “EBM-4” describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol. “EBM-21” describes seeded embryoid bodies harvested 21 days following initiation of the protocol.

Legend:

- ES cell
- EBM-4
- EBM-12
- EBM-21

Figure 4.8: Human α-mannosidase activity in pCAGα-mann transfected clones: differentiation experiment 2

rhαM activities were quantitated as described in Section 2.2.2.2. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with pCAGα-mann. “EBM-4” describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol. “EBM-21” describes seeded embryoid bodies harvested 21 days following initiation of the protocol.

Legend:

- ES cell
- EBM-4
- EBM-12
- EBM-21

255
Figure 4.9: Total α-mannosidase activity in pEFIRESa-mann transfected clones: differentiation experiment 2
Total α-mannosidase activities were quantitated as described in Section 2.2.2.1. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with pEFIRESa-mann. “EBM-4” describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol. “EBM-21” describes seeded embryoid bodies harvested 21 days following initiation of the protocol.

Legend:
- ES cell
- EBM-4
- EBM-12
- EBM-21

Figure 4.10: Human α-mannosidase activity in pEFIRESa-mann transfected clones: differentiation experiment 2
rhαM activities were quantitated as described in Section 2.2.2.2. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate. D3 ES cells were untransfected. All other clones described were D3 ES cells transfected with pEFIRESa-mann. “EBM-4” describes embryoid bodies formed using the MEDII differentiation system, harvested four days following initiation of the protocol. “EBM-12” describes embryoid bodies formed in a similar fashion, harvested 12 days following initiation of the protocol. “EBM-21” describes seeded embryoid bodies harvested 21 days following initiation of the protocol.

Legend:
- ES cell
- EBM-4
- EBM-12
- EBM-21
Table 4.1: Clone condition throughout differentiation: differentiation experiment 1

Legend:

s: smoothness of embryoid bodies
n: cavitation/neurectoderm of embryoid bodies
x: necrosis within embryoid bodies
b: beating areas within embryoid bodies
m: maturity of embryoid bodies

(see specific methods for further description of these terms)
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Table 4.2: Differentiation experiment 2

Legend:

s: smoothness of embryoid bodies
n: cavitation/neurectoderm of embryoid bodies
x: necrosis within embryoid bodies
b: beating areas within embryoid bodies
m: maturity of embryoid bodies

(see specific methods for further description of these terms)

Table 4.3: Differentiation experiment 2

Legend:

n: neurites extending out from embryoid body
f: fibroblastic cells growing around embryoid body
d: density of outgrowths from embryoid body

(see specific methods for further description of these terms)
Table 4.2: Differentiation experiment 2

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Table 4.3: Differentiation experiment 2

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</table>
4.3.6 Testing of clone EF3: lineage analysis

To confirm that ES cells transfected with pEFIREα-mann (clone EF3) retained a similar differentiation potential to untransfected ES cells, a qualitative assessment of immunoreactivity for a number of lineage markers was carried out in cell populations derived from EF3 ES cells, with untransfected D3 ES cells used as a control. Differentiation to EBM-21 stage was carried out as described previously, except that EBMs were seeded onto sterile coverslips (as per Section 2.2.3.4d). Immunofluorescence was carried out as described in Section 2.2.5.3.

Undifferentiated embryonic stem cells from (both untransfected D3s and clone EF3) were first analysed for Oct4 (Octamer-4, a marker of pluripotent cells, Rosner et al., 1990) immunoreactivity. Almost all cells from Both D3 and EF3 populations were found to be Oct4 positive (see Figure 4.11), with no differences observed between D3 and EF3 cells in this respect.

Following differentiation of cells from both D3 and EF3 cell populations to the EBM-21 stage, Oct4 immunoreactivity was again analysed, along with Nestin (a marker of neural progenitors, Zimmerman et al., 1994). Both cell populations displayed largely Oct4 negative embryoid bodies, with the occasional pocket of Oct4 positive cells observed within some embryoid bodies (see Figure 4.12). No notable differences were observed between the overall incidence of Oct4 immunoreactive cells in either cell population. Additionally, both D3 and EF3 populations showed strong Nestin immunoreactivity, in both the bodies themselves and the cell outgrowths from these bodies. Again, no qualitative differences were observed between D3 and EF3 EBMs.

Further lineage analysis involved immunofluorescence for GFAP (glial fibrillary acidic protein, a marker expressed by both glial precursors and differentiated astrocytes, Landry et al., 1990), NF200 (neurofilament protein, 200kDa subunit, a marker for mature neuronal processes; Straznicky et al., 1992), and SMA (smooth muscle actin, a marker for smooth muscle cells, Skalli et al., 1986). For each of these markers, positive cells were observed within the embryoid bodies and in cell outgrowths (see Figure 4.13). No notable differences were observed between untransfected D3- or EF3-derived embryoid bodies in any of these markers.
Figure 4.11: Lineage analysis of D3 and EF3 ES cells
A and C show untransfected D3 ES cells. B and D show EF3 ES cells. A and B are negative controls (no l<sup>0</sup> antibody), C and D show Oct<sub>4</sub> positivity. For all pictures, blue: DAPI (nuclear fluorescence), red: Oct<sub>4</sub>. 
Figure 4.12: Lineage analysis of D3 and EF3 embryoid bodies
A, C and E are embryoid bodies derived from untransfected D3 ES cells, B, D and F are embryoid bodies derived from EF3 ES cells. For all pictures, blue: DAPI (nuclear fluorescence). A and B are negative controls (no 1° antibody). C and D- red: Oct4. Arrows indicate Oct4 positive regions. E and F- red: Nestin.
Figure 4.13: Lineage analysis of D3 and EF3 embryoid bodies
A, C and E are embryoid bodies derived from untransfected D3 ES cells, B, D and F are embryoid bodies derived from EF3 ES cells. For all pictures, blue: DAPI (nuclear fluorescence). A and B- green: GFAP. C and D- green: NF200. E and F- red: SMA.
The lack of qualitative differences observed in immunoreactivity between D3- and EF3-derived embryoid bodies suggests that the genetic modification carried out to generate the cell line EF3 did not noticeably affect the absolute differentiation potential of these cells. Embryoid bodies derived from this cell line form cells of multiple lineages during differentiation, in a similar fashion to that observed in embryoid bodies derived from untransfected D3 ES cells.

4.3.7 Testing of clone EF3: cross-correction of α-mannosidosis skin fibroblasts

To demonstrate a therapeutic effect in vivo, cells derived from the cell line EF3 would need to produce and secrete functional α-mannosidase (human and/or mouse) able to be taken up by deficient cells and targeted to the lysosome through a process of M6P receptor-mediated endocytosis. To confirm that enzyme produced by the ES cell line EF3 satisfied this criterion, a cross-correction experiment was undertaken (similar to that described in Berg et al., 2001), using conditioned media from seeded embryoid body cell populations (72 hours of conditioning, culminating at day 21 of the differentiation protocol) of both EF3 and D3 cells. Untransfected D3 cells were included to determine whether endogenous expression and secretion levels were sufficient to mediate a reduction in the level of stored oligosaccharide, within the limits of the cross-correction experiment performed.

Oligosaccharides Man2NacGluc and Man3NacGluc were analysed for this experiment, as described in Section 2.2.3.5b. Mass spectrometric assays were performed by Dr. Maria Fuller. These oligosaccharides have been shown to be reduced in α-mannosidosis skin fibroblasts following treatment with recombinant enzyme in previous cross-correction studies (Berg et al, 2001). The addition of 4x10^4U/ml α-mannosidase (consisting of both endogenous mouse and recombinant human enzyme) to the culture media in the present study resulted in a significantly reduced level of both oligosaccharides (sample E, Figures 4.14 and 4.15). This reduction was found to be dose-dependent, since addition of a lower dose of enzyme (through dilution of the culture media) from the same source (8.6x10^6U/ml α-mannosidase, sample D) resulted in a lesser reduction in oligosaccharide levels. Indeed, this reduction was found to be greater 48 hours following addition of the conditioned media sample than at 96 hours following addition of the conditioned media; this suggests that this lower dose of enzyme may have dropped below the threshold of enzyme activity required to mediate
Figure 4.14: Cross correction of α-mannosidosis skin fibroblasts: Man$_2$NAcGluc levels
The levels of oligosaccharides are expressed as a ratio of intensity (counts per second) relative to the internal standard methylactose. See below for details of samples A-G.

Figure 4.15: Cross correction of α-mannosidosis skin fibroblasts: Man$_2$NAcGluc levels
The levels of oligosaccharides are expressed as a ratio of intensity (counts per second) relative to the internal standard methylactose. See below for details of samples A-G.

Legend:

- 48 hours
- 96 hours

<table>
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<tbody>
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<td>Unconditioned</td>
</tr>
<tr>
<td>B</td>
<td>Normal skin fibroblasts (SF4976R8)</td>
<td>Unconditioned</td>
</tr>
<tr>
<td>C</td>
<td>α-Mannosidosis skin fibroblasts (SF3949R1R2R4)</td>
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</tr>
<tr>
<td>D</td>
<td>α-Mannosidosis skin fibroblasts (SF3949R1R2R4)</td>
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</tr>
<tr>
<td>E</td>
<td>α-Mannosidosis skin fibroblasts (SF3949R1R2R4)</td>
<td>4x10$^{-4}$U/ml α-mannosidase (EF3 conditioned, rhαM and endogenous enzyme)</td>
</tr>
<tr>
<td>F</td>
<td>α-Mannosidosis skin fibroblasts (SF3949R1R2R4)</td>
<td>4x10$^{-4}$U/ml α-mannosidase (EF3 conditioned, rhαM and endogenous enzyme) + 5mM M6P</td>
</tr>
<tr>
<td>G</td>
<td>α-Mannosidosis skin fibroblasts (SF3949R1R2R4)</td>
<td>8.6x10$^{-4}$U/ml α-mannosidase (D3 conditioned, endogenous enzyme only)</td>
</tr>
</tbody>
</table>
and maintain reduction in the levels of these stored oligosaccharides, allowing further accumulation. The addition of $8.6 \times 10^6$ U/ml $\alpha$-mannosidase in the form of conditioned media derived from untransfected D3 embryoid bodies (endogenous mouse enzyme only) also resulted in a reduction in the level of stored oligosaccharides (sample G), although to a lesser extent than the high dose conditioned media (sample E) derived from the cell line EF3. This result confirms that along with recombinant human $\alpha$-mannosidase, endogenous mouse $\alpha$-mannosidase can also be secreted by mouse cells, taken up by deficient skin fibroblasts, and mediate a reduction in the amount of stored oligosaccharides. Over-expression of rh$\alpha$M (as in cell line EF3) results in a higher level of $\alpha$-mannosidase activity secreted from the cell, and thus the potential to mediate a greater reduction in the levels of stored oligosaccharides in $\alpha$-mannosidosis skin fibroblasts, as assessed in this cross-correction experiment.

The addition of 5mM M6P to the conditioned media sample, along with $4 \times 10^{-4}$ U/ml $\alpha$-mannosidase (sample F), resulted in notably less reduction in the levels of the oligosaccharides Man$_2$NAcGluc and Man$_3$NAcGluc compared to enzyme alone. This result verifies that $\alpha$-mannosidase uptake and oligosaccharide reduction in this experiment occurs in a M6P-dependent fashion. This cell line can thus produce functional $\alpha$-mannosidase that is secreted and can be taken up and targeted to the lysosome via M6P receptor-mediated endocytosis.

4.3.8 Testing of clone EF3: demethylation experiment

As shown in Figures 4.5, 4.6, 4.9 and 4.10, pEFIR$\alpha$-mann transfected clones display elevated levels of total $\alpha$-mannosidase activity, which are observed to decline throughout differentiation. Both methylation-dependent and independent mechanisms have been shown to be responsible for transgene repression in ES cells (Cherry et al., 2000). To determine whether methylation-mediated transgene inactivation is partly responsible for this observed decline in expression, an experiment was carried out using the methyltransferase inhibitor 5-azadeoxycytidine (5-azadC), similar to that described in a previous study (Cherry et al., 2000). This demethylation agent was added to cultured cells at a concentration of 0.15$\mu$M on days 13 and 15 of the differentiation protocol. Cells were then fed day 18, prior to harvest at day 21 (EBM-21 stage) of the differentiation protocol, as described in Section 2.2.3.4.
Two separate culture dishes of EBM-21 cells were generated from EF3 ES cells, and when treated with 5-azadC were found to provide variable results (see Figure 4.16, E and F). Although an increase in both total and human activity was observed in one of the 5-azadC treated replicates (E) relative to untreated EF3 controls (C and D), this increase was only marginal compared to the total reduction in activity seen throughout differentiation of cell line EF3 (see Figure 4.10 for comparison). Additionally, the second 5-azadC treated EF3 cell population (F) did not show any increase in activity following treatment.
Figure 4.16: Demethylation experiment of EBM-21 cells from cell line EF3: α-mannosidase activity.
A and B are seeded EBM-21 cells derived from untransfected D3s, treated with 5-azadC. C and D are seeded EBM-21 cells derived from cell line EF3 (no 5-azadC treatment). E and F are seeded EBM-21 cells derived from cell line EF3 treated with 5-azadC. Total α-mannosidase activities were quantitated as described in Section 2.2.2.1, rhαM activities were quantitated as described in Section 2.2.2.2. Activities were expressed as nmol/min/mg of total cell protein. Error bars shown were +1SD. Activities and proteins were measured in triplicate.

Legend:

- Total Activity
- Human Activity
Figure 4.16

Specific Activity (nmol/min/mg)

A  B  C  D  E  F
4.4 Discussion

This chapter describes the generation and characterisation of the mouse ES cell line EF3, which expresses recombinant human α-mannosidase at levels approximately 15-fold above endogenous expression levels (measured at pluripotent ES cell stage). However, these expression levels decrease throughout differentiation to a level approximately four-fold above endogenous levels after 21 days of differentiation. Through analysis of various lineage markers in undifferentiated ES cells and their derivatives, this cell line was found qualitatively to have retained a similar differentiation potential to untransfected ES cells.

Differentiated derivatives of the cell line EF3 secreted enzyme (both rhαM and endogenous mouse α-mannosidase) that was able to mediate cross-correction of human α-mannosidosis skin fibroblasts in a dose-dependent and M6P-dependent fashion (as assessed by a reduction in the levels of two stored oligosaccharides). Although present at lower doses than enzyme secreted by the cell line EF3, endogenous mouse α-mannosidase (produced and secreted by untransfected D3 ES cells) also showed a cross-correction effect. The results of this experiment suggested that for a given dose of α-mannosidase activity provided to the deficient fibroblasts, endogenous enzyme (derived from untransfected D3 ES cells) was able to mediate a greater reduction in oligosaccharide levels than EF3-derived rhαM (see Figures 4.14 and 4.15, samples D and G). One possible explanation for this observation is that rhαM may have been less efficiently mannose-6-phosphorylated than endogenous mouse α-mannosidase, resulting in less efficient delivery of this enzyme to the lysosomes of deficient fibroblasts, and thus a smaller reduction in the levels of stored oligosaccharides. This has been previously reported to be of concern in the recombinant production of lysosomal enzymes such as α-N-acetylgalactosaminidase (Weber et al., 2001). Metabolic labelling experiments involving radiolabelled phosphate ions (similar to experiments described in Weber et al., 2001) could be employed to examine the efficiency of phosphorylation of rhαM by ES cells and their derivatives in this system.

The CMV enhancer/ chicken β-actin promoter (CAG) and the EF1α promoter have previously been evaluated to facilitate transgene expression in ES cells and their derivatives (Chung et al., 2002). This previous study reported a higher activity for the EF1α promoter compared to the CAG promoter in transiently transfected ES cells, and
showed that the activity of the promoter EF1α was found to drop (and was reported to be more variable) during differentiation. This previous study only analysed activity out to six days of differentiation, and only in cells transiently transfected two days prior to this analysis. In contrast to this previous study, the study described in this thesis involved stable transfection of ES cells, followed by isolation and evaluation of multiple clonal cell lines. Additionally, the evaluation of expression out to 21 days of differentiation (directed using the MEDII differentiation protocol) has not previously been reported for both of these vectors. To date, the creation of stably transfected mouse ES cell lines for the expression of a lysosomal enzyme has been reported only once previously (Lau et al., 2004).

In the present study, pCAGα-mann was found to facilitate sustained expression in select clones (such as clone G8, shown in Figure 4.8), although at moderate levels relative to endogenous α-mannosidase expression levels. In contrast, pEFIREα-mann was shown to facilitate significant over-expression of α-mannosidase. Despite the observation that these levels decline throughout differentiation, significant over-expression was still noted after three weeks of in vitro differentiation. These results suggest the need to evaluate appropriate expression vectors for each individual gene where over-expression is the ultimate aim of constructing the cell line. Indeed, a similar vector to pCAGα-mann driving expression of the enzyme sulphamidase (using the same promoter as the vector described in this study) has been shown to effectively facilitate sustained (out to 15 days of differentiation) over-expression in ES cells and their derivatives, relative to endogenous enzyme levels (Lau et al., 2004).

Studies involving the use of viral vectors (such as a mouse stem-cell virus, or MSCV) to facilitate transgene expression have shown a similar decline in the levels of transgene expression over time, which has been attributed in part to methylation-mediated transgene inactivation (Cherry et al. 2000). An experiment (similar to that described in Cherry et al., 2000) involving the use of a demethylation agent was carried out to determine if methylation-mediated transgene inactivation was in part responsible for the reduction in rhαM levels observed in pEFIREα-mann transfected clones through differentiation. The results of this demethylation experiment did not conclusively indicate methylation to be a factor in rhαM expression in the cell line EF3, and suggest that factors other than methylation may be contributing to the
decrease in activity observed in these cells throughout differentiation. Additionally, the 5-azadC dosage and treatment protocol utilised in this experiment may have resulted in incomplete demethylation in these highly differentiated cell populations. This has been previously suggested as a consideration for terminally differentiated cells, in comparison to ES cells (Cherry et al., 2000). However, this experiment would need to be repeated, perhaps with a higher dosage or more frequent treatments of 5-azadC, to confirm that methylation is not a contributing factor.

Other systems of gene regulation such as histone deacetylation have also been implicated in transgene silencing (Rosenqvist et al., 2002), although these mechanisms were not investigated in this thesis. The phenomenon of transgene inactivation is of particular concern to the ES cell-based approach described in this thesis, because it potentially undermines the use of a cell-based approach to mediate long-term therapeutic effect through expression of a deficient enzyme. The dramatic changes in gene expression that a population of stem cells will necessarily undergo during differentiation from a pluripotent phenotype (at which genetic modification has occurred) to an ultimately terminally differentiated phenotype additionally introduce further possibilities of transgene shutdown. The site of vector integration may thus have a profound effect on transgene expression, possibly because regions of the genome involved solely in early development may no longer be actively involved in gene expression in more mature cell phenotypes. Integration of the transgene into sites such as these during pluripotent ES cell stage could potentially result in markedly decreased transgene expression during differentiation. Additionally, the proximity of transgene insertion sites to other regulatory sequences such as those of ubiquitously expressed "housekeeping" genes has previously been shown to influence the expression of these transgenes (Cohen-Tannoudji, 2000). This, in part, could also explain the variation in expression seen in this study at various stages of differentiation for each of the individual cell lines (see Figures 4.8 and 4.10).

Additionally, by differentiating ES cells through generation and seeding of embryoid bodies, morphologically distinct, phenotypically heterogeneous populations of cells are created (as shown by the different immunoreactivity of cells observed, described in Section 4.3.6). Different differentiated phenotypes of cells may have variable responses to transgene expression (both in terms of inactivation mechanisms and promoter activity), which would further contribute to the variation observed in
expression, not only between different cell lines but also between different populations of embryoid bodies generated from the same cell line. The activity of the EF1α promoter might also be reduced in more differentiated or less metabolically active phenotypes such as those described at the EBM-4, EBM-12 and EBM-21 stages, relative to rapidly dividing undifferentiated ES cells. In the study mentioned above (Chung et al., 2002), the activity of the EF1α promoter was found to be more highly variable in the different cell populations analysed (such as differentiated and undifferentiated D3 and R1 ES cells) than the activity of the CAG promoter.

One of the primary advantages of pursuing a stem cell-based approach to therapy is the possibility of ultimately achieving sustained therapeutic benefit through long term graft survival and gene expression. The cell line EF3 was found to sustain elevated levels of expression of human α-mannosidase when differentiated for up to 21 days. Further analysis of longer-term expression would be prudent, however, before undertaking longer-term animal experiments using these cells. More extensive and defined differentiation protocols (such as those described in Barberi et al., 2003) could be employed to analyse expression levels in phenotypically defined and diverse populations of cells (for example, neural precursor as well as mature neuronal and glial phenotypes).

Sustained expression in multiple cell phenotypes would be an important validation of this cell line, since this therapeutic approach would ideally require α-mannosidase expression by all of the phenotypes that might ultimately arise in a differentiated stem cell graft. Additionally, teratoma experiments involving generation of sub-cutaneous teratomas in mice (similar to those described in Pfeifer et al., 2002) could be carried out to provide an in vivo differentiation environment. Histological analysis at various time points could then be employed to determine whether transgene expression is additionally maintained in vivo over these periods.

Ultimately, if transgene inactivation proves to be a significant concern for the cell line generated in this thesis (following further evaluation), alternative approaches to achieving gene expression could be considered. One possible approach could be the use of lentiviral vectors to facilitate gene expression. These vectors have been shown to evade some of the cellular mechanisms responsible for transgene inactivation in other systems, resulting in stable gene expression in both ES cells and transgenic mice (Pfeifer et al., 2001), and also in human ES cells (Ma et al., 2003). Further
understanding of the mechanisms involved in shutdown and the conditions required for stable gene expression will ultimately expand the potential for *ex vivo* gene therapy approaches involving ES cell lines.

Previous studies utilising the MEDII differentiation protocol have reported generation of homogeneous EBMs comprised of cells differentiated to neuroectodermal lineages, with low-level *Oct4* expression sustained by the majority of cells within the population, rather than sustained high-level expression by a small population of residual pluripotent cells within the bodies (analysed at EBM-9, Rathjen et al., 2002). Although the present study also found widespread differentiation to cells of a neuroectodermal lineage (see Figure 4.12E and F, and 4.13A-D), small pockets of *Oct4* immunoreactive cells (Figure 4.12C and D), and numerous *SMA* immunoreactive cells (of a mesodermal lineage, see Figures 4.13E and F) were also observed, even after 21 days of differentiation. Since the factors responsible for inductive activity in MEDII are yet to be fully characterised; it is possible that variation between batches in MEDII could result in variable inductive activity in this protocol. Thus, the EBMs generated in this study may have received sufficient induction to form neuroectoderm and further differentiated cells of this lineage, but insufficient induction for this directed differentiation to be homogeneous (in contrast to the previously described study). The full characterisation of the factors involved in this induction would allow for more tightly defined culture conditions and thus greater reproducibility in this protocol.

At present, it is unknown exactly how much graft-derived α-mannosidase would be required *in vivo* in order to see a therapeutic effect on neurological pathology. The observation of clinical benefit to neurological disease in α-mannosidosis from BMT alone (Walkley et al., 1994, Wall et al., 1998) suggests that very high expression levels may ultimately not be required to achieve the desired reduction in lysosomal storage. Indeed, the cross-correction experiment carried out also showed that differentiated untransfected D3 ES cells could secrete enzyme that could be taken up by α-mannosidosis skin fibroblasts to mediate a reduction in stored oligosaccharide levels. Therapeutic benefit as a result of stem cell implantation would be expected to depend on the dose of α-mannosidase delivered, and therefore the inclusion of untransfected control cells in implantation studies is not only important as a control for the genetic modification of over-expressing cell lines, but potentially as a therapeutically useful treatment group in itself. If untransfected cells were found to secrete sufficient levels of
α-mannosidase to achieve a therapeutic effect following implantation, this would also avoid the potential problems associated with transgene inactivation, as this enzyme would be expressed under control of endogenous regulatory elements. Additionally, avoiding the need for a specific genetic modification could potentially expedite the application of these cells to other LSD with neurological pathology. In a similar fashion, techniques generated using an unmodified cell approach could also be more readily applicable to human ES cells, which could further expedite the application of a stem cell-based therapeutic approach to larger animal models and ultimately humans.
Chapter 5:
Evaluation of survival and engraftment of mouse ES cell
derived implants in the guinea pig brain
5.1 Introduction

Stem cell therapy approaches have previously shown promise for treatment of LSD (Snyder et al., 1995; Lacorazza et al., 1996; Meng et al., 2003). These studies all used conditionally immortalised neural progenitor cells, but demonstrate proof of principle for these approaches; multipotent cells can be transplanted, engraft and migrate throughout the host brain, and secrete enzyme to mediate a reduction in lysosomal storage in the CNS.

As previously discussed, the studies described in this thesis involve the use of mouse ES cells as a source of cells for implantation. The characteristic of ES cells being readily expandable in culture (without conditional immortalisation) has facilitated the generation of α-mannosidase over-expressing cell lines with only minimal genetic modification. This addresses potential concerns about the theoretically tumorigenic potential of conditionally immortalised cell lines (Martinez-Serrano and Björklund, 1997) and the potential of disrupting functions critical to stem cell engraftment, as may occur following more extensive genetic manipulation (Flax et al., 1998). In combination with appropriate in vitro differentiation protocols, mouse ES cells have been shown to have the potential to form numerous mature neural cell phenotypes, including multipotent NSCs, which exhibit similar differentiation potential to previously used conditionally immortalised neural progenitors (Rathjen et al., 2002; Stavridis and Smith 2003; Barberi et al., 2003). However, current protocols for the in vitro neural differentiation of ES cells may also result in contamination with other pluripotent or non-neural cell types, as indicated in Figure 1.8.

In evaluating an ES cell-based therapeutic approach, following in vitro characterisation of the differentiation protocol and cells to be used, conditions for the stable and safe in vivo engraftment of cells must be investigated. To achieve this aim, this study involved implantation of cells derived from untransfected D3 mouse ES cells. Numerous studies have previously shown that pluripotent cells have the potential to form teratomas following implantation in the brain (Brüstle et al., 1997, Deacon et al., 1998, Björklund et al, 2002). Thus, any therapeutic approach involving pluripotent cells should involve in vitro differentiation prior to implantation.

Previous studies have suggested that differentiation to a neural phenotype may be the “default” differentiation state for ES cells, in the absence of external signals (Tropepe et al., 2001). This has been further proposed to be due to allowing autocrine
FGF signalling, in the absence of extrinsic signals (Ying et al, 2003). Additionally, ectoderm has been shown to differentiate into neural tissue when cell-cell interactions are disrupted by dissociation of the cells (Grünz and Tacke, 1989; Sato and Sargent, 1989), perhaps through a similar mechanism. As a result, others hypothesised that the differentiation of pluripotent cells to extra-neural lineages could be inhibited by ensuring that cell-cell connections are effectively disrupted prior to implantation of low density cell suspensions (Björklund et al., 2002).

This thesis has pursued a similar approach, involving implantation of low cell numbers (1.5x10^3 cells per side), with the addition of the MEDII differentiation protocol to the experimental design. As discussed previously, culture of ES cells in MEDII-conditioned media induces differentiation to a primitive ectoderm-like phenotype (Rathjen et al., 1999), then ultimately to neuroectoderm following further conditioning (Rathjen et al., 2002). The Björklund et al. (2002) study still observed teratomas in 20% of the animals implanted, despite the cell preparation protocol used. Other studies have hypothesised that stochastic elements or a community effect may also influence differentiation in cell populations (Ying et al, 2003). Thus, compared to ES cells differentiated for four days in fetal calf serum alone (such as in Björklund et al., 2002), we hypothesised that a greater proportion of EBM-4 cells generated for this study (see Section 2.2.3.4) will be of an ectodermal lineage as a result of this induction, which, when coupled with the dissociation protocol described by Björklund et al. (2002), should result in robust engraftment, neural differentiation and a decreased incidence of teratomas following implantation in the guinea pig brain.

The implantation site chosen for these studies was the dentate gyrus of the hippocampus. The hippocampus plays a major role in memory and learning in the brain (Mihailoff and Haines, 1997). Additionally, the rate of neurogenesis in the dentate gyrus of the hippocampus has been shown to vary between different mouse strains and correlate directly with the performance of these strains in the acquisition phase of the Morris water maze (Kempermann and Gage, 2002), suggesting the importance of hippocampal neurogenesis for effective memory and learning. Previously, lysosomal storage has been observed in the hippocampus of α-mannosidosis guinea pigs (Crawley et al., 1999); Chapter 3 of this thesis detailed significant differences in performance in the Morris water maze between normal and α-mannosidosis guinea pigs. These differences might in part be accounted for by lysosomal storage in the hippocampus.
disrupting various aspects of hippocampal development and/or function, although a causative link has yet to be established. Thus, providing \( \alpha \)-mannosidase to the hippocampus through stem cell implantation might result in a reduction in the cognitive deficits observed in \( \alpha \)-mannosidosis.

The aim of this study is thus to evaluate the potential of low cell number EBM-4 implants to engraft, differentiate to neural lineages and migrate throughout the guinea pig brain, with a view ultimately to using this approach to secrete enzyme in the brain, to therapeutic effect.
5.2 Specific methods

All methods were as described in Section 2.2.

5.3 Results

5.3.1 Striatal implants of EBM-9 cells

A preliminary study was carried out as an initial investigation into the survival of mouse ES cell-derived EBM-9 cells (see Section 2.2.3.4) in the guinea pig brain. These implants were carried out during the establishment of implantation protocols, and surgery was carried out by Natalie Hulbert (BresaGen Ltd.). Additionally, immunofluorescent techniques for the detection of mouse cells had not yet been established, and, as such, grafts in this preliminary study were evaluated by morphology alone. The striatum was chosen for these initial implants because it is a large, morphologically distinct structure, easily identifiable under light microscopy. Additionally, lysosomal storage has been observed in the striatum of α-mannosidosis guinea pigs (Crawley et al., 1999). In the absence of an appropriate atlas, injection coordinates were first confirmed by injection of 1μl toluidine blue dye in euthanased one week old animals. EBM-9 cells (a population equivalent to a neurectoderm-like monolayer; Rathjen et al., 2002) were chosen for this preliminary study, in the absence of further reports (Björklund et al., 2002) that led to the hypothesis outlined above for the use of EBM-4 cells. Guinea pigs were sacrificed at one week (n=3), one month (n=2) and two months (n=1) post-implantation. See Appendix 1 for further details of animals implanted in this preliminary study. All guinea pigs were treated with CSA as described in Section 2.2.4.4.

Surviving morphologically distinct grafts were observed out to two months following striatal implantation of 10^5 mouse EBM-9 cells (see Figures 5.1 and 5.2). Although the graft shown in Figure 5.2 may have been a teratoma (due to the presence of morphologically diverse structures within the graft itself), specific graft-derived cell phenotypes were not investigated to confirm this. However, no behavioural abnormalities were observed in any of these animals following implantation. Host immune response to implanted cells was also not investigated. The results of this preliminary study confirmed that these mouse cell grafts were able to survive in the host guinea pig brain out to two months following implantation.
Figure 5.1: One week striatal implants
Haematoxylin/eosin stained coronal section one week following implantation of $10^5$ EBM-9 cells. A shows the cortical surface (cs), needletract (nt) and the graft within the striatum. B is a higher magnification image of the graft within the striatum.
Figure 5.2: Two month striatal implants
Haematoxylin/Eosin stained coronal section two months following implantation of $10^5$ EBM-9 cells. A shows the cortical surface (cs), and the graft within the striatum. B is a higher magnification image of the graft within the striatum.
Following this result, further studies were carried out involving longer time points, lower numbers of cells and investigation of graft-derived cell phenotypes.

5.3.2 Hippocampal implants of EBM-4 cells

5.3.2.1 Low dose cell implants

Following the establishment of techniques for implantation and immunofluorescent detection of implanted mouse cells, a larger study was undertaken. This study involved implantation of $1.5 \times 10^3$ EBM-4 cells (see Section 2.2.3.4) bilaterally into the dentate gyrus of one week old guinea pigs. In the absence of an appropriate atlas, injection coordinates were first confirmed by injection of 1µl toluidine blue dye in euthanased one week old animals, followed by examination of cryostat-cut brain sections (this procedure was carried out by Dr. Kim Hemsley). Animals were all treated with CSA (see Section 2.2.4.4) commencing the day before surgery, and evaluated weekly using the neurological examination described in Section 3.3. Cell implanted normal animals were sacrificed at two days, two weeks, four weeks, eight weeks and 13 weeks following implantation, and each brain was prepared as described in Section 2.2.5.1. See Table 5.1 for details of the number of brains analysed from each group. Each brain was examined for the presence of surviving graft cells using the immunofluorescence techniques described in Section 2.2.5. Sagittal sections were analysed using mouse microsatellite in situ hybridisation and GFAP immunofluorescence, until at least two sections per animal were visualised showing the glial scar (observed locally at the injection site) caused by the injection procedure. Thorough analysis of all regions of these sections was carried out to ascertain whether each brain had surviving mouse cells. Surviving cells were defined as cells with fluorescent nuclear puncta, indicative of positivity for the mouse microsatellite in situ hybridisation. This marker has been shown to detect all mouse graft-derived cells, regardless of phenotype (Brüstle et al. 1995; Bruce Davidson, personal communication).

Surviving cells were detected in brains analysed at two days (Figure 5.3), two weeks (Figure 5.4), four weeks (Figure 5.5A) and eight weeks (Figure 5.5B). No surviving cells were detected in animals sacrificed 13 weeks following implantation (see Table 5.1 for numbers of animals with surviving cells detected compared to total numbers analysed). Only one of the cell-implanted animals was found to display a
Table 5.1: Number of cell implanted guinea pigs with graft cells detected at various time points

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<tr>
<td>2 days</td>
<td>Normal low cells</td>
<td>3</td>
<td>2 (66%)</td>
</tr>
<tr>
<td></td>
<td>Normal sham</td>
<td>2</td>
<td>0 -</td>
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<tr>
<td>2 weeks</td>
<td>Normal low cells</td>
<td>5</td>
<td>2 (40%)</td>
</tr>
<tr>
<td></td>
<td>Normal sham</td>
<td>3</td>
<td>0 -</td>
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<tr>
<td>4 weeks</td>
<td>Normal low cells</td>
<td>6</td>
<td>2 (33%)</td>
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<tr>
<td></td>
<td>Normal sham</td>
<td>3</td>
<td>0 -</td>
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<tr>
<td></td>
<td>Normal high cells</td>
<td>3</td>
<td>2 (66%)</td>
</tr>
<tr>
<td></td>
<td>α-Mannosidosis high cells</td>
<td>1</td>
<td>0 (0%)</td>
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<tr>
<td></td>
<td>α-Mannosidosis sham</td>
<td>2</td>
<td>0 -</td>
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<tr>
<td>8 weeks</td>
<td>Normal low cells</td>
<td>3</td>
<td>1 (33%)</td>
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<tr>
<td></td>
<td>Normal sham</td>
<td>2</td>
<td>0 -</td>
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<tr>
<td>13 weeks</td>
<td>Normal low cells</td>
<td>3</td>
<td>0 (0%)</td>
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<tr>
<td></td>
<td>Normal sham</td>
<td>3</td>
<td>0 -</td>
</tr>
<tr>
<td></td>
<td>α-Mannosidosis low cells</td>
<td>3</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>α-Mannosidosis sham</td>
<td>3</td>
<td>0 -</td>
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“low cells” were $1.5 \times 10^3$ EBM-4 cells per side. “high cells” were $10^5$ EBM-4 cells per side. “sham” was vehicle only (DMEM).
Figure 5.3: Mouse cells detected two days after implantation following hippocampal injections
A and B show cells in the needle tract from a normal low cell (1.5x10^3 per side) injected guinea pig sacrificed two days after implantation. C and D were from another normal low cell (1.5x10^3 per side) injected guinea pig sacrificed two days after implantation. C shows a single microsatellite positive cell (white arrow) in the hippocampus. D- shows microsatellite positive cells (white arrows) in the cortex. Red: Mouse microsatellite in situ hybridisation. Green: GFAP. Blue: DAPI (nuclear fluorescence).
Figure 5.4: Mouse cells detected two weeks after implantation following hippocampal injections

A was a normal sham injected guinea pig, showing microsatellite positive cells in the thalamus. B and C were from another normal low cell (1.5x10^3 per side) injected guinea pig, showing microsatellite positive cells in the cortex. White arrows indicate microsatellite positive cells. Red: Mouse microsatellite in situ hybridisation. Green: GFAP. Blue: DAPI (nuclear fluorescence). Note that Sections shown in B and C were not mounted in DAPI containing media, and thus did not exhibit nuclear fluorescence in the blue channel.
Figure 5.4

A

thalamus

50µm

B

cortex

100µm

C

cortex

100µm
Figure 5.5A: Mouse cells detected four weeks after implantation following hippocampal injections
A was a normal low cell ($1.5 \times 10^3$ per side) injected guinea pig, with microsatellite positive cells detected in the hippocampus. White arrows indicate microsatellite positive cells. Red: Mouse microsatellite in situ hybridisation. Green: GFAP. Blue: DAPI (nuclear fluorescence). Note that the section shown was not mounted in DAPI containing media, and thus did not exhibit nuclear fluorescence in the blue channel.

Figure 5.5B: Mouse cells detected eight weeks after implantation following hippocampal injections
B was a normal low cell ($1.5 \times 10^3$ per side) injected guinea pig showing microsatellite positive cells that had migrated to the superior colliculus. White arrows indicate microsatellite positive cells. Red: Mouse microsatellite in situ hybridisation. Green: GFAP. Blue: DAPI (nuclear fluorescence).
notable change in neurological presentation (as assessed using the neurological examination described in Section 3.3, see Figure 6.1), accompanied by a drop in body weight. Histological analysis of tissues from this animal suggested that it died of a pulmonary infection as a consequence of immunosuppression, rather than the presence of a teratoma (no surviving graft was detected). This animal is discussed further in Section 6.3.1. All other cell-implanted animals showed no notable changes in neurological presentation throughout the study (see Figure 6.1), and gained weight at a similar rate to sham-injected guinea pigs (data not shown).

In animals where surviving mouse cells were detected, they were most often observed in the cortex or hippocampus (see Figure 5.3). Although the hippocampus (specifically, the dentate gyrus of the hippocampus) was the target of these implants, cells were often found in the cortex above the hippocampus, corresponding to the path of the needle during implantation. Cells were observed in the thalamus of one animal two weeks following implantation (Figure 5.4A), although this was probably due to the implantation procedure delivering cells too deeply in this individual. No microsatellite-positive cells were observed in any of the sham-injected brains analysed.

Consideration of all implant data (see Table 5.1 and Figure 5.6) suggested the progressive death of mouse cell grafts following implantation.
Figure 5.6: Proportion of guinea pig brains analysed with surviving mouse cells
This figure shows a graphical representation of data summarised in Table 5.1, for normal animals implanted bilaterally with $1.5 \times 10^3$ EBM-4 cells, sacrificed at various time points following implantation.
Figure 5.6

Percentage of guinea pig brains analysed containing surviving mouse cells

Days post implantation

Percentage

0 10 20 30 40 50 60 70 80 90 100

0 20 40 60 80 100 120

2 days
2 weeks
4 weeks
8 weeks
13 weeks
5.3.2.2 High dose cell implants

Due to the poor survival observed following implantation of $1.5 \times 10^3$ EBM-4 cells per side, further implants were carried out involving implantation of $10^5$ cells per side, bilaterally into the dentate gyrus of the hippocampus. These implants were carried out to assess migration and differentiation following implantation of EBM-4 cells in the dentate gyrus of one week old guinea pigs, using a cell dosage previously shown to result in graft survival for at least two months (see Section 5.3.1). Implants were carried out as described above, including a total injection volume of 1µl per side. Animals were treated with CSA (see Section 2.2.4.4) commencing the day before surgery, and evaluated weekly using the neurological examination described in Section 3.3. Both normal (n=3) and α-mannosidosis (n=1) animals were sacrificed as described previously, four weeks following implantation. Immunofluorescent techniques for detection of graft cells were carried out as described previously.

As with the animals implanted with $1.5 \times 10^3$ cells per side, animals implanted with $10^5$ cells per side were not found to display any notable change in neurological presentation (as assessed using the neurological examination described in Section 3.3), and gained weight at a similar rate to sham-injected guinea pigs (data not shown). Following sacrifice and histological analysis, two of the three normal animals implanted with $10^5$ cells per side showed clear evidence of teratomas four weeks after transplantation (see Figure 5.7), as assessed by the presence of pluripotent cells and cells of multiple germ lineages within the grafts (see Section 5.3.2.2b for phenotypic analysis). These teratomas had a diameter of approximately 2-4mm, resulting in noticeable distortion of the hippocampus (Figure 5.7a and 5.7b), suggesting growth of these grafts had occurred during the time following implantation. One of the normal animals displayed a morphologically distinct structure in the lateral ventricle just rostral to the hippocampus, appearing to be a cell graft (Figure 5.8A), although no living microsatellite-positive cells were detected in this structure. No morphological changes or microsatellite-positive cells were found in the α-mannosidosis guinea pig implanted with $10^5$ cells (Figure 5.8B).
Figure 5.7: Teratomas observed in high cell injection, four weeks post implantation

A and B are normal guinea pigs injected bilaterally with $10^5$ EBM-4 cells per side, sacrificed four weeks post implantation. Red: Mouse microsatellite *in situ* hybridisation. Green: *GFAP*. Blue: DAPI (nuclear fluorescence). Note displacement of hippocampus in B, and distension and displacement of hippocampus in A, due to the size of the teratomas.
Figure 5.7

A

B

hippocampus

teratoma

1000μm

1000μm
Figure 5.8: Lack of graft survival in high cell injection, four weeks post implantation
A is a normal guinea pig injected bilaterally with $10^5$ EBM-4 cells per side, sacrificed four weeks post implantation. B is an $\alpha$-mannosidosis guinea pig injected with cells and sacrificed in a similar fashion. Red: Mouse microsatellite in situ hybridisation. Green: GFAP. Blue: DAPI (nuclear fluorescence). White arrow in A indicates graft structure not found to be immunoreactive for mouse microsatellite or GFAP.
Figure 5.8

A

B

dentate gyrus

needle tract

dentate gyrus

1000 µm
1000 µm
5.3.2.2a Migration of implanted cells

One of the normal animals with a teratoma four weeks following implantation was chosen for further analysis of migration and differentiation potential of the implanted cells. Analysis of all areas of the sagittal section showed that in addition to the large microsatellite-positive graft present at the hippocampus, graft-derived cells had migrated to various sites throughout the brain, as far as approximately 10mm rostrally and 8mm caudally (see Figure 5.9). Migration in the lateral axis was not examined. The migration of implanted cells was relatively infrequent, as the majority of implanted cells remained in the graft at the injection site. This suggested that a small proportion of implanted cells differentiated to a phenotype that had the potential to migrate to distal sites throughout the brain over this four week period. Implanted cells observed in sites distal to the injection site were not found to be immunoreactive for GFAP and thus could not be classified as glial precursors or differentiated astrocytes. The phenotype of these cells was thus not determined in these studies.

5.3.2.2b Differentiation of implanted cells

Sections from the brain of the above-mentioned animal were also used for further phenotypic analysis of engrafted cells. This was carried out to assess differentiation of implanted cells to various phenotypes.

Immunofluorescent confocal microscopy techniques (as described in Section 2.2.5) showed graft-derived cells positive for Nestin (see Figures 5.10A, B and C), GFAP (see Figures 5.10D, E and F) and MAP2 (Microtubule associated protein 2, a marker for mature neurons, see Figures 5.10G, H and I). Graft-derived cells were also found to be positive for TH (tyrosine hydroxylase, a putative marker for dopaminergic neurons, see Figures 5.11A, B and C), Oct4 (see Figures 5.11D, E and F) and SMA (see Figure 5.11G, H and I). Genuine expression of each phenotypic marker (shown in green) by graft-derived cells was confirmed by co-localisation (within the same cell) with mouse microsatellite puncta (red) in both the X-Y plane, and in a reconstruction of the Z-axis through part of the X-Y plane.
Figure 5.9: Migration of implanted cells in the guinea pig brain
Surviving mouse cells in a normal guinea pig injected bilaterally with $10^5$ EBM-4 cells per side, sacrificed four weeks post implantation. Arrows indicate microsatellite positive mouse cells. Red: Mouse microsatellite *in situ* hybridisation. Green: *GFAP*. Blue: DAPI (nuclear fluorescence).
Figure 5.10: Confocal microscopy for lineage analysis from high cell implanted animal, four weeks post implantation

Figure 5.11: Confocal microscopy for lineage analysis from high cell implanted animal, four weeks post implantation

5.3.3 Analysis of mouse-specific antibodies in sera from cell implanted and sham-injected guinea pigs

Serum samples were taken from a number of guinea pigs at sacrifice, and incubated with a suspension of mouse EBM-4 cells (see Section 2.2.3.4e) before washing and incubating with a biotinylated anti-guinea pig secondary antibody, followed by a fluorophore (Cy5) conjugated tertiary antibody (as described in Section 2.2.4.10). This method was provided by Professor Tony Ferrante (WCH). Cells were then run in a fluorescence-activated cell sorter (FACS), to determine if EBM-4 cells incubated with the serum from cell-implanted animals demonstrated an increase in fluorescence that could indicate elevated levels of circulating antibodies specific to the implanted mouse cells, relative to sham-injected controls. Figure 5.12a shows a representative plot of forward and side scatter characteristics of these cell populations, along with the gated region of cells analysed (R1). Gating conditions were set by Dr. Greg Hodge (WCH) to provide a live, single cell population for analysis of fluorescence. Figure 5.12b shows a representative overlay of fluorescence from a serum-incubated cell population, compared to a control population incubated with secondary and tertiary antibodies alone.

EBM-4 cells incubated with serum from sham-injected controls showed an increase in binding compared to the control cell population, reflecting the background level of preformed antibodies against mouse epitopes present in guinea pig serum (Figure 5.13). Cells incubated with serum from animals implanted with both low (1.5x10³ per side) and high (10⁵ per side) cell numbers showed an increase in fluorescence compared to cells incubated with serum from sham-injected controls. However, due to the small sample size (n=2 sham-injected animals, n=2 low cell-injected animals, and n=3 high cell-injected animals), these differences were not found to be significant when analysed with a student t-test (p=0.28 and p=0.17, for comparisons between sham and low cells or high cells, respectively).
Figure 5.12a: Scatter plot of EBM-4 cell population
Representative scatter plot of EBM-4 cell population incubated with guinea pig serum taken from an animal injected bilaterally with $1.5 \times 10^3$ cells, four weeks after implantation. FSC: forward scatter. SSC: side scatter. R1 is the region analysed, with forward and side scatter characteristics indicative of live cells.

Figure 5.12b: Fluorescence intensity of EBM-4 cell populations
Fluorescence intensity from populations of EBM-4 cells incubated with serum taken from an animal injected bilaterally with $1.5 \times 10^3$ cells four weeks after implantation (blue) and a control population incubated with secondary antibody alone (orange) are shown. FL4-H: Fluorescence intensity for each event. M1 was set such that 2% of the cells of the control population (orange) were gated.
Figure 5.13: Binding of antibodies from sera of guinea pigs against mouse cells
Data shown is the increase in median fluorescence intensity (MFI) for each group of cell populations, relative to a control population (secondary antibody only, no serum), ±1SEM. Serum was taken from animals four weeks post-implantation. “Sham”: Animals implanted with vehicle alone (n=2). “Low cells”: Animals implanted bilaterally with $1.5 \times 10^3$ cells per side (n=2). “High cells”: Animals implanted bilaterally with $10^5$ cells per side (n=3). All animals were of a normal phenotype. “ns” indicates no significant increases in cell implanted populations, relative to sham injected controls.
Figure 5.13

The bar chart shows the increase in MFI for different conditions: sham, low cells, and high cells. The y-axis represents the increase in MFI, ranging from 0 to 14. The x-axis lists the conditions: sham, low cells, and high cells. The chart indicates no significant difference (ns) for sham and high cells (p=0.28 and p=0.17, respectively).
5.4 Discussion

Preliminary studies investigating the implantation of $10^4$ mouse ES cell-derived EBM-9 cells into the striatum of neonatal guinea pigs showed surviving grafts out to two months following transplantation, with no observed detrimental effects on these animals. These studies established that implanted mouse cells could survive for at least two months in the CSA-treated guinea pig brain. As a result, further cell implantation studies were undertaken, involving lower doses of cells.

Implantation of low numbers (1.5x10³) of mouse ES cell-derived EBM-4 cells in the dentate gyrus of the guinea pig brain resulted in poor survival of grafts, with a progressive death observed over the 13 week period following implantation. The few surviving grafts observed consisted of isolated pockets of very few cells. This extremely low survival precluded the use of tissue from these animals for both detailed phenotypic analysis and stereological techniques for quantitation of implanted cells, due to the logistics of these experiments. As such, implantation studies involving higher cell numbers ($10^5$ cells per side) were carried out in order to gain insight into the differentiation and migration potential of these cells and their derivatives in vivo.

Investigation of graft survival and differentiation following high cell ($10^5$ per side) implants of mouse ES cell-derived EBM-4 cells showed that implanted cells had the potential to differentiate into neural precursor, mature neuronal and mature glial lineages, with a proportion of implanted cells (of an undetermined phenotype) migrating to various sites throughout the brain. The observation of surviving graft-derived cells in such distal areas to the implantation site is particularly exciting for the potential treatment of LSD such as α-mannosidosis, where neurological pathology is present in many disparate sites in the brain (Crawley et al., 1999). Ultimately, implantation of a cell phenotype with the propensity to engraft and migrate throughout various structures within the brain could allow delivery of deficient enzyme to multiple brain regions, potentially requiring relatively few injection sites to achieve therapeutic effect. Similar cell migration has been observed in other studies, following implantation of either pluripotent or adult stem cells into various sites such as the cerebral lateral ventricles or the sub-ventricular zone (Flax et al., 1998; Yandava et al., 1999; Fricker et al., 1999; Park et al., 2002; Erdo et al., 2003), the striatum (Fricker et al., 1999) or the dentate gyrus of the hippocampus (Fricker et al., 1999), although the conditions and signals contributing to this phenomenon are at present poorly
understood. Further studies into these conditions will ultimately improve the prospects for stem cell therapies in LSD. Additionally, a greater understanding of the cell phenotypes able to migrate in this fashion, in combination with in vitro techniques for the derivation of these cells from ES cells, should expand the prospects of ES cell based-therapies for treatment of LSD.

Despite the disruption of cell-cell connections in the preparation of the single cell suspension implanted, teratomas were still found in half of the animals studied, injected with $10^5$ cells per side. The graft dynamics immediately following implantation of this high cell dose would be expected to be significantly different to those of a dilute low cell dose ($1.5 \times 10^3$ cells per side), with a potentially much higher frequency of interactions between implanted cells, rather than between implanted cells and the local microenvironment of the host. These interactions might have contributed to the formation of teratomas in animals implanted with $10^5$ EBM-4 cells per side.

Further phenotypic analysis of one of these grafts was carried out. The presence of cells positive for the marker Nestin suggests that some of the implanted cells had differentiated into Nestin immunoreactive precursor cells such as neural precursors. Additionally, the observation of mouse cells immunoreactive for GFAP, MAP2 and TH suggests that implanted cells were able to further differentiate into neurons and glia following implantation. A similarly broad differentiation potential has been reported following previous implantation studies involving ES cell-derived cells (Björklund et al., 2002).

The presence of Oct4 immunoreactive pluripotent cells further suggests that the in vitro cell preparation (including MEDII conditioning) and high cell dose implantation protocol utilised in this study did not sufficiently restrict the differentiation potential of these cells, resulting in the formation of teratomas in some of the animals implanted (see Figure 1.8). Additionally, the presence of SMA immunoreactive smooth muscle cells (“extra-neural” cells, of a mesodermal lineage) in this group further supports this conclusion. The safety issue of teratomas formed following implantation of $10^5$ EBM-4 cells per side thus precludes the use of this specific protocol for longer-term investigations of implants in the guinea pig.

Previously, studies involving implantation of similar numbers ($1.5 \times 10^3$) of differentiated ES cells into the rat striatum have been described (Björklund et al., 2002). This study involved implantation of EB-4 cells, which are embryoid bodies
grown in the presence of serum for four days (rather than in the presence of any specific inductive signals). In this study, teratomas were found in 20% of cell-implanted animals, while 24% of animals were found to have no surviving grafts. The remaining 56% of implanted animals were reported to have well-integrated grafts, which after nine weeks were found to have differentiated into stable, terminally differentiated, non-dividing phenotypes. The present study involved implantation into a different implantation site within the brain (the dentate gyrus of the hippocampus, compared to the striatum) and a considerably more distant species (guinea pig, compared to the aforementioned study involving implantation of mouse cells into rat).

The markedly lower graft survival observed following implantation of $1.5 \times 10^3$ EBM-4 cells per side in this thesis could be due to a number of factors. Firstly, the inherent variability due to the technical considerations of implantation of low numbers of cells may have contributed to poor graft survival in some of the animals. This has been discussed as one explanation for the lack of surviving grafts in a proportion of animals in the previous study (Björklund et al., 2002). Secondly, the local environment of the implantation site may have had an influence on the fate of graft cells following implantation. In contrast to the striatum, the dentate gyrus of the hippocampus is a neurogenic zone in the brain (Eriksson et al., 1998). Previous studies have shown that the local environment can have an influence on the differentiation of implanted stem cells in the brain (Fricker et al., 1999), although studies directly comparing the influence of different implantation sites on survival of implanted ES cell-derived cells are yet to be published. Future experiments could thus involve implantation of stem cells of a more tightly defined phenotype into a range of sites, with a view to determining implantation sites most conducive to achieving widespread and stable engraftment within the brain.

Thirdly, (and perhaps most importantly), the significant species differences between guinea pigs and implanted mouse cells may have influenced graft survival. Previous studies have shown that implantation of embryonic mouse neural tissue into the brain of guinea pigs resulted in no surviving grafts in implanted animals, in contrast to poorly surviving grafts observed when implanted in rats (Larsson et al., 1999). In this same study, transplantation of embryonic rat neural tissue resulted in good survival in both implanted rat and guinea pig brains. Although this study used an entirely different source of tissue and implantation site compared to the study described in this
thesis, it serves to highlight the importance of species differences in animal models, with regard to the potential for rejection of xenotransplanted tissue. Part of the decreased survival in guinea pigs could be attributed to the presence of preformed antibodies to mouse epitopes in the guinea pig (a discordant xenograft) that are not present in the rat (a concordant xenograft) (Larsson et al., 1999). The observation of surviving grafts following implantation of $10^5$ cells either in the striatum or in the dentate gyrus could be due to the fact that an immune response may not have been sufficient to mediate the death of all implanted cells within the time periods analysed.

Although all animals were immunosuppressed with CSA in this study, the extent to which CSA is required to achieve survival of engrafted cells in the brain is at present uncertain. Indeed, previous studies have found that CSA treatment may have little influence on short-term NSC graft survival (Modo et al., 2002), although others have shown that immunosuppression (such as with CSA treatment) is necessary to ensure survival in long-term fetal neural allografts (Sloan et al., 1991). The immunosuppression protocol employed in the present study was designed in order to achieve a level of immunosuppression in neonatal (and older) animals, while allowing acceptable weight gain, particularly in α-mannosidosis animals, which appeared to be more susceptible to weight loss while immunosuppressed (data not shown). Due to the fact that a sustained dose of 20mg/kg/day was found to be highly detrimental to the health of these neonatal guinea pigs, the maintenance dose used in this thesis had to be reduced to 10mg/kg/day (administered orally), after two weeks at 20mg/kg/day. Other references have reported the effective oral dose in humans to be 10-15mg/kg daily initially, decreasing to a maintenance dose of 2-6mg/kg daily (MIMS Annual, 1997). Mouse studies involving intra-cranial implantation of NSCs into neonates have however reported treatment with a constant dosage of 10mg/kg daily, administered intraperitoneally (Flax et al., 1998), thus resulting in a higher effective dose than oral administration. Studies involving intra-cranial implantation of stem cells into adult rats have employed a 10mg/kg/day CSA dose for maintenance of immunosuppression, following initial treatment with a 15mg/kg/day for the first 10 weeks following implantation (Björklund et al., 2002). Guinea pigs treated in the present study were tested to ensure that serum CSA concentrations fell within a consistent range during the maintenance period (approximately 100-200ng/ml trough level, see Appendix 3). However, in the absence of established reference ranges for CSA in guinea pigs, it is
uncertain whether this dosage regime resulted in an effective level of immunosuppression in all animals treated. Immune response may thus have been a factor contributing to the death of implanted cells in this study.

Accordingly, a small experiment was conducted as a preliminary investigation into the possibility of an immune response contributing to the poor graft survival observed. Although these data provided inconclusive results due to a limited sample size, they suggested that immune response could indeed have been a contributing factor. Further implantation studies involving xenotransplantation of cells into the guinea pig brain should thus involve more concerted analysis of immune response as a potential factor influencing graft survival. Previously, studies investigating the rejection of neural grafts have included lymphocyte proliferation assays, and analysis of upregulation of immunological markers at the implantation site, such as MHC class I and II, CD45, and CD11b (Modo et al., 2002). Along with further investigations (involving a greater sample size) into the presence of circulating antibodies specific to mouse cells (as described above), a similar lymphocyte proliferation assay (involving analysis of lymphocytes derived from cervical lymph nodes at sacrifice) could be employed to investigate whether implanted cells were sufficiently immunogenic to induce a significant response (Modo et al., 2002). Histological analysis of immunological markers indicating a response against grafted cells would logistically require sufficient graft survival to provide tissue sections for these techniques. Unfortunately, the scarcity of tissue sections containing surviving cells from the low cell dose implants performed in this thesis precluded this method of analysis.

The cell phenotype and dosage protocol used in this thesis is clearly not ideal for transplantation studies. Low cell number implants (1.5x10^3 cells per side) resulted in poor graft survival, while high cell number implants (10^5 cells per side) resulted in large teratomas in implanted animals. Although a titration of cell numbers in future studies could establish an optimum dose between these two extremes (in order to provide better outcomes in terms of graft survival) the issue of pluripotent cells contaminating cell populations generated using the MEDII differentiation protocol (see Section 4.3.6) ultimately raises questions regarding the safety and consistency of this approach at any cell dose. Previous studies have shown that EBM-4 cell aggregates are comprised of largely pluripotent primitive ectoderm-like cells (Rathjen et al., 2002). These studies found that further conditioning of these EBMs with MEDII induced the
homogeneous formation of neurectoderm and ultimately neural precursors in a fashion recapitulating embryonic development (Rathjen et al., 2002), including the elimination of pluripotent cells during this differentiation protocol (Rathjen et al., 2002). In contrast to these results, however, data generated in this thesis (discussed in Chapter 4) has shown that even after 21 days of differentiation, nine days of which consisted of MEDEI conditioning, isolated pockets of pluripotent (Oct4 positive) cells remained in these aggregates (see Section 4.3.6). The contamination of differentiated cell populations with pluripotent cells is of concern, since without the elimination of the tumorigenic potential of these cells, studies into ES cell-based therapeutic approaches in animal models will continue to face safety issues, particularly when considering the use of higher cell numbers. In vitro protocols for the generation of transplantable cell phenotypes from ES cells need to be thoroughly evaluated prior to application in animal models to ensure the efficient elimination of pluripotent cells. Recently, more extensive differentiation protocols have been developed for the derivation of NSCs and other mature neural phenotypes from ES cells (Barberi et al., 2003). These protocols have allowed the implantation of $10^5$ ES cell-derived cells for up to two months, with no evidence of teratomas following histological analysis. The use of more extensive differentiation protocols to generate more mature cell phenotypes (and the exclusion of other pluripotent cells, as represented in Figure 1.8) may improve prospects for achieving stable and reproducible grafts in the $\alpha$-mannosidosis guinea pig. Ultimately, this should increase the potential of stem cell therapy for treatment of brain disease in $\alpha$-mannosidosis, and other LSD with CNS pathology.
Chapter 6:
Evaluation of the efficacy of cell implantation for treatment of CNS pathology in the α-mannosidosis guinea pig
6.1 Introduction

Previous studies into BMT for α-mannosidosis (Walkley et al., 1994) have shown that enzyme provided to the CNS by even a relatively low number of allogeneic bone marrow-derived cells can mediate a significant reduction in CNS pathology. Other studies involving implantation of bone marrow stromal cells (MSCs) into the hippocampus and cerebellum of mice with Niemann-Pick disease (NPD) found that, despite low cell survival, treated mice displayed an increased lifespan, improved body weights and improved coordination (relative to untreated controls; Jin et al., 2002). Thus, even a relatively low dose of enzyme delivered to the brain of the α-mannosidosis guinea pig may mediate a reduction in storage sufficient to affect the progression of CNS pathology. Although no surviving cells were detected at 13 weeks post-implantation in either α-mannosidosis or normal animals, these animals were tested to determine if the implantation of these cells had influenced the progression of their disease as a result of enzyme secretion during the time grafts were still surviving. This was assessed by examining behavioural characteristics of the animals (using tests detailed in Chapter 3), as well as lysosomal storage in the CNS (using histological techniques).

Additionally, the multiple-component neurological examination (as described in Chapter 3) served an important safety purpose in this study, by providing an effective means of observing the neurological characteristics in both cell-implanted and sham-injected animals. This allowed the early diagnosis of any rapid changes in neurological status that may have occurred as a result of cell implantation. This is important where safety concerns exist, such as in evaluating the cell-based therapy described in this thesis, due to the possibility of these cells forming teratomas.

6.2 Specific methods

All methods were as described in Section 2.2.

6.3 Results

All cell-implanted animals were weighed and treated daily with CSA, along with Bactrim during the Morris water maze testing period, as described previously for sham-injected animals.
6.3.1 Neurological Examinations

Both normal and α-mannosidosis guinea pigs implanted with 1.5x10^3 cells per side bilaterally were tested weekly using the neurological examination described in detail in Section 3.3 (see Figure 3.10). The progression of neurological examination score (total score, see Figure 6.1) in both normal and α-mannosidosis cell-implanted animals was found to be unchanged compared to sham-injected controls. All control data shown (normal and α-mannosidosis sham-injected animals) are from the studies described in Chapter 3 (Figure 3.11a).

All cell-implanted animals (implanted with either 1.5x10^3 or 10^5 cells per side) were tested weekly with this neurological examination to ensure that cell-implanted animals did not present with any rapid neurological changes that could indicate the presence of a teratoma. Throughout the testing period, a rapid change in neurological status was only observed in one animal, as indicated in Figure 6.1 by the label “Pl”. This animal died six days after the last neurological test shown, despite antibiotic treatment commencing the day after the last neurological examination. Following extensive histological evaluation, it was determined that this animal died of a pulmonary infection as a consequence of immunosuppression (data not shown). No surviving mouse cell graft or teratoma was detected. The observation of this rapid change in condition using this test supports the use of these examinations as a safety feature in ES cell-derived cell implantation studies in guinea pigs. If rapid changes in neurological presentation were caused by the growth of a teratoma they could hopefully be detected early using this test, allowing early euthanasia.
Figure 6.1: Neurological examination: % Affected
Test items 1-25, inclusive (see Figure 3.10). Data shown is the score for each animal expressed as a percentage of the total possible score. "α-mannosidosis sham" and "normal sham" data was as reported in Chapter 3 (Figure 3.11a). "Normal" groups include both normal and heterozygous guinea pigs. "PI" indicates the animal that died during this study as a result of a pulmonary infection.

Legend:

- α-mannosidosis sham
- normal sham
- α-mannosidosis low cells
- normal low cells
- α-mannosidosis high cells
- normal high cells
6.3.2 Gait analysis

Gait length and width were measured and analysed in cell-implanted α-mannosidosis (n=3) and normal (n=14) guinea pigs as described in Chapter 3. The aim of these analyses was to determine if bilateral implantation of 1.5x10^3 EBM-4 cells (as described in Chapter 5) resulted in observable normalisation of gait in treated α-mannosidosis animals.

Analysis of gait length (normalised to body weight) showed no significant differences between sham-injected and cell-implanted α-mannosidosis animals, at either two or three months (p=0.369 and p=0.951 for two and three months, respectively, see Figure 6.2).

Analysis of stride width in cell-implanted α-mannosidosis animals (compared to sham-injected α-mannosidosis controls) additionally showed no significant differences (p=0.168 and p=0.149 for two and three months, respectively, see Figure 6.3). Similarly, length/width ratio was not found to be significantly different between α-mannosidosis cell-implanted and sham-injected animals (p=0.083 and p=0.205 for two and three months, respectively, see Figure 6.4).

Collectively, these data suggest that implantation of cells in these animals did not have any significant effect on the progression of gait changes observed in α-mannosidosis guinea pigs.
Figure 6.2: Stride length of guinea pigs at two and three months of age.

Normal sham-injected guinea pigs n=11 (two months), n=12 (three months)
α-Mannosidosis sham-injected guinea pigs n=5 (two months), n=8 (three months)
Normal low cell-injected guinea pigs n=17 (two months), n=14 (three months)
α-Mannosidosis low cell-injected guinea pigs n=4 (two months), n=3 (three months)

Data shown is the mean stride length for all animals normalised to the weight of each animal at testing, ±1SEM. “α-mannosidosis sham” and “normal sham” data were as reported in Chapter 3 (Figure 3.9a).

Legend:

- normal sham
- α-mannosidosis sham
- normal low cells
- α-mannosidosis low cells

Statistical analysis

Two months
α-mannosidosis low cells vs. α-mannosidosis sham p=0.369
α-mannosidosis low cells vs. normal low cells p=0.141
α-mannosidosis sham vs. normal sham p=0.151
normal low cells vs. normal sham p=0.080

Three months
α-mannosidosis low cells vs. α-mannosidosis sham p=0.951
α-mannosidosis low cells vs. normal low cells p=0.393
α-mannosidosis sham vs. normal sham p=0.008
normal low cells vs. normal sham p=0.141
Figure 6.2

(see figure legend for results of statistical analysis between groups)
Figure 6.3: Stride width of guinea pigs at two and three months of age.

Normal sham-injected guinea pigs n=11 (two months), n=12 (three months)
α-Mannosidosis sham-injected guinea pigs n=5 (two months), n=8 (three months)
Normal low cell injected guinea pigs n=17 (two months), n=14 (three months)
α-Mannosidosis low cell-injected guinea pigs n=4 (two months), n=3 (three months)

Data shown is the mean stride width for all animals normalised to the weight of each animal at testing, ±1SEM. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.9b).

Legend:

- Normal sham
- α-mannosidosis sham
- Normal low cells
- α-mannosidosis low cells

Statistical analysis

Two months
α-mannosidosis low cells vs. α-mannosidosis sham p=0.168
α-mannosidosis low cells vs. normal low cells p=0.055
α-mannosidosis sham vs. normal sham p<0.001
Normal low cells vs. normal sham p=0.600

Three months
α-mannosidosis low cells vs. α-mannosidosis sham p=0.149
α-mannosidosis low cells vs. normal low cells p=0.011
α-mannosidosis sham vs. normal sham p<0.001
Normal low cells vs. normal sham p=0.148
Figure 6.3

(see figure legend for results of statistical analysis between groups)
Figure 6.4: Stride length/width ratio of guinea pigs at two and three months of age.

Normal sham-injected guinea pigs n=11 (two months), n=12 (three months)
α-Mannosidosis sham-injected guinea pigs n=5 (two months), n=8 (three months)
Normal low cell-injected guinea pigs n=17 (two months), n=14 (three months)
α-Mannosidosis low cell-injected guinea pigs n=4 (two months), n=3 (three months)

Data shown is the mean stride length divided by the mean stride width, ±1SEM. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.9c).

Legend:

- normal sham
- α-mannosidosis sham
- normal low cells
- α-mannosidosis low cells

Statistical analysis

Two months
α-mannosidosis low cells vs. α-mannosidosis sham p=0.083
α-mannosidosis low cells vs. normal low cells p=0.003
α-mannosidosis sham vs. normal sham p=0.001
normal low cells vs. normal sham p=0.438

Three months
α-mannosidosis low cells vs. α-mannosidosis sham p=0.205
α-mannosidosis low cells vs. normal low cells p<0.001
α-mannosidosis sham vs. normal sham p=0.003
normal low cells vs. normal sham p=0.016
Figure 6.4

(see figure legend for results of statistical analysis between groups)
6.3.3 Morris water maze

A group of normal animals (n=14) and α-mannosidosis animals (n=3) were implanted with EBM-4 cells at one week of age, and taken through to Morris water maze testing at three months of age. These groups were included both to investigate whether this therapy had an effect on memory and learning ability in α-mannosidosis guinea pigs, and additionally (in the case of normal animals) to ensure that this procedure had no negative effects on cognitive ability in normal treated animals. Data generated following testing of these animals were compared to data generated for three month old naïve sham-injected animals, described in Chapter 3 (n=12 normal guinea pigs, and n=8 α-mannosidosis guinea pigs).

6.3.3a Acquisition Phase

Both normal and α-mannosidosis cell-implanted treatment groups began the acquisition phase with similar seek times to their sham-injected counterparts, between approximately 30 and 35 seconds (Figure 6.5a). Over the course of the acquisition phase, α-mannosidosis cell-implanted animals did display a marginal improvement in search times compared to sham-injected α-mannosidosis animals, particularly at days five and six of the test. Statistical analysis of the acquisition phase as a whole did not show these improvements to be significant, however (see Figure 6.5a, p=0.838). Additionally, the rate of failure of treated (cell-implanted) α-mannosidosis animals was not significantly decreased in this period, compared to sham injected controls (Figure 6.5b, p=1).

No significant differences were observed between the groups, in the proportion of trials the animal was observed to swim off the platform following mounting (Figure 6.5c). The other measurements reported in Chapter 3 (attempting to climb up the sides of the pool, vocalising and jumping out of the pool) were excluded from analysis due to the fact that no significant differences were observed between normal and α-mannosidosis animals following studies detailed in Chapter 3 (see Table 3.2 for summary).

The performance of normal sham-injected and normal cell-injected animals was not found to be significantly different during this period (see Figure 6.5a, p=0.237), suggesting that the implantation of cells themselves did not have a significant effect on
the ability of the animals to perform the Morris water maze test.

### 6.3.3b Reversal phase

Statistical analysis on the reversal phase of this test did not show any significant differences between seek times of animals in any of the groups tested (Figure 6.5a). Additionally, fail rates (Figure 6.5b) and all the proportion of trials the animal swam off the platform following mounting (Figure 6.5c) were not found to be significantly different between any of the treatment groups.

### 6.3.3c Probe test

Both sham-injected and treated normal animals showed a strong preference for the target quadrant during the probe test, spending an average of 20 seconds and 23 seconds in this quadrant, respectively (see Figure 6.5d). This represented a statistically significant increase ($p=0.018$ and $p<0.001$) compared to the time spent in the other quadrants (see Figure 6.5e). Both groups of normal animals thus demonstrated strong retention of the task of finding the hidden platform. Sham-injected $\alpha$-mannosidosis animals showed no significant increase in the time spent in the target quadrant ($p=0.507$) compared to the other quadrants (see Figure 6.5e), in contrast to normal animals described above. Treated $\alpha$-mannosidosis animals showed a marginally increased time in the target quadrant (approximately 17 seconds), although this was not found to be significant when compared to the time spent in the non-target quadrants ($p=0.525$). All treatment groups once again showed an increase in the time spent in the operator quadrant, although this was not found to be statistically significant for any of the groups (Figure 6.5f).

Statistical analysis between groups (time spent in the target quadrant) showed no significant differences between treated $\alpha$-mannosidosis animals and sham-injected $\alpha$-mannosidosis animals ($p=0.471$). The marginal increase in the time treated $\alpha$-mannosidosis animals spent searching the target quadrant did, however, result in this group not showing significant differences between normal sham-injected animals ($p=0.380$); this contrasts with comparisons between normal sham-injected and $\alpha$-mannosidosis sham-injected animals (as shown in Chapter 3, Figure 3.6.5g, $p=0.026$).

No significant differences were observed between treated $\alpha$-mannosidosis and
sham-injected α-mannosidosis guinea pigs in any of the stages of the Morris water maze. Thus, implantation of stem cells as described appears to have had no significant effect on neurological pathology as measured by this test.
Figure 6.5a: Morris Water Maze: Three month old naïve animals. Data shown is the mean of four trials performed per animal, for each phenotype group, on each day, ±1SEM. Maximum duration of each trial was 45 seconds, after which time the animal was guided to the platform. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.6.5a).

Legend:

- normal sham (n=12)
- α-mannosidosis sham (n=8)
- normal low cells (n=14)
- α-mannosidosis low cells (n=3)

Statistical analysis

**Acquisition Phase (days 1-7), Repeated measures analysis**
- α-mannosidosis low cells vs. α-mannosidosis sham p=0.838
- α-mannosidosis low cells vs. normal low cells **p=0.023**
- α-mannosidosis low cells vs. normal sham p=0.060
- α-mannosidosis sham vs. normal low cells **p=0.001**
- α-mannosidosis sham vs. normal sham **p=0.009**
- normal low cells vs. normal sham p=0.237

**Reversal Phase (days 13-14), Kruskal-Wallis test**
Between all groups p=0.107
Figure 6.5a

* (see figure legend)

ns p=0.107
Figure 6.5b: Proportion of trials failed: Three month old naïve animals. Failed trials were defined as when the animal did not find the platform during the 45 second trial period. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.6.5b).

**Legend:**

- normal sham (n=12)
- α-mannosidosis sham (n=8)
- normal low cells (n=14)
- α-mannosidosis low cells (n=3)

**Statistical analysis**

**Acquisition Phase (days 1-7): Repeated measures analysis**
- α-mannosidosis low cells vs. α-mannosidosis sham p=1
- α-mannosidosis low cells vs. normal low cells p=0.019
- α-mannosidosis low cells vs. normal sham p=0.070
- α-mannosidosis sham vs. normal low cells p<0.001
- α-mannosidosis sham vs. normal sham p=0.005
- normal low cells vs. normal sham p=0.267

**Reversal Phase (days 13-14): Mann-Whitney test**
- α-mannosidosis low cells vs. α-mannosidosis sham p=0.091
- α-mannosidosis low cells vs. normal low cells p=0.099
- α-mannosidosis low cells vs. normal sham p=0.410
- α-mannosidosis sham vs. normal low cells p=0.120
- α-mannosidosis sham vs. normal sham p=0.302
- normal low cells vs. normal sham p=0.843
Figure 6.5b

* (see figure legend)

ns
Figure 6.5c: Proportion of trials subject rejected platform: Three month old naïve animals.

“Subject rejecting platform” was defined as when the animal, having found or having been guided to the platform, swam back into the pool instead of waiting on the platform. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.6.5c).

Legend:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>◆</td>
<td>normal sham (n=12)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>α-mannosidosis sham (n=8)</td>
<td></td>
</tr>
<tr>
<td>◆</td>
<td>normal low cells (n=14)</td>
<td></td>
</tr>
<tr>
<td>□</td>
<td>α-mannosidosis low cells (n=3)</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

**Acquisition Phase (days 1-7): Repeated measures analysis**
- α-mannosidosis low cells vs. α-mannosidosis sham p=0.683
- α-mannosidosis low cells vs. normal low cells p=0.057
- α-mannosidosis low cells vs. normal sham p=0.058
- α-mannosidosis sham vs. normal low cells p=0.060
- α-mannosidosis sham vs. normal sham p=0.095
- normal low cells vs. normal sham p=0.187

**Reversal Phase (days 13-14): Mann-Whitney test**
- α-mannosidosis low cells vs. α-mannosidosis sham p=0.278
- α-mannosidosis low cells vs. normal low cells p=0.019
- α-mannosidosis low cells vs. normal sham p=0.164
- α-mannosidosis sham vs. normal low cells p=0.149
- α-mannosidosis sham vs. normal sham p=0.726
- normal low cells vs. normal sham p=0.031
Figure 6.5c
Figure 6.5d: Probe test (overview):
n=8 α-mannosidosis sham injected guinea pigs, n=12 normal/heterozygous sham
injected guinea pigs, n=3 α-mannosidosis low cell injected guinea pigs, and n=14
normal/heterozygous low cell injected guinea pigs. Values shown were the mean time
searching for the platform in each quadrant for each group, ±1SEM. Quadrant 4 was the
target quadrant (where platform was located in previous trials), Quadrant 1 was where
the operator was located. “α-mannosidosis sham” and “normal sham” data was as
reported in Chapter 3 (Figure 3.6.5g).

Results of ANOVA for time spent in Quadrant 1, across all groups
p=0.874 (no significant differences in the time spent in Quadrant 1 between any groups)

Results of multiple comparisons following ANOVA for time spent in Quadrant 4,
across all groups
α-mannosidosis low cells vs. α-mannosidosis sham p=0.471
α-mannosidosis low cells vs. normal low cells p=0.091
α-mannosidosis low cells vs. normal sham p=0.380
α-mannosidosis sham vs. normal low cells p=0.001
α-mannosidosis sham vs. normal sham p=0.026
normal low cells vs. normal sham p=0.185

Legend:

- Quadrant 1
- Quadrant 2
- Quadrant 3
- Quadrant 4
Figure 6.5d

(see figure legend for results of statistical analysis between groups)
**Figure 6.5e: Probe test (Target vs. Non-Target Quadrants).**

n=8 α-mannosidosis sham injected guinea pigs, n=12 normal/heterozygous sham injected guinea pigs, n=3 α-mannosidosis low cell injected guinea pigs, and n=14 normal/heterozygous low cell injected guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Target Quadrant” was the mean time spent searching for the platform in Quadrant 4, “Non-Target Quadrants” was the mean time spent in Quadrants 1, 2 and 3. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.6.5h). Statistical analysis was a t-test performed between the time spent in target quadrant vs. time spent in non-target quadrants, for each group.

**Legend:**

- Target Quadrant
- Non-Target Quadrants

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**Figure 6.5f: Probe test (Operator vs. Non-Operator Quadrants).**

n=8 α-mannosidosis sham injected guinea pigs, n=12 normal/heterozygous sham injected guinea pigs, n=3 α-mannosidosis low cell injected guinea pigs, and n=14 normal/heterozygous low cell injected guinea pigs. Values shown were the mean time searching for the platform in each quadrant for each group, ±1SEM. “Operator Quadrant” was the mean time spent searching for the platform in Quadrant 1, the quadrant closest to the operator (see Figure 3.1). “Non-Operator Quadrants” was the mean time spent in Quadrants 2, 3 and 4. “α-mannosidosis sham” and “normal sham” data was as reported in Chapter 3 (Figure 3.6.5i). Statistical analysis was a t-test performed between the time spent in the operator quadrant vs. time spent in non-operator quadrants, for each group.

**Legend:**

- Operator Quadrant
- Non-Operator Quadrants
6.3.4 Lysosomal storage in the hippocampus of treated and sham-injected normal and α-mannosidosis animals

Hippocampus samples were taken from α-mannosidosis and normal sham-injected animals, as well as an α-mannosidosis animal implanted with $10^5$ cells per side, four weeks post-surgery. Samples were also taken from α-mannosidosis and normal sham-injected animals, and the three α-mannosidosis animals implanted with $1.5\times10^3$ cells per side, thirteen weeks after surgery. All samples were prepared and embedded for electron microscopy, as described in Section 2.2.5. Sections were cut at a thickness of 0.5μm by Lyn Waterhouse (Adelaide Microscopy) and stained with toluidine blue.

Analysis under light microscopy revealed clearly visible lysosomal inclusions in the hippocampal neurons of α-mannosidosis sham-injected animals at both four and 13 weeks post-surgery (see Figures 6.6c and 6.6d, and 6.7c and 6.7d). Normal animals at both time points showed no evidence of lysosomal storage (Figures 6.6a, 6.6b, 6.7a and 6.7b). α-Mannosidosis animals implanted with $10^5$ cells also displayed clearly visible lysosomal inclusions four weeks post-surgery, qualitatively indistinguishable from sham-injected age-matched α-mannosidosis animals (Figures 6.6e and 6.6f). At 13 weeks post-surgery, α-mannosidosis animals implanted with $1.5\times10^3$ cells per side showed clearly visible lysosomal inclusions in neurons (see Figures 6.7e and 6.7f).

These were also qualitatively indistinguishable from those observed in the neurons of a sham-injected α-mannosidosis control animal (Figures 6.7a and 6.7b). Thus, implantation of differentiated cells derived from untransfected mouse ES cells did not appear to have any impact on lysosomal storage observed in the hippocampus of affected animals.
Figure 6.6: Toluidine Blue stained sections of hippocampus four weeks post implantation
A and B are from a normal sham injected guinea pig, C and D are from an α-mannosidosis sham injected guinea pig, E and F are from an α-mannosidosis guinea pig implanted with $10^5$ EBM-4 cells per side. All animals were sacrificed four weeks following surgery. Black arrows indicate neurons displaying lysosomal storage.
Figure 6.7: Toluidine Blue stained sections of hippocampus 13 weeks post implantation

A and B are from a normal sham injected guinea pig, C and D are from an α-mannosidosis sham injected guinea pig, E and F are from an α-mannosidosis guinea pig injected with $1.5 \times 10^3$ EBM-4 cells per side. All animals were sacrificed 13 weeks following surgery. Black arrows indicate neurons displaying lysosomal storage.
Figure 6.7

A

B

C

D

E

F

50µm

50µm

50µm

50µm
6.4 Discussion

Neurological examination tests suggested no change in the progression of neurological disease observed in α-mannosidosis animals implanted with stem cells. However, this test was subjective in nature and thus was intended for consideration along with gait analysis and the Morris water maze for assessing the progression of pathology in the α-mannosidosis guinea pig. Analysis of gait in α-mannosidosis guinea pigs (along with Morris water maze testing) additionally showed no significant improvements in cell-implanted animals, compared to sham-injected α-mannosidosis controls, described in Chapter 3. The small sample size of cell implanted α-mannosidosis animals (n=3) suggests the need for caution when interpreting the results of all the behavioural tests described in this thesis.

Histological analysis of hippocampus from the brain of treated α-mannosidosis animals showed no change in the level of lysosomal storage compared to untreated α-mannosidosis animals. This supports, at a histological level, the lack of significant improvement observed in treated animals when considering any of the other behavioural tests.

The observation that untransfected cells can mediate cross-correction of deficient fibroblasts (Section 4.3.7) suggests that even implantation of untransfected cells could possibly result in therapeutic effect. However, the observation of the progressive death of cell implants in the weeks following implantation (see Section 5.3.2.1 and Figure 5.6) suggests that, in these animals, any reduction in storage that may have occurred in the hippocampus as a result of implanted cells secreting functional α-mannosidase in their local environment may not have been significant enough in magnitude or duration to have a therapeutic effect.

To date, only one study has investigated the behavioural effects of stem cell implantation for treatment of LSD, involving a rotarod test of coordination, in Niemann-Pick mice (Jin et al., 2002). The question thus remains whether stem cell transplantation for α-mannosidosis is able to not only correct the enzyme deficiency and mediate a reduction in lysosomally-stored compounds in the brain, but whether this is a viable long-term treatment strategy: will implanted cells survive, engraft and continue to provide enzyme to deficient host CNS cells, resulting in behavioural correction, cognitive improvement and ultimately an increase in life expectancy in
treated animals? Clearly, implantation experiments involving a larger sample size would be required to effectively evaluate a stem cell-based approach for treatment of CNS pathology in α-mannosidosis, and LSD generally. As discussed in previous chapters, in vitro techniques for the reproducible production of differentiated ES cell-derived cells for implantation would need to be developed before these can be undertaken.
Chapter 7:
Conclusions and future work
7.1 Introduction:

\(\alpha\)-Mannosidosis is a progressive and debilitating LSD that presents with a range of clinical symptoms. One of the most difficult aspects of \(\alpha\)-mannosidosis to treat is CNS pathology, due to the presence of the BBB separating the CNS from the rest of the circulation. At present, there is no universally available safe and effective therapy for brain disease in all \(\alpha\)-mannosidosis patients. Numerous strategies are being pursued to circumvent the BBB, with a view to developing effective therapies for CNS pathology in \(\alpha\)-mannosidosis and LSD as a group. This thesis has investigated one such strategy involving the implantation of stem cells to facilitate the secretion of deficient enzyme in the brain, to be taken up by host cells to therapeutic effect.

7.2 Summary:

Since discovery (Muntz et al., 1999) and initial characterisation (Crawley et al., 1999), the \(\alpha\)-mannosidosis guinea pig has proven to be a valuable animal model of LSD for investigations into both the pathogenesis of and therapeutic approaches for this disease. \(\alpha\)-Mannosidosis is a promising disease model in which to evaluate stem cell therapies for LSD, due to the biochemical and pathological convergence with numerous other LSD displaying CNS pathology, in both patients and animal models. This thesis has described the further characterisation of this animal model, involving the Morris water maze, gait analysis and a multiple-component neurological examination. The Morris water maze was found to be an effective test to quantify cognitive deficits in \(\alpha\)-mannosidosis guinea pigs, with significant differences observed between \(\alpha\)-mannosidosis and normal guinea pigs at three months of age. Additionally, \(\alpha\)-mannosidosis guinea pigs were found to exhibit certain characteristic behaviours during the Morris water maze test, such as erratic performance and (occasionally) rejecting the platform as an escape by swimming off once mounted. The Morris water maze has previously been shown to be highly effective for the evaluation of ERT in mouse models of MPS VII (O’Connor et al., 1998) and MPS IIIA (Gliddon and Hopwood, 2004), as well as for evaluating the effects of prenatal ethanol exposure in guinea pigs (Richardson et al., 2002). This thesis describes the first report of cognitive deficit analysis in a guinea pig model of LSD, using the Morris water maze.

Characterisation of gait changes in \(\alpha\)-mannosidosis guinea pigs (compared to
normal animals) showed significant and progressive changes in gait at two and three months of age. Previous studies have demonstrated the utility of gait analysis for the evaluation of pathology in the MPS IIIA mouse (Hemsley and Hopwood, in preparation).

The multiple-component neurological examination carried out in this thesis was found to be specific for α-mannosidosis pathology, by virtue of the fact that normal animals were found to fluctuate around a baseline score throughout the course of the period analysed (approximately 100 days), while α-mannosidosis guinea pigs displayed a progressive increase in score using this test, reflecting the progressive nature of pathology in this LSD model. This neurological examination was also found to be effective at detecting rapid changes in the neurological presentation of animals in this study, as was observed prior to one animal dying of a pulmonary infection. In addition to being useful for the evaluation of therapies for α-mannosidosis, this test is thus also useful for inclusion as a safety measure where concerns exist regarding the possibility of teratomas, as is the case with ES cell-based therapeutic approaches.

Previous studies into stem cell therapies for LSD have involved genetic modification of the stem cells utilised, in order to achieve over-expression of the relevant deficient enzyme for the disease in question (Snyder et al., 1995; Lacorazza et al., 1996; Jin et al., 2002; Meng et al., 2003), and have demonstrated proof of principle for this therapeutic strategy. Following a similar approach, this thesis has involved the production and characterisation of mouse ES cell lines for the sustained over-expression of α-mannosidase.

Characterisation of the highest expressing cell line created in this study (cell line EF3) showed expression (driven by the EFlα promoter) of high levels of rhαM throughout differentiation, with over-expression sustained at least out to 21 days of in vitro differentiation. Cross-correction of human α-mannosidosis skin fibroblasts showed a dose-dependent and M6P-dependent correction effect (reduction in the levels of two stored oligosaccharides) mediated by enzyme (both rhαM and endogenous mouse α-mannosidase) secreted by differentiated populations of the cell line EF3. To our knowledge, this thesis describes the first report of characterisation of transgene expression out to 21 days of directed differentiation in a stably transfected mouse ES cell line. To date, the creation of stably transfected mouse ES cell lines for the
expression of a lysosomal enzyme has been reported only once previously (Lau et al., 2004).

To investigate the prospects for stem cell therapy using the α-mannosidosis guinea pig, conditions and protocols for the in vitro generation and implantation of cells in order to provide good survival and widespread engraftment in vivo are required. Towards establishing such conditions, this thesis investigated the implantation of a dilute single cell suspension of 1.5x10^3 EBM-4 cells, generated using the MEDII differentiation protocol (Rathjen et al., 1999), bilaterally into the dentate gyrus of the hippocampus. This cell preparation protocol and implantation dosage was similar to that described by Björklund et al. (2002), with the addition of the MEDII conditioned media differentiation system to provide specific induction to primitive-ectoderm like cells (Rathjen et al., 1999). Cells implanted in this fashion were not detectable in guinea pig brains beyond eight weeks and were not found to survive and engraft in significant numbers, contrasting with results of a previous study (Björklund et al., 2002), where implantation of similar numbers of cells into the rat striatum resulted in surviving, well integrated grafts in the majority of animals.

When cell numbers were increased to 10^5 cells per side (in order to investigate the migration and differentiation potential of this cell type), large teratomas were found after four weeks in 50% of the implanted animals. Additionally, despite the induction to EPL cells provided by the MEDII in the differentiation protocol and the preparation of a single cell suspension prior to implantation (in order to minimise interactions between graft cells and thus facilitate further neural differentiation), pluripotent cells and extra-neural phenotypes such as smooth muscle were still found in these grafts after four weeks. Although implanted cells were found dispersed throughout the brain in these animals, the vast majority of implanted cells remained within the graft itself. Due to the poor survival observed following low cell implants and the teratomas observed following high cell implants, this cell type (EBM-4) was not ideal for transplantation studies of this sort. The implantation studies described in this thesis thus represent a set of preliminary experiments towards establishing conditions that would allow the safe and stable engraftment of stem cells for treatment of CNS pathology in α-mannosidosis.

Numerous studies have suggested that even a small or brief dose of enzyme can possibly have a therapeutic effect on CNS pathology in LSD. Despite the progressive
death rate of EBM-4 cells observed following implantation, three α-mannosidosis animals implanted with $1.5 \times 10^3$ EBM-4 cells per side were thus evaluated using the Morris water maze, gait analysis, and multiple-component neurological examination, described in Chapter 3. Analysis of these three animals suggested that cell implantation had no effect on behavioural pathology. Following sacrifice, histological analysis of brain tissue confirmed that cell implantation had no effect on the lysosomal storage observed in these animals. Although this treatment was not found to have a significant effect on α-mannosidosis pathology, testing of cell-implanted α-mannosidosis animals further demonstrated the utility of the behavioural tests described in Chapter 3.

7.3 Conclusions and implications:

The establishment of the Morris water maze test and further characterisation of CNS pathology using gait analysis and neurological examinations has expanded the scope of analyses available for the evaluation of therapies in the α-mannosidosis guinea pig, adding prospects for behavioural evaluation to an existing battery of biochemical and histological tests. This has further contributed to the potential utility of this valuable animal model for the evaluation of therapies for LSD.

Despite the need for further characterisation, the results reported in this thesis do suggest the potential utility of the mouse ES cell line EF3 (and untransfected D3 mouse ES cells) for the generation of cell types for stem cell therapy approaches to treatment of CNS pathology in α-mannosidosis. Since EF3 is a mouse ES cell line, it could potentially be used in concert with a variety of in vitro differentiation protocols (such as described in Barberi et al., 2003) to generate a range of potentially therapeutically useful cell phenotypes. This cell line thus provides the flexibility to allow us to take advantage of future developments in protocols for the directed differentiation of ES cells, and provides another potential therapeutic strategy to be investigated for the treatment of α-mannosidosis.

Although preliminary implantation studies carried out in this thesis did not result in the development of conditions for the safe, stable and reproducible engraftment of ES cell-derived stem cells in the guinea pig brain, these studies are an important first step towards establishing such conditions.
7.4 Future work:

Analysis of the progression of behavioural pathology in the α-mannosidosis guinea pig has raised the possibility of correlation with future histological studies, in order to better understand the cellular changes that underlie this pathology in specific brain regions. These studies could provide valuable insight into the pathological processes contributing to brain disease in α-mannosidosis and LSD generally.

Before use in implantation studies, further characterisation of the cell line EF3 would be required. Additional cross correction studies could be carried out, perhaps involving immunopurification of secreted rhαM, in order to confirm that secreted rhαM was similarly functional to endogenous enzyme.

Due to the lack of cell survival and engraftment observed in implanted guinea pigs in this study, the proximal aim of stem cell therapy studies in α-mannosidosis requires the establishment of conditions to achieve this engraftment without the formation of teratomas. Future studies should thus focus on the utilisation of more extensive and defined differentiation protocols for the generation of mature phenotypes that would allow safe implantation of higher cell numbers. The future use of more refined differentiation protocols should increase the prospects for in vivo studies utilising ES cell-derived cells, and thus the prospects for ES cell-based therapies for α-mannosidosis and LSD generally.

With techniques established for the generation of reproducible stem cell grafts, further studies could then focus on refining conditions to address other factors such as cell and enzyme dosage and distribution, the efficacy of cell implantation in different brain regions, and immune response and the issue of immunosuppression.

While immune response to implanted cells may be a significant consideration when investigating a mouse stem cell-based approach for treatment of neurological disease in the α-mannosidosis guinea pig, it is important to recognise that the ultimate aim of these studies is to develop effective therapies for humans; animal models of LSD provide a means to this end. Thus, the immune response considerations that complicate transplantation in a discordant xenograft paradigm (mouse cells into guinea pig) may not accurately reflect challenges that would be encountered in an allogeneic transplantation situation (human ES cell-derived cells into humans). With this consideration in mind, future studies might initially be better suited to mouse ES cell-
derived cell implants into mouse models of LSD, before applying the techniques developed to human ES cells in a mouse model, larger animal models and (results permitting) ultimately humans. The immediate challenges associated with ES cell-based therapies require extensive in vitro studies and perhaps further studies involving smaller and more logistically permissive animal models (such as mice) before further application of these approaches to the α-mannosidosis guinea pig.
References:


Berg T., King B., Meikle P.J., Nilssen O., Tollersrud O.K., Hopwood J.J. (2001) Purification and characterization of recombinant human lysosomal alpha-


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Jackson, K.A., Mi, T. and Goodell, M.A. (1999) Hematopoietic potential of


Office of the White House Press Secretary, August 9, 2001


http://www.bioethics.gov/background/outkpaper.html


Appendix 1: Cohort of guinea pigs used in this study
Tables showing all animals used in this study, experimental groups to which they were assigned, and behavioural analyses they underwent. The genotype of all α-mannosidosis animals was confirmed by PCR, as described in Section 2.2.4.1. “MWM”: Morris water maze. “NE”: Neurological examinations. “G (2)” and “G (3)” indicate gait analysis at two and three months, respectively. “VAT”: Viability of cells after transplantation, where applicable. “Cells?”: Indicates whether surviving grafts were detected in these animals.
## Appendix 1:

### Preliminary Striatal Implants Study:

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Appendix 2: Vectors used in this study

*pCAGINeoGW* and *pENTRII* (used to construct *pCAGα-mann*) were provided by Dr. Stephen Wood, Child Health Research Unit, WCH. *pENTRII* was the Gateway entry vector used (Invitrogen). *pCAGINeoGW* was modified by Stephen Wood and Poon-Yu Khut from the original vector *pCAGIPuro* (constructed by Ian Chambers and Morag Robertson, April 1997) by the addition of the Gateway cassette (to facilitate recombination) in the multiple cloning site upstream of the IRES sequence, as well as replacing the *puro* gene with *neo*, derived from the vector *pEFIRESPneo* (Hobbs et al., 1998).

*pEFIRESPuro-6* was provided by Dr. Dan Peet (Department of Molecular Biosciences, University of Adelaide). This vector was modified from the original *pEFIRESPuro* (Hobbs et al., 1998) by the addition of a new multiple cloning site, to provide restriction sites as shown (Dan Peet, personal communication).
Appendix 3: Guinea pig whole blood CSA levels

All animals shown were treated daily with 10mg/kg/day Neoral (CSA), administered orally. Whole blood CSA levels shown were measured at approximately one to two months of age and were found to vary considerably between individual animals (individual data not shown).

No general therapeutic CSA range has been reported for guinea pigs. Therapeutic ranges for humans have been reported as 150-400ng/ml (Yee and Salomon, 1992), although these ranges refer to 12 hour trough levels, rather than the 24 hour trough levels graphed above. The trough level of CSA required to provide effective immunosuppression in guinea pigs is at present unknown.
Addenda

Page 12, line 6: The final sentence in this paragraph should read, “One such strategy involves the implantation of stem cells into the brain, to facilitate delivery of enzyme to affected cells in vivo, to therapeutic effect.”

Page 115, line 4: This sentence should read, “All statistical analyses were performed under the guidance of professional statisticians (Craig Hirte and Janine Jones), at the Public Health Research Unit, WCH.”

Page 141, line 29: “MPS II A” should be changed to, “MPS III A”.

Page 270, line 23: The fourth sentence in the third paragraph should read, “The addition of 4x10⁴ U/ml α-mannosidase (consisting of both endogenous mouse and recombinant human enzyme) to the culture media in the present study resulted in a reduced level of both oligosaccharides (sample E, Figures 4.14 and 4.15).” (The word “significantly” was removed).

Page 277, line 20: This sentence should read, “One possible explanation for this observation is that rhoM may have been less mannose-6-phosphorylated than endogenous mouse α-mannosidase, resulting in less efficient delivery of this enzyme to the lysosomes of deficient fibroblasts, and thus a smaller reduction in the levels of stored oligosaccharides.” (The word “efficiently” was removed).

Page 279, line 1: “Robinson, unpublished observations” should be inserted at the end of this first sentence.

Page 281, line 14: The semicolon in this line should be changed to a comma.

Page 320, line 10: The sentence at the end of this line should read, “Although detailed phenotypic analysis and the use of stereological techniques could theoretically be employed to characterize and quantify surviving cells, the scarcity of tissue sections containing surviving cells from the low cell number implants performed in this study precluded these methods of analysis.”

Page 322, line 6: After “non-dividing phenotypes” should follow “(Björklund et al., 2002).”

Page 324, line 18: The last two sentences of this paragraph should be replaced with, “The low cell number implants carried out in this study provided only a limited number of tissue sections containing microsatellite-positive mouse cells. Although histological analysis of immunological markers could have provided valuable data about a possible immune response to these implanted cells, these analyses were not performed due to the scarcity of these sections. Higher levels of cell survival in future studies should thus enable improved characterization of other factors such as immune response.”

Page 360, line 3: The citation “(Hemsley and Hopwood, in preparation)” should read, “(Hemsley and Hopwood, unpublished observations”).

Page 364, line 7: The following paragraph should be inserted, “Following further investigation, should stem cell-based therapies show promise for the long-term treatment of CNS pathology in LSD, it will be important to conduct larger studies in animal models to evaluate these approaches in parallel with other methods for delivering deficient enzyme to the CNS (such as the use of viral vectors and modified enzymes). A thorough assessment of all available strategies for delivering enzyme to the brain will hopefully lead to a safe, reliable therapy for LSD patients.”


Page 375: All references cited in this bibliography should list the journal name in abbreviated form. For example, the fifth reference on this page should cite the journal as “Proc Natl Acad Sci, USA.”