

ENZYME REPLACEMENT THERAPY
IN A MURINE MODEL OF
MUCOPOLYSACCHARIDOSIS
TYPE IIIA

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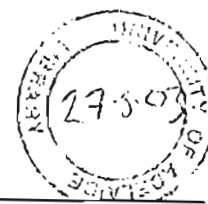
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LIST OF ABBREVIATIONS

BBB	blood brain barrier
BMT	bone marrow transplantation
CHO	Chinese hamster ovary
CNS	central nervous system
CS	chondroitin sulphate
D31N	aspartic acid to asparagine change at codon 31
DMSO	dimethylsulphoxide
DS	dermatan sulphate
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ERT	enzyme replacement therapy
FCS	fetal calf serum
G418	Geneticin
GAG	glycosaminoglycan
GUSB	β -glucuronidase
HA	hyaluronan
hNS	human sulphamidase
HS	heparan sulphate
hr	hours
HRP	horseradish peroxidase
KS	keratan sulphate
LSD	lysosomal storage disorder
mNS	murine sulphamidase
min	minutes
MPS	mucopolysaccharidoses
MPS IIIA	mucopolysaccharidosis type IIIA
M6P	mannose-6-phosphate
MWM	Morris water maze
NS	sulphamidase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PS	penicillin/streptomycin
rhNS	recombinant human sulphamidase
rmNS	recombinant murine sulphamidase
rAAV	recombinant adeno-associated virus
SDS	sodium dodecyl sulphate
sec	seconds
WCH	Women's and Children's Hospital

THESIS ABSTRACT

Mucopolysaccharidosis type IIIA (MPS IIIA, Sanfilippo A syndrome) is an autosomal recessive lysosomal storage disease, with a prevalence in Australia of 1 in 114,000. MPS IIIA is caused by a deficiency of the lysosomal enzyme sulphamidase which is needed together with other exohydrolases and a N-acetyltransferase to break down the glycosaminoglycan heparan sulphate to sulphate and monosaccharides. Patients are characterised by severe central nervous system (CNS) degeneration together with mild somatic involvement; this disproportionate correlation is unique amongst the mucopolysaccharidoses (MPS). Features include severe behavioural disturbances, such as hyperactivity and aggressiveness, coarse hair and mild hepatosplenomegaly. Death is usually in the mid- to late-teenage years. Enzyme replacement therapy (ERT) by intravenous administration of recombinant human NS (rhNS) has been proposed as a potential therapy for MPS IIIA.

A naturally occurring mouse model for MPS IIIA has been described and a colony of these mice has been established. The mice exhibit many of the debilitating features found in human MPS IIIA patients. The MPS IIIA mouse provides an excellent model for developing and testing treatment strategies.

The mutation causing MPS IIIA in the mice was identified as a base substitution at codon 31 in the sulphamidase gene, altering an aspartic acid to an asparagine (D31N). This aspartic 31 (D31) is involved in binding of the divalent metal ion needed for catalytic function. When overexpressed in CHO-K1 cells the intracellular D31N sulphamidase protein corresponded to 1.5% of wild-type sulphamidase levels as determined using a radiolabelled tetrasaccharide substrate. Recombinant murine sulphamidase (rmNS) was expressed in CHO-K1 cells to levels of 4 mg/L of culture medium and purified to apparent homogeneity using a two-step ion exchange method. Denaturing and reducing SDS/PAGE revealed a subunit size of 62 kDa. Kinetic analysis demonstrated that rmNS had kinetic parameters similar to rhNS and human sulphamidase purified from tissue. RmNS could be taken up by MPS IIIA skin fibroblasts via a mannose-6-phosphate receptor mediated process.

Before therapy trials were initiated MPS IIIA mice were tested to assess cognitive functions. The Morris water maze (MWM) assesses spatial learning abilities in rodents. The MWM was performed on MPS IIIA and normal control mice at various ages throughout development. At seven-weeks of age no significant difference was observed between MPS IIIA and normal mice. At 15-weeks of age MPS IIIA mice performed notably worse than normal mice, suggesting that neurological deterioration had commenced. By 20-weeks of age a statistically significant difference was observed between the MPS IIIA and normal mice, with the MPS IIIA mice performing significantly worse than the normal mice in every phase of the testing. From this study it was concluded that severe neurological deterioration of the MPS IIIA mice, as measured in the MWM, occurs by 20-weeks of age.

For many inborn errors of metabolism, early treatment is vital in preventing long-term clinical pathology. The major site of pathology in the MPS IIIA mice is the CNS. Therefore therapy protocols must address the problem of being able to target sufficient amounts of enzyme to the CNS in order to reverse or prevent the cognitive deficits associated with the disease. Transport of molecules from the blood into the CNS is greatly restricted due to the presence of very tight junctions between the endothelial cells of the cerebrovascular capillary walls, known as the blood-brain barrier (BBB). Mice are born with an incompletely formed BBB, which is not fully intact until 10- to 14-days of life. Enzyme distribution studies, by intravenous administration of 1mg/kg of rhNS were performed on MPS IIIA mice on day one of life and at six-weeks of age. Enzyme was detected in the CNS of mice who received enzyme from birth, but not in mice injected with enzyme at six-weeks of age.

Long-term ERT was performed on MPS IIIA mice, initiated either at birth or at six-weeks of age. MPS IIIA and normal control mice received weekly intravenous injections with 1 mg/kg of rhNS until 20-weeks of age. Treatment initiated at birth was more effective than treatment initiated in young adults. In general male MPS IIIA mice housed together become aggressive and fight by 10-weeks of age. MPS IIIA mice who began treatment at six-weeks also began fighting at 10-weeks of age, whereas male MPS IIIA mice, treated from birth were not aggressive until at least 18-weeks of age. Throughout the 20-week study, control mice did not show this aggressive behaviour. Neurological function was measured using the MWM. MPS IIIA mice, treated from birth, and normal

mice performed equally and significantly better, in every phase of this test, than MPS IIIA mice treated from six-weeks of age or left untreated. Histology showed no storage vacuoles in neurons of the cerebellum and cerebral cortex in three-week MPS IIIA mice treated from birth. By eight-weeks of age minimal storage vacuoles had started to return to these cells. Open membranous Storage vacuoles were present in age matched MPS IIIA mice, untreated or treated from six-weeks of age. By 14-weeks of age the neurons in MPS IIIA mice, untreated or treated from six-weeks of age contained abundant storage vacuoles, which were membranous whorls and stacks, a pattern also observed at 18- and 23- weeks of age. In contrast by 14-weeks of age open membranous storage vacuoles were present in MPS IIIA mice enzyme treated from birth; it was not until 18-weeks of age that membranous whorls and stacks were observed in the neurons of these mice. These experiments suggest that rhNS, entering the brain in the first few weeks of life, before the BBB matures, is able to retard the development of behaviour and learning difficulties in MPS IIIA mice.

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:.....20/12/02.....

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The research presented in this thesis was funded by a Sam Lister PhD scholarship. Sam Lister is a young boy from Queensland, Australia, who was diagnosed with MPS IIIA. The lack of an effective therapy prompted his family to raise money for research into therapies for this disorder. The money raised formed the Sam Lister PhD Scholarship. One of the highlights of my PhD was the opportunity to present the work in this thesis at the 2002 Australian MPS Society conference in Canberra. It was at this conference that I had the absolute pleasure of meeting and personally thanking Sam's mum, Penny Lister, for providing me with this scholarship. I hope that this work brings us one step closer to finding an effective therapy for MPS IIIA patients

Special thanks go to my supervisor, Professor John Hopwood, for introducing me to the world of lysosomal storage disorders and for giving me the opportunity to complete my PhD on a very interesting and at times challenging project in the Lysosomal Diseases Research Unit. I am very grateful for his encouragement, guidance and enthusiasm throughout the last four years.

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CHAPTER 1.0

INTRODUCTION AND REVIEW

1.1 GENERAL INTRODUCTION AND PRELIMINARY COMMENTS

Lysosomal storage disorders (LSD) are inborn errors of metabolism characterised by intralysosomal accumulation of unmetabolised compounds. Some 50 different lysosomal disorders are known in humans, each genetically distinct, biochemically related, inherited diseases (Wraith, 2002). Eleven of the disorders, the mucopolysaccharidoses (MPS), result from defects in the catabolism of glycosaminoglycans (GAG). The prevalence values for individual LSD classify these as rare genetic disorders. However, collectively, LSD are far more common, with a prevalence of 1 per 7,700 births (Meikle et al., 1999). Each year in Australia there are, on average, 28 LSD diagnoses made, with an additional four- to five prenatal diagnoses (Meikle et al., 1999).

The work presented in this thesis was performed with the aim of characterising and evaluating a recently discovered murine model of mucopolysaccharidosis type IIIA (MPS IIIA) (Bhaumik et al., 1999). The main focus of the study was the evaluation of enzyme replacement therapy in this model.

This introduction aims to comprehensively cover the process of normal degradation of GAG and lysosomal enzyme biosynthesis and trafficking processes that are defective in MPS IIIA. The historical, clinical, biochemical and genetic aspects of MPS IIIA are also reviewed. Current and future approaches to therapy for the MPS, and animal models for these disorders are the major focus of this review.

1.2 LYSOSOMES AND LYSOSOMAL STORAGE DISORDERS

The lysosome was originally defined as a cytoplasmic particle containing acid hydrolases (De Duve et al., 1955). Novikoff et al (1960) later characterised these organelles at the electron microscope level, showing them to be membrane-bound vacuoles, which contained one or more acid hydrolases. Lysosomes can be distinguished from other intracellular organelles by their acidification properties, their high density on Percoll gradients and by the presence of recognised lysosomal proteins (Hopwood and Brooks, 1997).

The concept of “lysosomal storage disorders” was introduced by Hers (1965) to explain how the genetically determined absence of an enzyme, α -glucosidase, could lead to the fatal condition known as Pompe disease. The deficiency of this enzyme led to the accumulation of undegraded substrate within the lysosome, resulting in an increase in the size and number of these organelles. The cellular pathology resulted in the malfunction of the affected organ. The identification of additional LSD occurred from this discovery, the majority resulting from the deficiency of a single lysosomal enzyme.

1.3 THE MUCOPOLYSACCHARIDOSES

The MPS are a family of heritable disorders caused by deficiencies of lysosomal enzymes required for the degradation of GAG. The undegraded or partially degraded GAG are stored in lysosomes and excreted in urine. Each of the known MPS involves the deficiency of one of 11 enzymes required for the stepwise degradation of dermatan sulphate (DS), heparan sulphate (HS), keratan sulphate (KS), chondroitin sulphate (CS) or hyaluronan (HA), singly or in combination.

The deficiency of a single enzyme has complex and far-reaching effects, ranging from abnormalities of the skeletal and vascular systems and, in some instances, to the impairment and deterioration of mental function.

The first clinical observation of patients with MPS was by Charles Hunter (1917) and Gertrude Hurler (1919). Hunter described two brothers exhibiting unique facial appearance, hepatosplenomegaly, short stature, deafness, heart disease and joint stiffness. Hurler described two unrelated males exhibiting the same features with the addition of corneal clouding and mental retardation. In the medical literature the two entities were rarely separated and became known as Hurler-Hunter syndrome. In 1952 Brante devised the term 'mucopolysaccharidoses' for the disorders after finding a CS-like substance storing in the liver and meninges of a Hurler patient.

Dorfman and Lorincz (1957) noted the increase in urinary excretion of GAG, whilst van Hoof and Hers (1957) were the first to demonstrate large vacuoles containing granular material from the liver of a Hurler patient. They suggested that the undigested GAG were distending the lysosomes to cause a lysosomal storage disease. McKusick (1965) systematised the classification of the MPS into six eponymic types based on the types of GAG excreted in the urine, mode of inheritance and clinical features.

Fratantoni et al (1968) proposed that the basic defect in MPS was due to a decrease in the breakdown of GAG. A hypothesis prior to this report had been of overproduction of MPS and sequestration of the material in the lysosome. In the ensuing period Fratantoni et al (1969) discovered that the abnormal metabolism of GAG in cultured fibroblasts from Hurler or Hunter patients could be corrected if cells of these two genotypes were mixed with each other or with normal cells. They showed that cell contact was not necessary because the effect was mediated by substances or "corrective factors" released into the culture medium.

During the 1970's most of the enzymatic steps involved in the degradation of the GAG were described and MPS patients catalogued according to enzyme deficiency.

The two major sites of pathology in the MPS are the central nervous system (CNS) causing mental retardation, and the skeletal system, resulting in dwarfism. Each MPS type

exhibits one or both of these disease outcomes (Table 1.1). MPS I (Hurler/Scheie syndrome) is due to the deficiency of α -L-iduronidase. Two forms are distinguished clinically, but do in fact result from the same enzyme deficiency. Hurler syndrome (MPS IH) is the clinically severe form and has become the prototype of the whole group. The clinically milder form, Scheie syndrome (MPS IS), was initially considered a separate disorder, MPS V, however, it was shown that cells grown from Hurler syndrome patients were not corrected by co-cultivation with culture media from MPS V cells. The enzyme defect in MPS V led to the reclassification of MPS V as MPS IS. MPS II (Hunter syndrome) results from the deficiency of iduronate-2-sulphatase. MPS II is the only MPS transmitted by an X-linked recessive mode of inheritance, while the others are inherited as autosomal recessive diseases. MPS III (Sanfilippo syndrome) comprises four distinct subtypes A, B, C and D based on different enzyme deficiencies, which will be discussed later in Section 1.8. MPS IV (Morquio syndrome) is similarly recognised as two distinct enzyme deficiencies, A and B. MPS VI (Maroteaux-Lamy syndrome) is due to the deficiency of N-acetylgalactosamine-4-sulphatase. MPS VII (Sly syndrome) is due to the deficiency of β -glucuronidase. Finally, MPS IX is due to a deficiency of the enzyme hyaluronidase.

Before a detailed review of MPS-III, the normal processes of GAG synthesis and degradation, and the process of lysosomal enzyme synthesis and maturation will be reviewed.

1.4 GLYCOSAMINOGLYCANS AND PROTEOGLYCANS

1.4.1 STRUCTURE AND FUNCTION

GAG are a group of negatively charged polysaccharides with a wide tissue distribution. In DS, HS and CS, the polysaccharide chains are composed of alternating residues of uronic acid and sulphated hexosamine. KS differs from the other GAG in that the uronic acid residues are replaced by galactose (Yanagishita, 1993). HA is a non-sulphated GAG which consists of repeating disaccharides of glucuronic acid and N-acetylglucosamine. It is synthesised at the plasma membrane (other GAG are synthesised in the Golgi and

Table 1.1: Classification of the mucopolysaccharidosis*.

* From Hopwood and Morris, 1990.

DS, dermatan sulphate; HS, heparan sulphate; KS, keratan sulphate; CS, chondroitin sulphate; HA, hyaluronan.

^ within each cell type, disease severity is variable.

M = mental retardation

S = skeletal deformities

- = absent

Table 1.1: Classification of the mucopolysaccharidoses*

MPS type	Eponym	Enzyme deficiency	Stored substrates #	Major clinical features ^
I	Hurler/ Scheie	α -L-Iduronidase	HS & DS	M S (Hurler) +/-M +/-S (Hurler/Scheie) - +/-S (Scheie)
II	Hunter	Iduronate-2- sulphatase	HS & DS	M S
IIIA	Sanfilippo A	Sulphamidase	HS	M -
IIIB	Sanfilippo B	α -N-Acetyl- glucosaminidase	HS	M -
IIIC	Sanfilippo C	Acetyl-CoA: α - Glucosaminide N-acetyltransferase	HS	M -
IIID	Sanfilippo D	Glucosamine-6- sulphatase	HS	M -
IV A	Morquio A	Galactose-6- sulphatase	KS & CS	- S
IV B	Morquio B	β -D-Galactosidase	KS	- S
VI	Maroteaux- Lamy	N-Acetyl- galactosamine- 4-sulphatase	DS & CS	- S
VII	Sly	β -D-Glucuronidase	DS, HS& CS	M S
IX	None present, Triggs-Raine, et al., 1999	Hyaluronidase	HA	- -

trans-Golgi network). The hexuronic acid and hexosamine composition and the configuration of the glycosidic linkages contribute to the diversity in the chemical organisation of GAG molecules. The hexosamine can be glucosamine or galactosamine. The hexuronic acid can be D-glucuronic acid or its epimer, L-iduronic acid, or both. Also, heterogeneity within each GAG is dictated by chain length and the degree and position of sulphate groups (Cantz and Gehler, 1976; Roden, 1980).

Most GAG exist as an integral component of proteoglycans where they are covalently bound to a protein core (Yanagishita, 1993). Only one type of GAG, HA, appears to be normally expressed as free chains without a core protein (Toole, 1990).

The biological functions of proteoglycans are influenced by their structure. Proteoglycans are ubiquitous in all mammalian tissues; at the cellular level they have been found in the nucleus, the cell membrane and in the extracellular matrix; they are a major component of connective tissue. Proteoglycans play a role in cell-cell recognition and adhesion, cell proliferation, matrix formation and cell-matrix interactions (Yanagishita, 1993).

1.4.2 GLYCOSAMINOGLYCAN SYNTHESIS

The first step in synthesis for glycoproteins and proteoglycans is the formation of a core protein to which glycan chains are subsequently attached. The core protein is synthesised on membrane-bound ribosomes and the nascent polypeptide chain is translocated into the endoplasmic reticulum (ER) (Yanagishita, 1993). GAG chain synthesis occurs during the passage of the core protein through the ER and Golgi compartments, and is initiated by the addition of a D-xylosyl moiety to specific serine residues in the core protein, followed by the extension of this xyloside with two β -linked galactose residues. After the addition of a glucuronic acid residue to the second galactose to form the linkage region serine-xylose-galactose-galactose-glucuronic acid, the GAG chain is then assembled onto the acceptor sequence by the repeated and alternating addition of a N-acetylhexosamine and a glucuronic acid to the non-reducing end of the growing GAG chain (Roden, 1980; Fransson, 1987). For example, for DS and CS, the hexosamine is N-acetylgalactosamine

and the hexuronic acid is glucuronic acid; once the GAG chain is formed some β -D-glucuronic acid is epimerised to α -L-iduronic acid for DS; all β -D-glucuronic acid residues remain in CS.

Sulphation of the GAG chains (except HA) can occur at selected N (for HS only) and O positions; the reaction is catalysed by sulphotransferases and takes place in the Golgi complex. The frequency and position of sulphation is variable, resulting in the structural heterogeneity evident in each GAG chain type (Figure 1.1). The proteoglycans are transported to the cell surface once the elongation and modification of the carbohydrate chains has been completed (Yanagishita, 1993).

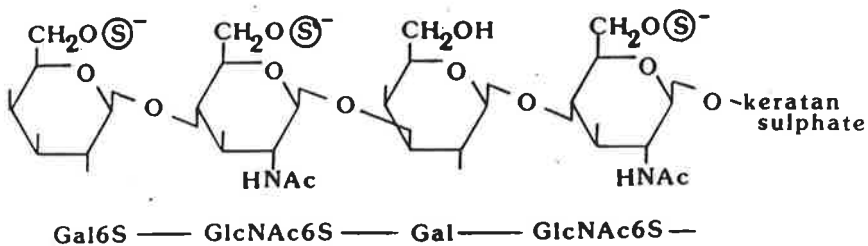
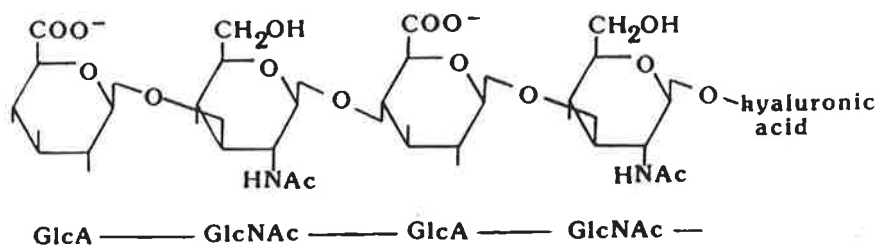
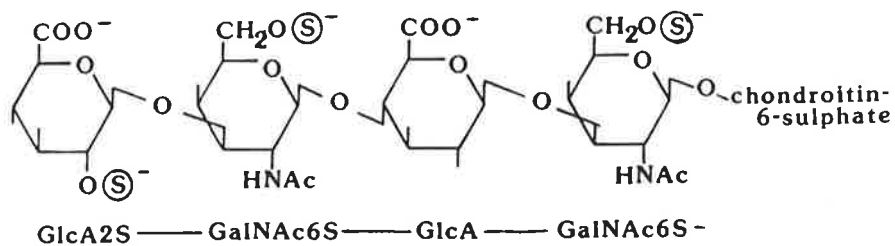
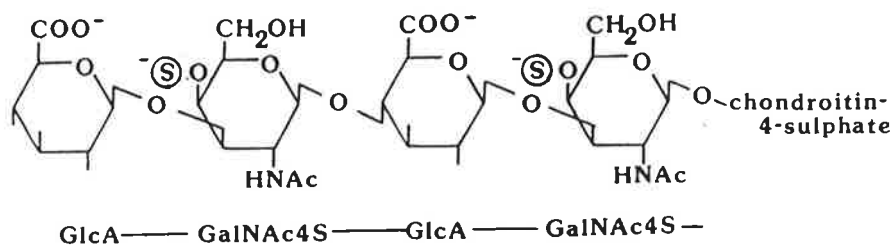
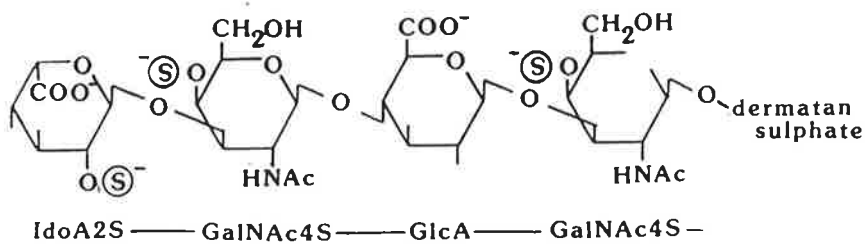
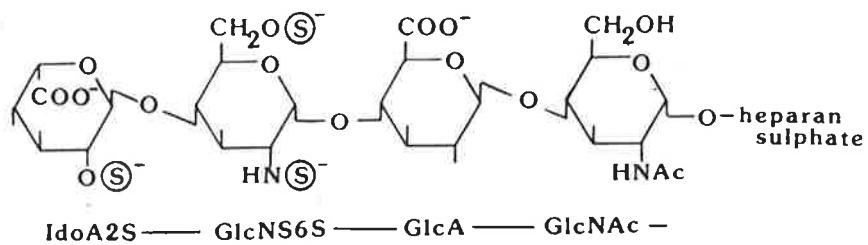
1.4.3 GLYCOSAMINOGLYCAN DEGRADATION

Proteoglycans and proteoglycan fragments are completely degraded to monosaccharides, sulphate and amino acids in the lysosome. At first proteoglycans are subjected to limited degradation at the cell surface, matrix or in endosomes by proteases to reduce the protein core to peptides, and by endoglycosidases that reduce GAG chains into oligosaccharide fragments (Hopwood and Morris, 1990). These GAG fragments are transported to the lysosome and are further degraded by the sequential action of a series of exohydrolases (glycosidases and sulphatases) and, for HS also a N-acetyltransferase, to reduce the oligosaccharide to free monosaccharides and sulphate (Hopwood and Morris, 1990). MPS disorders result when GAG degradation in the lysosome is defective. In the MPS, deficiencies in the activity of a single glycosidase, N-acetyltransferase or sulphatase perturbs the degradative pathway resulting in the intralysosomal storage and urinary excretion of partially degraded GAG (Hopwood and Morris, 1990).

The degradation of HS is affected in MPS III and also MPS I, II and VII patients. The normal process of synthesis and degradation of HS is discussed below.

Figure 1.1: Structures of the GAG.

Heparan sulphate (HS), dermatan sulphate (DS), chondroitin sulphate (CS), hyaluronic acid (HA) and Keratan sulphate (KS).



1.5 HEPARAN SULPHATE

HS is synthesised as a proteoglycan (consisting of at least two HS chains covalently linked to a protein core) and has ubiquitous distribution on cell surfaces and in the extracellular matrix (Salmivirta et al., 1996). Interactions between HS and specified proteins are being increasingly implicated in a variety of physiological processes such as cell adhesion, enzyme regulation and cytokine action (Lindahl et al., 1994).

HS is made up of uronic acid residues that alternate with α -linked glucosamine residues (Roden, 1980; Fransson, 1989; Hopwood, 1989). The uronic acid may be glucuronic or iduronic; these may occasionally or regularly be 2-sulphated and the glucosamine may be N-sulphated or N-acetylated, 6-sulphated and 3-sulphated. Some of the newly synthesised HS proteoglycan is intercalated into the plasma membrane, endocytosed and catabolised with a half-time of approximately four- to six-hours (Yanagishita and Hascall, 1984a, b; 1985).

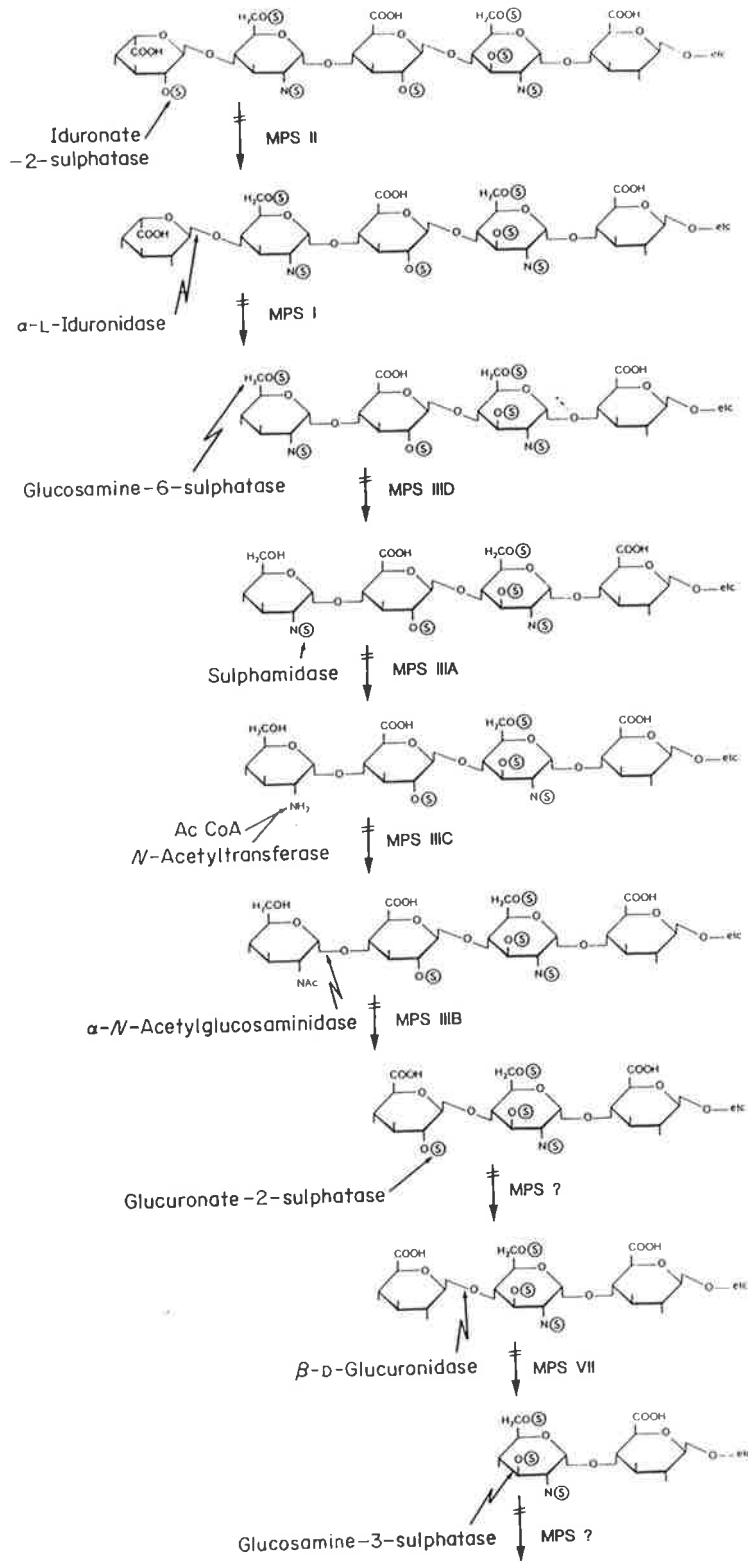
Degradation of HS begins in the early endosome, where it is subjected to proteolysis producing single HS chains (approximately 30 kDa), which are degraded by substrate structure-specific endo- β -glucuronidase to oligosaccharides (approximately 5 kDa) in various endosomic compartments (for a review see Hopwood, 1989). These oligosaccharides are exported from acid endosomes to the lysosomes where degradation continues from the non-reducing end by the action of up to three glycosidases, five sulphatases and a biosynthetic enzyme that transfers an acetyl group from acetyl CoA to glucosamine. The resultant monosaccharides and sulphate are removed from the lysosome by specific transport systems and may then be re-used in biosynthetic pathways (Hopwood and Morris, 1990).

Except for N-acetyltransferase and glucosamine-3-sulphatase, the enzymes involved in the degradation of HS (Figure 1.2) have been purified to homogeneity and their functions studied. Iduronate-2-sulphatase, the defective enzyme in MPS-II, hydrolyses non-reducing end iduronate-2-sulphate esters in HS and DS. α -L-Iduronidase then acts on the

Figure 1.2: Stepwise degradation of HS.

(from Hopwood and Morris, 1990)

A diagrammatic representation of the stepwise degradation of a hypothetical HS oligosaccharide in the lysosome by nine exoenzymes. An inability to degrade components of the HS chain due to defective activity of any one of these enzymes results in a specific MPS disorder, indicated alongside the broken arrows.



oligosaccharide product of iduronate-2-sulphatase, resulting in free iduronic acid and an oligosaccharide product that then becomes the substrate, degraded by glucosamine-6-sulphatase. The next enzyme in the pathway is sulphamidase which acts on the oligosaccharide product of glucosamine-6-sulphatase. Sulphamidase de-N-sulphates the non-reducing end glucosamine-N-sulphate (GlcNS) oligosaccharide. A biosynthetic step then takes place to N-acetylate the non-reducing end glucosamine product to GlcNAc oligosaccharide, allowing degradation to continue (Klein et al., 1978). The next enzyme, α -N-acetylglucosaminidase acts on the acetylated oligosaccharide product of the N-acetyltransferase. Glucuronate-2-sulphatase is then required to desulphate glucuronate-2-sulphate. The following enzyme, β -D-glucuronidase, is found in lysosomes and microsomes. β -D-Glucuronidase associates with egasyn, an accessory protein to hold a fraction of the total enzyme in microsomes. β -D-Glucuronidase is transported to the lysosome when it is in excess of egasyn (Luisis and Paigen, 1977).

To summarise, the degradation of HS GAG oligosaccharide fragments is carried out by the sequential action of lysosomal hydrolases and a biosynthetic enzyme, N-acetyltransferase. The synthesis, processing and intracellular trafficking of lysosomal enzymes is reviewed below.

1.6 BIOSYNTHESIS AND PROCESSING OF LYSOSOMAL ENZYMES

Lysosomal enzymes, along with secretory proteins and plasma membrane proteins, are synthesised on membrane-bound polysomes in the rough endoplasmic reticulum (ER) (Kornfeld, 1986). Each of these proteins contain a hydrophobic amino terminal signal peptide that interacts with a signal recognition particle, an 11S ribonucleoprotein, that initiates the vectorial transport of the nascent protein across the ER membrane into the lumen of that organelle (Erickson et al., 1981,1983; Rosenfeld et al., 1982).

1.6.1 ADDITION OF OLIGOSACCHARIDES

Lysosomal enzymes undergo cotranslational glycosylation of selected Asn residues. Glycosylation involves the transfer of preformed oligosaccharide that may contain three glucose, nine mannose and two N-acetylglucosamine residues from a lipid-linked intermediate to the nascent polypeptide (Kornfeld and Kornfeld, 1985). The signal peptide is cleaved within the ER, and the processing of the Asn-linked oligosaccharide begins by the excision of three glucoses and one of the mannose residues from the oligosaccharide. The proteins are then transported through the ER to the Golgi complex by membrane-bound vesicles where they are subjected to a variety of post-translational modifications and are sorted for targeting to a final destination, the lysosome, secretory granule or plasma membrane. During passage through the Golgi, the oligosaccharides on secretory and membrane glycoproteins are processed to have sialic acid-containing complex-type units. All sulphatases have their active site cysteine modified to a α -fomylglycine residue, this modification is essential for an active sulphatase product (reviewed by Hopwood and Ballabio, 2001). The critical modification for most lysosomal enzymes is the acquisition of phosphomannosyl residues; these serve as the essential component of a recognition marker which enables the binding to high affinity receptors known as mannose-6-phosphate (M6P) receptors and allow for the subsequent translocation to the lysosome (Kaplan et al., 1977).

1.6.2 ADDITION OF MANNOSE-6-PHOSPHATE

The M6P marker is generated in two steps by the sequential action of two Golgi enzymes (Kornfeld, 1986). Firstly, N-acetylglucosaminylphosphotransferase transfers N-acetylglucosamine-1-phosphate from the nucleus sugar uridine diphosphate-N-acetylglucosamine to selected mannose residues on lysosomal enzymes to give rise to a phosphodiester intermediate. Secondly, N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase removes the N-acetylglucosamine residue resulting in the M6P moiety. The M6P moiety can then bind to M6P receptors in the trans-Golgi network (TGN) which in turn targets the enzyme to the lysosome (Pfeffer, 1988).

1.6.3 MANNOSE-6-PHOSPHATE RECEPTORS

Two distinct M6P receptors have been isolated. One requires the presence of divalent cations for M6P binding and is therefore referred to as the cation-dependent M6P (CD-M6P) receptor. The other binds to M6P containing ligands independent of divalent cations, and as such is referred to as the cation-independent M6P (CI-M6P) receptor. The structure and function of M6P receptors have been reviewed by Pfeffer (1988), von Figura and Hasilik (1986), and Dahms et al., (1989). The CI-M6P receptor is a type I integral membrane glycoprotein of about 270-300 kDa. This receptor has been shown to be structurally and functionally identical to the insulin-like growth factor receptor¹ and is therefore sometimes referred to as the M6P/IGF-II receptor (Goda and Pfeffer, 1988). The human CI-M6P receptor is divided into two domains, a 226.5 kDa extracellular domain and a small (164 amino acid) cytoplasmic domain. The CD-M6P receptor is a smaller type I integral membrane glycoprotein of about 46 kDa. This receptor contains a 257 amino acid extracellular domain and a 67 amino acid cytoplasmic domain. The dimerisation of CD-M6P receptors may be required for their correct intracellular transport between the TGN, endosome and cell membrane (Pfeffer, 1988). The need for two M6P receptors is unclear. Both receptors have been implicated in the sorting of proteins to lysosomes (Goda and Pfeffer, 1988), however only the CI-M6P receptor is capable of endocytosing extracellular lysosomal enzymes at the cell surface (Kornfeld, 1992). Both receptors are primarily located intracellularly, within endosomes and the Golgi and, to a small extent, at the cell surface. M6P receptors are transported readily between these compartments, whether or not the receptors are occupied (Pfeffer, 1987; Duncan and Kornfeld, 1988).

1.6.4 MANNOSE-6-PHOSPHATE-DEPENDENT AND INDEPENDENT TRANSPORT

The majority of newly synthesised mannose-6-phosphorylated enzyme is targeted to the lysosome via a direct intracellular route, the *biosynthetic pathway*. The lysosomal enzymes bind to a 47 kDa or 300 kDa M6P receptor in the TGN at pH 7.0. The receptor-enzyme complex then exits the Golgi and is transported to a prelysosome or intermediate

compartment, where dissociation of the ligand occurs by acidification of the compartment to below pH 6.0 (Griffiths et al., 1988). The M6P receptor is either recycled back to the Golgi for further targeting of endogenous *de novo* synthesised lysosomal enzymes, or transported to the plasma membrane where it functions to internalise exogenous lysosomal enzymes (Dahms et al., 1989). Alternatively, a proportion of mannose-6-phosphorylated lysosomal enzymes, usually 5-20%, escape lysosomal targeting via the biosynthetic pathway and are secreted from the cell. A fraction of these enzymes may bind to M6P receptors present on the cell surface and be internalised and delivered to lysosomes via an *endocytic pathway* (Willingham et al., 1981). In fibroblasts, this secretion-recapture pathway lysosomally targets about 5-10% of lysosomal enzymes (Vladutiu and Rattazzi, 1979). This is therapeutically important when considering treatment strategies for LSD. The concept that all cells require an active lysosomal system and all appear capable of taking up lysosomal enzyme proteins from the surrounding cells or body fluids by this receptor-mediated endocytic pathway, makes LSD good candidates for replacement therapy. Furthermore, only small amounts of enzyme (approximately 5% of normal levels) are needed to correct the defect in cells (Hopwood and Morris, 1990).

A number of lysosomal enzymes are targeted to the lysosome independently of the M6P receptor pathway (Kornfeld, 1986). Evidence for the existence of M6P-independent sorting pathways came from the characterisation of cells from patients with I-cell disease (mucopolipidosis II). The cells of these patients are unable to synthesise the 6-phosphomannosyl marker on the N-linked oligosaccharides of the lysosomal hydrolases; as a result, newly synthesised lysosomal enzymes are unable to bind the M6P receptor. Fibroblasts from I-cell patients contain normal levels of some lysosomal enzymes such as β -D-glucuronidase and acid phosphatase. In contrast, other lysosomal enzyme activities in I-cell fibroblasts are significantly reduced, such as arylsulphatase A. Furthermore, lysosomal storage in I-cell patients is limited to only certain cell types (predominantly connective tissue). No lysosomal storage is observed in other tissues such as the liver, spleen, kidney and brain, all of which contain near normal levels of lysosomal enzymes. Some lysosomal enzymes can be transported to the lysosome independently of the M6P

receptor pathway via receptors specific for mannose, galactose, fucose and N-acetylgalactosamine (Koster et al., 1994).

1.6.5 PROCESSING OF LYSOSOMAL PROTEINS

In addition to oligosaccharide processing (trimming of the carbohydrate residues and dephosphorylation of the M6P oligosaccharides), lysosomal enzymes undergo further proteolytic processing, which includes fragmentation and trimming at the amino and carboxyl termini of the protein by the action of endo- and exo-proteinases that reside in the prelysosomal and lysosomal compartments (Kornfeld, 1986).

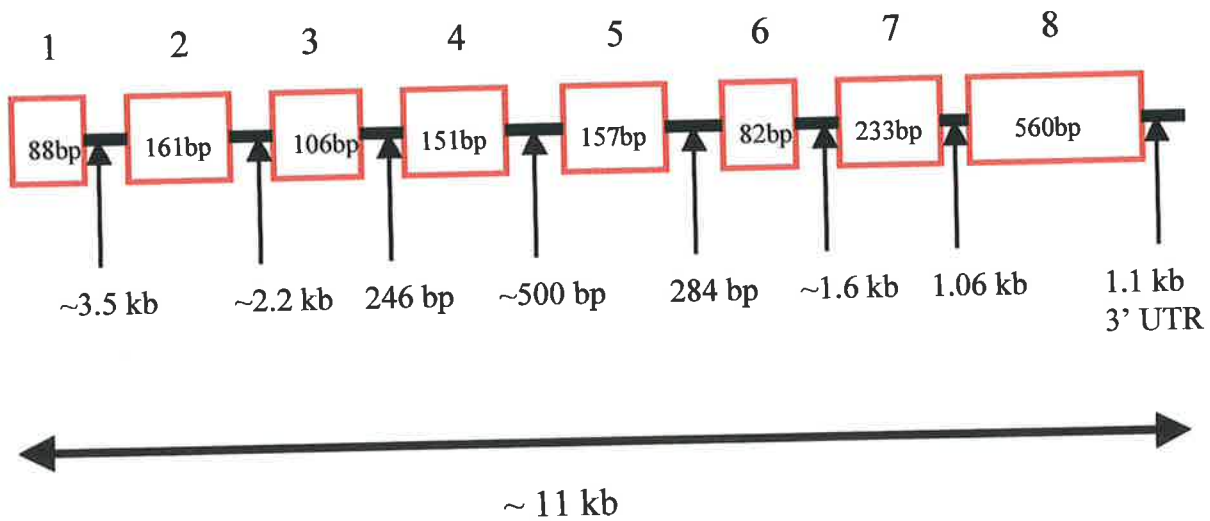
The following sections of this review analyse the enzyme deficiency in MPS IIIA and the clinical features, diagnostic and therapeutic aspects of this disorder.

1.7 SULPHAMIDASE

The lysosomal enzyme deficient in MPS-III A is sulphamidase (NS). NS is a lysosomal exohydrolase required for the degradation of HS in mammalian cells. The enzyme was first purified and characterised as the Sanfilippo A corrective factor it was partially purified from urine and shown to be able to correct storage of $^{35}\text{SO}_4^-$ metabolically labelled material in cultured fibroblast cells from the skin of MPS IIIA patients (Kresse and Neufeld, 1972).

The NS gene was localised by fluorescence in situ hybridisation to chromosome 17q25, the predicted localisation being 17q25.3 (Scott et al., 1995). The sequence required for enzymically active NS contains an open reading frame of 1,506 nucleotides, encoding a protein of 502 amino acids. Peptidase cleavage of the signal peptide takes place after amino acid 20, immediately before the start of the N-terminal amino acid sequence of mature NS (Scott et al., 1995). There are five potential N-glycosylation sites, at least one of which must carry the M6P lysosomal targeting signal (Scott et al., 1995). The NS gene consists of eight exons and spans approximately 11 kb (Karageorgos et al., 1996) (Figure 1.3). Northern blot analysis revealed transcripts of 3.1, 4.3 and 7.1 kb. These mRNA

Figure 1.3: Structure of the human sulphamidase gene*



* From Karageorgos et al., 1996.

The sulphamidase gene is split into 8 exons (red boxes) spanning approximately 11 kb. Introns are denoted by bars between each exon and approximate sizes are given.

species are likely to reflect NS mRNA produced using alternate polyadenylation sites; it is possible that they also result from overlapping genes and/or transcripts, or other NS mRNA processing events (Scott et al., 1995). NS mRNA has been detected by Northern blot analysis in most somatic tissues, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leucocytes, heart, placenta, lung, liver, skeletal muscle, kidney, pancreas and brain (Scott et al., 1995).

The native molecular weight of recombinant human NS (rhNS) secreted from CHO-K1 cells is 115 kDa (Bielicki et al., 1998), which is similar to the native size of mature NS purified from human liver (Freeman and Hopwood, 1986). Denaturing and reducing SDS/PAGE of recombinant NS revealed a 62 kDa precursor protein and a mature 56 kDa protein suggesting the native molecule exists as a dimer (Bielicki et al., 1998). Monoclonal and polyclonal antibodies toward recombinant human NS have been produced for the molecular characterisation of wild type and mutant NS (Perkins et al., 1999; Perkins et al., 2001).

1.8 MUCOPOLYSACCHARIDOSIS TYPE III

MPS III or Sanfilippo syndrome takes its name from Dr. Sylvester Sanfilippo who first described the condition (Sanfilippo et al., 1963). Eight patients with varying degrees of mental retardation and 'acid mucopolysacchariduria' of the HS type were studied at the University of Minnesota Hospitals between the period 1960-1963. Five of the children exhibited normal or non-specific facial characteristics, and three displayed facial characteristics indicative of those seen in Hunter-Hurler syndrome. The patients ranged from 1.5 to 10 years of age. Hepatosplenomegaly occurred in seven of the eight patients, corneal clouding was not observed, and two of the children were dwarfed. Paper chromatography assays were performed on GAG chemically isolated from the urine of the eight patients. Urine from four patients displayed paper chromatographic mobility characteristics of HS and DS, thus a diagnosis of Hunter-Hurler syndrome was made. However, urine from the other four patients displayed paper chromatographic mobility characteristics of HS only, no DS, a GAG found in excess in the urine of patients with

Hunter-Hurler syndrome, was evident. These studies indicated the existence of a new inborn error of mucopolysaccharidosis, coined MPS III or Sanfilippo syndrome.

Initially MPS III was classified as a single disorder based on clinical and urinary GAG analysis. Later experiments by cross-correction of fibroblasts isolated from MPS III patients, revealed two groups, each deficient in a different factor (Kresse et al., 1971). Eventually MPS III was divided into four distinct subtypes (reviewed by Neufeld and Muenzer, 1995). The enzymes deficient in the Sanfilippo sub-types include sulphamidase (type A); α -N-acetylglucosaminidase (type B); acetyl CoA: α -glucosaminide N-acetyltransferase (type C) and N-acetylglucosamine-6-sulphatase (type D). All four enzymes are required for the degradation of HS. Although Sanfilippo syndrome makes up a biochemically diverse group, the four sub-types are clinically indistinguishable.

MPS III is the most common form of MPS. Estimates of incidence range from 1:24,000 in The Netherlands (van de Kamp et al., 1981) to 1:66,000 in Australia (Meikle et al., 1999), to approximately 1:324,000 in British Columbia (Lowry et al., 1990). MPS IIIA is the most common subtype in Northern Europe, whereas MPS IIIB is more prevalent in Italy and Greece (Betris et al., 1986; Michelakakis et al., 1995).

1.8.1 CLINICAL DESCRIPTION

MPS III is a progressive disorder in which patients are characterised by severe CNS degeneration together with mild somatic involvement; this disproportionate correlation is unique amongst the MPS. Features may include hyperactivity, delayed development, coarse hair, hirsutism, sleep disorders and mild hepatosplenomegaly (Figure 1.4). The onset of clinical features is variable but is usually evident between the ages of two- to eight-years after a period of normal development (Neufeld and Muenzer, 1995). Due to the relatively late onset of clinically obvious symptoms, diagnosis is often made very late.

Figure 1.4: Clinical phenotype of MPS IIIA.

(From Clarke, 1997b).

Serial photographs of a patient with MPS IIIA at (a) 4 ½, (b) 6 ½, (c) 10, (d) 11, and (e) 12 years. A progressive coarseness of facial features and hair is observed.



(a)



(b)



(c)



(d)



(e)

In general, the disorder tends to have three main stages. Firstly, between the ages of one- and four-years the clinical pattern is one of developmental delay; children may be overactive and difficult to control. Many children may present with recurrent ear, nose and throat infections or diarrhoea (Cleary and Wraith, 1993).

The second phase of illness, which often begins around the ages of three- to seven-years, is characterised by severe behavioural disturbance. This stage of the disorder is the most difficult; the normal growth and well-developed muscular strength of an affected child makes them hard to control. Generally patients are extremely hyperactive and have frequent temper tantrums; there is also a rapid diminution in attention span (Cleary and Wraith, 1993).

In the third and final phase of the syndrome, which begins at about 10-years of age, frequent falls are common as balance is lost; eventually affected patients lose the ability to walk. Feeding becomes difficult due to an impaired swallowing mechanism resulting in increased episodes of aspiration; most children need a nasogastric tube to obtain a sufficient energy intake. Joint stiffness resulting from connective tissue deterioration and increasing spasticity impairs mobility, confining most patients to a wheelchair by their mid-teenage years. Seizures may also be prevalent during this phase (Cleary and Wraith, 1993).

General physical health and strength deteriorates with age and many children become wasted despite sufficient energy intake. Death most commonly occurs in mid-to late-teenage years, usually as a result of a respiratory infection.

1.8.2 ALLELIC VARIANTS

Consistent with many genetic diseases, a large number of mutant alleles exist in the MPS IIIA patient population, although a few alleles may predominate. Mutation analysis of MPS IIIA patients has revealed several common mutations. A total of 62 mutations have now been defined for MPS IIIA, including 42 missense mutations, four nonsense

mutations, one splice site mutation, eight small deletions and seven small insertions (Yogalingam and Hopwood, 2001).

Blanch et al (1997) found that six of 10 Australian and American patients with MPS IIIA had at least one allele of the NS gene which carried a G to A transition at nucleotide position 746, resulting in a missense mutation of arginine 245 to histidine (R245H). This missense mutation was present in seven of 20 alleles from the six patients, including one patient homozygous for R245H. A study of MPS IIIA patients from The Netherlands (Weber et al., 1997) found that the R245H mutation accounted for 56.7% of the MPS IIIA alleles. There was a higher prevalence of the R245H allele in Western Netherlands, as compared to the Eastern regions of The Netherlands. From the 39 patients analysed in this study, 13 were homozygous for this common mutation; they exhibited a more uniform but severe clinical phenotype compared to the remaining 21 or five patients, who, respectively, had one or no R245H alleles.

The missense mutation arginine 74 to cysteine (R74C), which disrupts an evolutionarily conserved amino acid in the active site of the NS enzyme, was found to be present in 56% of alleles of 16 Polish MPS IIIA patients (Bunge et al., 1997). However, this mutation was present in only 21% of disease alleles among German patients. Interestingly, the R245H mutation represented 35% of disease alleles in German patients, but only 3% in Polish patients.

In a study of 24 Italian MPS IIIA patients, Di Natale et al (1998) identified a serine 66 to tryptophan (S66W) missense mutation in the NS gene, which accounted for 33% of the total alleles.

Esposito et al (2000) transiently expressed a number of missense mutations (Y40N, A44T, G122R, P128L, L146P, R150Q, D179N, R182C, R206P and P227R) in COS cells. NS activity was not detected in COS cells over-expressing any of these mutations.

The four identified nonsense mutations include W210X, R233X, Q365X and E369X. (Yogalingam and Hopwood, 2001). A point mutation (IVS2-2A>G) has been identified in homozygous form in an MPS IIIA patient from Finland (Bunge et al., 1997). Eight deletions ranging from 1 bp to 11 bp and seven insertions ranging from 1 bp to 18 bp have been identified in the NS gene (Yogalingam and Hopwood, 2001).

1.8.3 DIAGNOSIS OF MPS III

Due to overlapping features between the different MPS types, the precise diagnosis of a given MPS disorder based solely on clinical and radiological criteria is difficult. Differential diagnosis must therefore be performed biochemically. Enzymatic assays can be performed on patient samples including leucocytes and cultured fibroblasts using various artificial and naturally derived substrates. Prenatal diagnosis may be performed in the first trimester by chorionic villus sampling and direct enzyme analysis of the villi, or by amniocentesis. MPS-III A can be detected by the demonstration of HS by two-dimensional electrophoresis of GAG. NS activity is usually assayed by the use of commercially available radiolabelled [³⁵S]-heparin but the use of radiolabelled disaccharides and tetrasaccharides has also been reported (Hopwood and Elliott, 1982).

Gradient PAGE is a very rapid and sensitive method for identifying the accumulated GAG in the MPS disorder. The combination of gradient PAGE and digestion with recombinant lysosomal hydrolases provides a definitive diagnosis of MPS types and has proven to be useful in distinguishing between MPS III subtypes (Byers et al., 1998). A shift in the banding pattern of GAG isolated from urine is observed after enzymatic digestion. The majority of lysosomal enzymes have been produced in recombinant systems or are available commercially (Byers et al., 1998). A fluorimetric enzyme assay for the diagnosis of MPS IIIA has also been developed (Kleijer et al., 1996), which utilises the synthetic substrate 4-methylumbelliferyl- α -D-N-suphoglucosaminide (MU- α -GlcNS). NS desulphates MU- α -GlcNS to MU- α -GlcNH₂; further hydrolysis with a α -hexosaminidase liberates 4-methylumbelliferone (MU) that can then be fluorometrically measured.

1.9 THERAPY FOR MPS III

1.9.1 CURRENT APPROACHES TO MPS III PATIENT MANAGEMENT

There is no specific therapy as yet for MPS III, hence current treatment is limited to ameliorative care. In the first phase of the illness attention to ear, nose and throat problems is necessary. Unlike other MPS the milder somatic phenotype in MPS III reduces the anaesthetic risk and therefore patients can undergo operative procedures. Management during the second phase of the illness is very difficult. Sleep disturbance is often managed by the use of drugs such as chloral hydrate and trimeprazine tartrate (Cleary and Wraith, 1993), however in some children this can produce overactivity and in others rebound sedation the next day. Maintaining the affected patients in a disciplined environment can help to manage the aggression and hyperactivity. Problems during the third and final stages of the disorder include feeding difficulties as a result of swallowing incoordination; food has to be progressively liquidised and episodes of aspiration and nasal regurgitation become more common (Cleary and Wraith, 1993). An inability to swallow saliva with constant choking heralds the need for nasogastric tube feeding combined with medication to inhibit saliva production. In most cases parents of affected children seek help through respite care.

For therapeutic protocols to be established for patients with MPS III and other disorders, they must first undergo evaluation in animal models. The various animal models available for LSD and MPS therapy evaluation are outlined below.

1.10 ANIMAL MODELS OF LSD AND MPS DISORDERS

A large number of genetically authentic animal models of LSD have been identified in a number of domestic, laboratory or farm animals, including dogs, cats, rats, mice, goats, guinea-pigs, emu and cattle (Patterson et al., 1982; Haskins et al., 1991; Crawley et al., 1999; Bermudez et al., 1995; Kim et al., 1996). Three well defined genetic LSD occur spontaneously in mice (MPS VII, Krabbe and MPS IIIA). However, with the advent of the combined homologous recombination and embryonic stem cell technology, directed

generation of mouse models with equivalent enzyme deficiencies to that found in human diseases has been possible. Nearly all of the known human sphingolipidoses, six MPS and aspartylglycosaminuria have thus far been reproduced in mice. Any prospective treatment method for the LSD should first undergo extensive and careful evaluation in animals before it can be clinically applied to humans. It is therefore necessary to have suitable animal models for these diseases, in which therapies can be evaluated and optimised. Animal colonies for many of these disorders have been established.

The same MPS type in several different animal species is useful in testing the consistency of responses to new therapies, thus giving greater knowledge of species differences and the predicted response if the same therapy is applied to humans.

Three animal models exist for MPS I, including a naturally occurring cat (Haskins et al., 1979) and dog (Shull et al., 1982), and a mouse model produced by targeted disruption of the murine α -L-iduronidase gene (Clarke et al., 1997a). The mutation causing MPS I in cats was identified as a 3 bp deletion resulting in the deletion of a single aspartate residue from the feline α -L-iduronidase protein (He et al., 1999). Molecular characterisation of the mutant canine α -L-iduronidase gene revealed a G to A transition in the donor splice site of intron 1, causing retention of intron 1 in the RNA and creating a premature termination codon at the exon-intron junction (Menon et al., 1992). Both canine and feline MPS I present with moderate clinical severity including coarse facial features, skeletal disease, heart valve disease and corneal clouding. Dwarfism and severe joint disease is present in the MPS I dog (Shull et al., 1982) and mouse (Clarke et al., 1997a) but neither are present in the MPS I cat (Haskins et al., 1981). Histological evidence of neurological lesions are present in all three animal models.

A mouse model to MPS II was created by targeted disruption of the gene encoding iduronate 2-sulphatase (Lamsa et al., 2002). The affected mice exhibit skeletal abnormalities, such as thickened digits, swollen hocks and distorted facies. Elevated urinary and tissue GAG are observed. By 60-weeks of age vacuolisation is evident in a

wide variety of tissues including heart valves, testes and cerebellar neurons, with neuronal necrosis of the brainstem and spinal cord (Lamsa et al., 2002).

Six animal models have been described with MPS III. The first was in a Nubian goat that presented at birth with neurological manifestations consistent with a LSD and a subsequent diagnosis of MPS IIIID was made (Thompson et al., 1992). The mutation causing MPS IIIID in the goat was identified as a nonsense mutation in codon 102 of the caprine N-acetylglucosamine 6-sulphatase protein (Jones et al., 1998). Primary neuronal accumulation of HS and the secondary storage of gangliosides were observed in the CNS of these animals. In addition, morphological changes in the CNS such as neurite expansions and other neuronal alterations have been noted. In the severe form of caprine MPS IIIID mild cartilaginous, bony and corneal lesions were observed (Jones et al., 1998).

MPS IIIA has been reported in two dog models, a wire haired Dachshund (Carmichael et al., 1996; Fischer et al., 1998) and a New Zealand Huntaway dog (Jolly et al., 2000). MPS IIIA in the Dachshund arises from a 3 bp deletion, 737to739delCCA, resulting in the loss of threonine at position 246 in the NS gene (Aronovich et al., 2000). The mutation giving rise to MPS IIIA in the New Zealand Huntaway dog was identified as a 708 to 709insA; the insertion of an adenosine at position 708 to 709 causes a frameshift and immediate chain termination at position 228 in the NS gene (Yogalingam et al., 2002). The wire haired Dachshunds display a comparatively milder form of MPS IIIA when compared with the New Zealand Huntaway dog model. The onset of clinical symptoms in the Dachshund starts at three-years of age and progresses gradually within two years to severe generalised spinocerebellar ataxia. In contrast, the clinical onset of disease in the Huntaway occurs at 1.5-years of age and progresses rapidly over a period of one-month. Histologically, both dog models are reported to contain neurons that accumulate abnormal lipids with features similar to gangliosidosis. Colonies have been established for both models. A spontaneous MPS IIIA mouse was identified as being deficient in NS activity and exhibiting HS uria (Bhaumik et al., 1999). Affected mice display many of the biochemical, pathological and clinical features found in children with this disease. Light

microscopic analysis of brain and other tissues revealed widespread intercellular storage in a variety of cell types. Neurons within the cerebral and cerebellar cortices, the deep cerebellar nuclei, and other brain areas exhibit cytoplasmic distention and contain typical “zebra body”-type storage material. Lysosomal storage is dramatically evident in the liver and, to a lesser extent, in the spleen. Cardiac muscle is markedly affected in chronic cases.

A mouse model to MPS IIIB was produced by targeted disruption of the gene encoding α -N-acetylglucosaminidase (Li et al., 1999). These mice had massive accumulation of HS in liver and kidney as well as secondary changes in activity of several other lysosomal enzymes in liver and brain, and elevation of gangliosides G_{M2} and G_{M3} in brain. MPS IIIB has also been confirmed in an emu model (Bermudez et al., 1995; Kim et al., 1996). In the first month of life, affected birds develop ataxia, tremor, circling, and lethargy; death ensues by six-months of age. Lysosomal pathology suggested a gangliosidosis disorder (Bermudez et al., 1997) with partial β -galactosidase deficiency (Freiscutz et al., 1997). However, later studies demonstrated accumulation of HS in hepatic cells and a deficiency of lysosomal α -N-acetylglucosaminidase was confirmed (Giger et al., 1997). This confirmation led to the diagnosis of MPS IIIB. The mutation leading to MPS IIIB in the emu was recently defined as a 2 bp deletion, 1098 to 1099delGG in exon 6 of the α -N-acetylglucosaminidase gene (Aronovich et al., 2001). The resulting frameshift predicts a longer open reading frame, encoding a polypeptide with 37 additional amino acids and 387 altered amino acids (Aronovich et al., 2001).

A mild form of MPS IVA was recently described in mice (Tomatsu et al., 2002a) and was produced by targeted disruption of the N-acetylgalactosamine-6-sulphatase gene. No obvious growth deficiency or mortality is observed within the first six-months of life. Affected mice accumulate GAG in liver, kidney and spleen and exhibit secondary elevations in the activities of several lysosomal enzymes. Lysosomal storage is evident at eight-weeks of age in reticuloendothelial cells such as Kupffer cells, and cells of the

sinusoidal lining of the spleen; the extent of storage does not dramatically increase with age (Tomatsu et al., 2002a).

Several animal models exist for MPS VI, the first of which was described in a Siamese cat in 1976/1977 (Cowell et al., 1976; Jezyk et al., 1977). Molecular characterisation of this colony revealed a severe and mild form of MPS VI. The severe form resulted from a substitution of leucine to proline at amino acid position 476 in the feline 4-sulphatase gene (Yogalingam et al., 1996). An additional mutation was later identified as a substitution of asparagine for glutamine at amino acid position 520. The D520N mutation is inherited independently of the L476P mutation, and is associated with a clinically mild MPS VI phenotype in D520/L476P compound heterozygous cats (Yogalingam et al., 1998). MPS VI has since been described in a Miniature Pinscher (Neer et al., 1992), in rats derived from the Ishibashi hairless strain (Yoshida et al., 1993), and a mouse model was developed after targeted disruption of the 4-sulphatase gene (Evers et al., 1996). The mutation giving rise to MPS VI in the rat was defined as a one base insertion at nucleotide 507 (507insC), resulting in a frameshift mutation and premature termination of the 4-sulphatase protein (Kunieda et al., 1995). All models of MPS VI display facial dysmorphism, skeletal abnormalities and widespread inclusions in connective tissues and in leucocytes (Haskins et al., 1980; Yoshida et al., 1993; Neer et al., 1995; Evers et al., 1996).

Naturally occurring MPS VII animal models include a dog, cat and mouse. All models present with facial dysmorphism, dwarfism and severe skeletal abnormalities and are classified as moderately severe (Haskins et al., 1984; Birkenmeier et al., 1989; Vogler et al., 1990; Haskins et al., 1991; Gitzelmann et al., 1994). Mutations in the respective β -glucuronidase (GUSB) gene giving rise to these models have been defined. The spontaneous mutation in murine MPS VII was identified as a 1 bp deletion, creating a frameshift mutation within exon 10, resulting in a premature stop codon at position 497 of the murine GUSB protein (Sands and Birkenmeier, 1993). A substitution of glutamate for lysine at amino acid position 351 results in the MPS VII cat (Fyfe et al., 1999), and

the MPS VII dogs are homozygous for an arginine to histidine substitution at amino acid 166 (Ray et al., 1998). Joint disease is obvious in the dog and mouse. Extensive lysosomal storage is present in the CNS and connective tissues in all MPS VII animal models.

A number of missense mutations found in MPS VII patients (E540A, E540Q and L176F) have recently been reproduced in mice by targeted mutagenesis of equivalent sites in the murine GUSB gene (E536A, E536Q and L175F) (Tomatsu et al., 2002b). Phenotypes of varying severity have been observed in the mice. The E536A mouse displayed a severe phenotype demonstrating no detectable GUSB activity in any tissues, while the E536A and L175F mice had low levels of residual enzyme activity and milder phenotypes. All three mutant models showed progressive lysosomal storage in many tissues but had different rates of accumulation (Tomatsu et al., 2002b).

1.11 PAST, PRESENT AND FUTURE THERAPIES IN HUMAN LSD AND MPS PATIENTS

1.11.1 HISTORICAL ASPECTS OF ENZYME REPLACEMENT THERAPY

The concept of treating LSD by enzyme replacement therapy (ERT) has been in existence for many years. The finding that the metabolic defect in cultured MPS patient fibroblasts was corrected by the addition of 'corrective factors' stimulated hope that the MPS disorders could be treated by this method. The early attempts at ERT were initially unsuccessful, due to the fact that insufficient enzyme was isolated and was inefficiently targeted to lysosomes, in part due to use of enzymes lacking appropriate oligosaccharide structures for efficient uptake by certain cell surface receptors (for example, as reviewed by Neufeld and Muenzer, 1995). Enzyme replacement was evaluated using two mechanisms including direct enzyme replacement and tissue/organ transplantation.

The concept of direct enzyme replacement was restricted due to the scarcity of sufficient amounts of purified enzyme. Plasma and leucocytes were studied as a direct source of enzyme (DiFerrante et al., 1971). Infusions into MPS I and MPS II patients resulted in only transient clinical responses (Erickson et al., 1972; Dekaban et al., 1972). Purifying enzyme from urine had also been attempted, and was used to inject intravenously or intrathecally into patients with lipid storage disorders. No clinical improvements were observed, as the enzyme was cleared from circulation far too rapidly (reviewed by Brady et al., 1982).

It was thought that tissue or organ transplantation would provide a renewable source of enzyme. Organs such as the liver, spleen and kidney were transplanted to provide a source of enzyme in severe lipid disorders. This, however, proved unsuccessful in treating the disorders, a result of insufficient amounts of enzyme being released into circulation to reach sites of pathology; immune reactions also complicated matters (Brady et al., 1982).

The identification of different glycoprotein-specific cell-surface receptors such as the M6P receptor, mannose receptors and galactose and fructose receptors has helped overcome some of the technical problems associated with the early ERT attempts. The lysosomal enzyme β -glucocerebrosidase purified from placenta was shown to contain galactose and N-acetylneuraminic acid terminated oligosaccharides (reviewed by Brady and Barton, 1991). Following intravenous administration of this enzyme to rats it was revealed that the majority of the enzyme was targeted to hepatocytes, consistent with galactose and fructose receptors being abundant on hepatocytes. However, after terminal mannose residues were exposed upon partial deglycosylation of the native enzyme, selective targeting the enzyme to the mannose receptors on macrophages resulted (Furbish et al., 1981). These studies proved that enzymes could be selectively targeted to some tissues.

In 1990, Barton et al demonstrated clinical efficacy of ERT in a type I Gaucher patient. Gaucher disease is a prevalent LSD resulting in a multisystem disorder including lipid-

filled macrophages, visceral enlargement and skeletal pathology (Cox et al., 1997). There are three types of Gaucher disease: type I is non-neuronopathic, type II is an acute neuronopathic form that presents within the first three-months of life. Whilst type III Gaucher disease is a subacute neuronopathic form, these patients have CNS involvement, but a later onset and a more protracted disease course than type II patients (Blom and Erikson, 1983). All three types result from a deficiency of β -glucocerebrosidase needed for the lysosomal degradation of β -glucocerebroside.

Initial treatment of type I Gaucher patients with mannose-terminated human placental β -glucocerebrosidase, resulted in a reduction in splenomegaly and increase in platelet numbers and haemoglobin concentration. A gradual improvement in skeletal pathology due to clearance of storage in Gaucher cells in the marrow was also observed (Barton et al., 1990;1991). Later, with the advances in molecular biology, recombinant protein was produced in large amounts for the treatment of Gaucher patients (see Section 1.11.2).

1.11.2 CURRENT THERAPIES IN HUMAN LSD AND MPS PATIENTS

Currently two main therapeutic approaches have been used to clinically treat human LSD patients. These include enzyme replacement therapy (ERT) and bone marrow transplantation (BMT).

ENZYME REPLACEMENT THERAPY

ERT has been successful in treating patients affected with Gaucher disease (see section 1.11.1). ERT using macrophage-targeted β -glucocerebrosidase is currently being used to treat patients with the type I form (Altarescu et al., 2000). The therapy is effective in reversing the characteristic anaemia, thrombocytopaenia and organomegaly, whilst also being successful in removing storage from the bone marrow. Normal bone structure and skeletal development has also been achieved (Altarescu et al., 2001). However, whilst being effective in treating type I Gaucher disease, ERT has its limitations and thus far it

remains ineffective in patients with the type II form. In these patients ERT is unable to modify the fatal neurological pathology.

Fabry disease is an X-linked LSD resulting from mutations in the α -galactosidase A gene (Desnick et al., 2001). Systemic accumulation of globotriaosylceramide and related glycosphingolipids occurs in vascular endothelial and smooth muscle cells, myocardium, renal epithelium, the dorsal root and autonomic ganglia in the peripheral nervous system and neurons in the CNS (Desnick et al., 2001). Patients are characterised by small fibre neuropathy and hypohydrosis followed by renal, cardiac and cerebrovascular deterioration (Desnick et al., 2001).

Two different pharmaceutical companies have been developing ERT for Fabry disease. One has been using α -galactosidase A produced in a genetically engineered continuous human cell line (Schiffmann et al., 2000; Schiffmann et al., 2001). A six-month, double-blind, placebo-controlled ERT trial using 0.2 mg/kg body weight α -galactosidase A resulted in a reduction in neuropathic pain leading to an improvement in pain-related quality of life. A significant improvement in renal pathology and a reduction in lysosomal inclusions in kidney vascular endothelial cells was also observed (Schiffmann et al., 2001). At the completion of the controlled ERT trial, patients in the placebo group began enzyme infusions and displayed the same improvements seen in patients in the original ERT group. Only mild infusion reactions occurred, which were easily controlled and tended to disappear with repeated infusions (Schiffmann and Brady, 2002). Currently, α -galactosidase A is successfully administered as home therapy to patients with Fabry disease (Schiffmann and Brady, 2002).

The second group has been using α -galactosidase A prepared in CHO cells (Eng et al., 2001a). A 20-week, double-blind, placebo-controlled study using this enzyme at a dose rate of 1 mg/kg body weight infused fortnightly was conducted in 58 Fabry patients (Eng et al., 2001b). At the completion of the study 20 out of the 29 patients receiving α -galactosidase A demonstrated no microvascular endothelial globotriaosylceramide

deposits, compared with none of the 29 patients in the placebo group. Similar results were obtained in skin and heart biopsies (Eng et al., 2001b). Mild to moderate infusion reactions were encountered in patients receiving α -galactosidase A (Eng et al., 2001b).

Pompe disease is a fatal LSD caused by a deficiency of lysosomal α -glucosidase (Hirschhorn, 1995). Infants are affected by hypotonia due to central and peripheral system pathology; lysosomal glycogen storage is observed in cardiac and skeletal muscle and is the main target accessible to ERT. Human lysosomal α -glucosidase purified from the milk of transgenic rabbits was administered to four infants with Pompe disease at a dose rate of 40 mg/kg body weight (van den Hout et al., 2000; van den Hout et al., 2001). A reduction in heart size and an improvement in function as well as a normalisation in muscle (van den Hout et al., 2001) was observed. A separate trial, this time using human lysosomal α -glucosidase produced in CHO cells was administered at a dose rate of 5 mg/kg to three infantile Pompe patients for up to 17-months. Once again an improvement was seen in cardiac size and function, and all patients lived past one-year of age without developing cardiac problems (Amalfitano et al., 2001).

ERT is also under investigation in a number of MPS. A recent study was published describing the use of ERT in patients with MPS I (Kakkis et al., 2001). MPS I patients present with delayed development, corneal clouding, airway obstruction, cardiac disease, hepatosplenomegaly and severe joint restriction. Ten MPS I patients were treated with recombinant human α -L-iduronidase (produced in CHO cells) at a dose of 125,000 U/kg of body weight once weekly for 52-weeks. Enzyme infusions were successful in significantly decreasing hepatosplenomegaly in all patients, the size of the liver was normalised for body weight and age, and growth rate increased as measured by height and weight. The mean maximal range of motion of shoulder flexion and elbow extension increased, and episodes of sleep apnoea and hypopnoea decreased. Urinary GAG excretion also significantly decreased after three- to four-weeks of treatment (Kakkis et al., 2001). A pivotal trial of ERT in more than 40 MPS I patients is also underway (Wraith et al., 2002).

Clinical trials are also underway to evaluate the use of recombinant enzyme, iduronate-2-sulphatase and N-acetylgalactosamine-4-sulphatase in patients with MPS II and MPS VI respectively (Muenzer et al., 2002; Harmatz et al., 2002).

BONE MARROW TRANSPLANTATION

Although the bone marrow is not a significant site of pathology, BMT has been used to treat many patients with LSD. The importance of BMT as a potential therapeutic approach for LSD is based on two major factors. Firstly, the bone marrow derived from the donor supplies enzymatically normal leucocytes to the patient. Normal lysosomal enzymes derived from these donor cells can be transferred to deficient cells by the mechanisms of direct cell-cell contact, and by endocytosis of secreted enzyme via M6P receptor-mediated pathway. Secondly, engraftment of the donor pluripotent haematopoietic stem cells differentiate into a number of different cell types which can then provide the deficient lysosomal enzymes to the various tissues in the patients (Hobbs et al., 1981).

Two main disadvantages of BMT limit its application. First, prior to transplantation the patient's bone marrow is destroyed with cytotoxic agents to permit engraftment of the donor bone marrow. Second, HLA-matched sibling donor marrow is only available for 25-30% of the patients. Consequently, haplo-identical or matched, unrelated BMT which has a much higher complication rate, is the only available option for some patients (Hoogerbrugge, 1997). One study determined the transplant-related mortality to be 10% in HLA-identical sibling donor marrow, and 20-25% if mismatched tissue was used (Hoogerbrugge et al., 1995). However, improved donor matching networks are now available reducing risks by providing better matched donor marrow.

Animal model BMT studies suggest that the age at transplantation has significant impact on overall outcome, particularly with regard to CNS disease where the greatest improvement is seen in those animal which are transplanted earlier in life. In untreated

fucosidosis dogs, obvious neurological deterioration was apparent by 12- to 18-months of age and gait deficits began at 12-to 15-months of age. Clinical changes became progressively more severe and euthanasia was necessary before 40-months (Taylor et al., 1987). In an animal BMT at four-months of age, only mild signs of fucosidosis were present three-years post-transplantation. In animals given BMT at slightly older ages, onset of neurological disease was delayed but not prevented (Taylor et al., 1989a). Fucosidase activity in the brain of animals with long-term marrow engraftment (> six-months) reached 20% of the levels found in normal control dogs, compared with less than 1% enzyme activity in untreated fucosidosis dogs (Taylor et al., 1989b, 1992). Lower levels of enzyme activity were observed in tissues including the CNS after less than two-months of marrow engraftment (Taylor et al., 1992). Substantial histological correction was also observed in the CNS in animals given BMT at an early age (Taylor et al., 1989a). These results indicated that enzyme transfer into the CNS had occurred, probably due to donor-derived cells crossing the blood brain barrier (BBB), and that the donor-derived macrophages were slow to establish themselves in the CNS compared with other tissues.

In the case of successful engraftment, BMT has a clinical effect in type I Gaucher disease, however it has no effect on the CNS pathology associated with type II (Schiffmann and Brady, 2002). MPS I patients transplanted at a young age generally maintain intellectual level or show a slower rate of decline compared with the anticipated progressive mental retardation (Hopwood et al., 1993; Whitley et al., 1993). Some improvements in CNS function may be a result of resolution of hydrocephalus, improved hearing and vision and other physical improvements (reviewed by Neufeld and Muenzer, 1995). In MPS VI patients, BMT has been shown to stabilise or slightly improve the general condition, cardiomyopathy and facial features, however the skeletal benefit is limited (HersHKovits et al., 1999).

BMT was successful in preventing CNS manifestations of globoid-cell leucodystrophy. Five children with globoid-cell leucodystrophy (one with infantile type and four with late-

onset disease) were treated with allogeneic stem-cell transplantation (Krivit et al., 1998). Engraftment of donor-derived hematopoietic cells occurred in all patients and was followed by restoration of normal leucocyte β -galactocerebrosidase levels. CNS deterioration was prevented in the four patients with late-onset disease and did not manifest in the patient with the infantile form of the disease (Krivit et al., 1998).

1.11.3 FUTURE THERAPIES FOR LSD AND MPS PATIENTS

A wide range of therapeutic concepts are under investigation either in animal models or in the very early stages of human clinical trials, including substrate deprivation, stem cell, and neural progenitor cell transplantation, a number of viral-mediated gene therapies as well as enzyme enhancement.

SUBSTRATE DEPRIVATION

The goal of substrate deprivation is to prevent the accumulation of storage materials by using small molecules to inhibit the enzymes involved in the biosynthesis of the accumulating storage material (Platt and Butters, 1998). One such small molecule in experimentation is N-butyldeoxynojirimycin (NB-DNJ); this molecule is water-soluble and non-cytotoxic over a broad range of concentrations and is able to cross the BBB. NB-DNJ inhibits glycosphingolipid biosynthesis at the first step (transfer of glucose to ceramide), therefore making it suitable for the treatment of any disease state in which the storage product is derived from glucosylceramide including Gaucher, Fabry, Tay-Sachs and Sandhoff diseases, and G_{M1} gangliosidosis (Platt et al., 1994).

NB-DNJ has been trialed in mouse models of Tay-Sachs and Sandhoff diseases with good effect. Tay-Sachs disease results from mutations occurring in the *HEXA* gene, resulting in a deficiency in the A isoenzyme needed to degrade G_{M2} ganglioside, leading to severe neurodegeneration in patients (Gravel et al., 2001). The Tay-Sachs mouse model was generated by targeted disruption of the mouse *Hexa* gene. The affected mice progressively store G_{M2} ganglioside in the CNS, however not at concentrations that exceed the threshold required to elicit neurodegeneration (Yamanaka et al., 1994; Taniike et al.,

1995). This is because a lysosomal sialidase in mice (not in humans) is sufficiently abundant or active to convert G_{M2} ganglioside to G_{A2} ganglioside, that can in turn be degraded by the hexosaminidase B isoenzyme, which is unaffected by the *Hexa* knockout (Sango et al., 1995). Therefore, the Tay-Sachs mice store G_{M2} ganglioside in the CNS, but never develop neurological symptoms. Substrate deprivation was evaluated in Tay-Sachs mice, who were reared on food containing NB-DNJ for 12-weeks (Platt et al., 1997). At the completion of the study a reduction in stored G_{M2} ganglioside was observed in all animals in the NB-DNJ treated group; storage in neurones was markedly reduced confirming that NB-DNJ could cross the BBB (Platt et al., 1997).

Sandhoff mice store both G_{M2} and G_{A2} gangliosides in the CNS and periphery; the mice exhibit rapid, progressive neurodegeneration and die at four- to five-months of age (Sango et al., 1995). When Sandhoff mice were treated with NB-DNJ their life expectancy was increased by 40% and glycosphingolipid storage was reduced in both peripheral tissues and in the CNS (Jeyakumar et al., 1999). NB-DNJ was also found to enhance the beneficial effects of BMT in Sandhoff mice (Jeyakumar et al., 2001).

Patients with type I Gaucher disease underwent a non-blinded study to evaluate the efficacy of NB-DNJ as a possible treatment (Cox et al., 2000; Platt et al., 2001). A decrease in liver and spleen size was observed as well as an increase in haemoglobin and platelet counts. Whilst promising, some adverse effects were found such as diarrhoea as a result of inhibition of intestinal disaccharidases, and peripheral neuropathy was also reported in some cases (Cox et al., 2000).

Inhibitors to the biosynthesis of GAG would be advantageous for the treatment of the MPS. Synthetic HS analogues such as 4-deoxy-4-fluoro analogues of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose have been evaluated for their effects on cellular GAG biosynthesis (Berkin et al., 2000). 2-Acetamido-1,3,6-tri-O-acetyl-2,4-dideoxy-4-fluoro-D-glucopyranose exhibited a reduction of tritium labelled D-glucosamine incorporation into hepatocyte cellular GAG to 12% of the control cells

(Berkin et al., 2000). Further investigation of this molecule, perhaps in the MPS IIIA mouse model, is required before its usefulness as a treatment can be determined.

GENE THERAPY

A desired treatment would be one that could be administered early in life, has no morbidity and leads to long-term reconstitution of enzyme activity. The aim of gene therapy is to complement the function of the patient's defective gene by the transfer of a normal gene copy, which enables the patient's cells to manufacture the normal gene product. The prospect of using gene therapy to treat LSD is being actively pursued in animal models.

The recent development of specific recombinant adeno-associated virus (rAAV) vectors has provided promising results in the correction of lysosomal storage in the CNS of LSD animal models, particularly in the mouse model to MPS VII. Newborn MPS VII mice were intravenously treated with a single injection of rAAV encoding the GUSB cDNA (Daly et al., 1999). By one-week of age therapeutic levels of GUSB were demonstrated in the liver, heart, lung, spleen, kidney, brain and retina, and were still present at the completion of the 16-week study. Cells of the CNS including neurons, microglia and meninges showed widespread reduction of lysosomal storage (Daly et al., 1999). Long-term expression of GUSB in multiple tissues and a reduction of lysosomal storage persisted for at least one-year in MPS VII mice given a single intravenous injection of virus at birth (Daly et al., 2001). Unfortunately, however, three out of the five rAAV-treated MPS VII mice displayed hepatocellular carcinomas by 72-weeks of age; the mechanisms of tumour formation is currently unknown (Donsante et al., 2001), but suggest that further investigation into the efficacy of rAAV vectors for therapeutic purposes is still needed. Gene transfer by intracranial injection of the rAAV-GUSB construct into newborn MPS VII mice resulted in GUSB levels which approached normality by 18-weeks of age; lysosomal distension was also reduced and an improvement in cognitive function was observed (Frisella et al., 2001).

Viral-mediated gene therapy has recently been shown to ameliorate established CNS storage and the related functional deficits in MPS VII mice. MPS VII mice were treated with the lentivirus feline immunodeficiency viral vector, which was effective in transducing terminally differentiated cells in the brain and mediating GUSB gene transfer into CNS cells of adult MPS VII mice (Brooks et al., 2002). GUSB was secreted from transduced cells and taken up by non-transduced cells, resulting in reduced brain disease that led to improved behaviour, spatial learning and memory (Brooks et al., 2002).

A remarkable improvement was observed in MPS VII dogs injected intravenously at two- to three-days of age with a retroviral vector expressing canine GUSB (Ponder et al., 2002). The body weights of untreated MPS VII dogs are 50% of normal at six-months, they cannot walk or stand after six months, and progressively develop eye and heart disease. The treated MPS VII dogs achieved 87% of normal body weight, could run at all times of evaluation from six- to 17-months, and demonstrated little or no corneal clouding and no mitral valve thickening.

Whilst gene therapy seems very promising for the treatment of LSD patients, potential limitations do exist including the development of immune response to the therapeutic enzyme or to other components of the vector delivery system. Consequently further evaluation is needed before gene therapy can be safely and efficiently performed in humans.

CELL-MEDIATED THERAPY

Cellular therapy of LSD may be defined as the incorporation of cells into a space in the patient's body. These cells would be able to produce enzyme able to correct the consequence of enzyme deficiency in the patients.

Cell therapy approaches have been investigated in the MPS VII mouse. Murine GUSB-secreting fibroblasts that were enclosed in alginate microcapsules to render them immunologically tolerant were injected into mutant MPS VII mice (Ross et al., 2000).

GUSB activity was detected in the liver and spleen for the duration of the eight-week experiment, lysosomal storage was dramatically reduced in liver and spleen tissue and urinary GAG content was normalised. Unfortunately, implanted mutant MPS VII mice developed antibodies against the murine GUSB, which was moderately overcome by co-injection of the purified CD4 antibody with the microcapsule (Ross et al., 2000).

MPS VII mice were transplanted with donor fetal liver cells *in utero* and were assessed after birth (Barker et al., 2001). Results demonstrated that donor fetal liver cells replaced host stem cell descendants and prolonged life dramatically by reducing bone dysplasia and lysosomal storage in all tissues, including a reduction in cortical neurons and glia (Barker et al., 2001).

Long-term expression of GUSB was observed in MPS VII mice transplanted with genetically modified human neural progenitor cells (HNPC) in the CNS (Buchet et al., 2002). When grafted into the mouse striatum, HNPC differentiated into neurons and astrocytes and expressed GUSB for at least six-months (Bucher et al., 2002).

ENZYME ENHANCEMENT

Another possible therapeutic strategy for LSD is the administration of competitive inhibitors that act as 'chemical chaperones' at sub-inhibitory intracellular concentrations, resulting in an increase in activity of the mutant enzyme. Such compounds under investigation include galactose and 1-deoxy-galactonojirimycin (DGJ). DGJ was found to be a potent competitive inhibitor of α -galactosidase A, the enzyme deficient in Fabry disease (Fan et al., 1999). A study of residual activities of mutant enzyme in many Fabry patients showed that some had kinetic properties similar to those for normal α -galactosidase A, but were significantly less stable. The mutant protein was shown to form aggregates in the ER, demonstrating that the enzyme deficiency in some mutants is mainly caused by abortive exit from the ER leading to excessive degradation (Fan et al., 1999). DGJ was able to enhance α -galactosidase A activity in Fabry lymphoblasts when administered at concentrations lower than that usually required for intracellular inhibition

of the enzyme. DGJ seemed to accelerate transport and maturation of the mutant enzyme. Oral administration of DGJ to transgenic mice over-expressing mutant α -galactosidase A substantially elevated the enzyme activity in some organs (Fan et al., 1999).

A patient with Fabry disease and severe cardiomyopathy was treated with galactose (Frustaci et al., 2001). Galactose was intravenously infused at 1 g/kg body weight, administered over a four-hour period every second day for over two-years. Clinical efficacy in cardiac function, endocardial pathology, and quality of life was observed in the patient. Furthermore, an increase in enzyme activity was demonstrated in peripheral blood white cells and cardiac tissue with galactose infusions (Frustaci et al., 2001).

Whilst results from these studies seem promising, this method of therapy is limited to patients with mutations susceptible to these molecules.

1.12 EXPERIMENTAL THERAPEUTIC APPROACHES TO MPS III

Any experimental approach to treating the MPS must address the problem of targeting sufficient enzyme to the diverse sites of pathology. Therapy for MPS III patients is complicated by the fact that MPS III is largely a progressive neurological disease. There are two main areas that need to be considered in developing an effective therapy for MPS III: (1) pre-clinical diagnosis, and (2) a therapeutic approach that is successful in the CNS. The first problem is under investigation by the development of general screening methods for LSD. The second problem includes the limiting effect of the BBB on the delivery of drugs into the CNS.

Several preliminary therapy options have been evaluated in animal models to MPS III and will be discussed below; following this is a description of the BBB.

1.12.1 THERAPIES IN ANIMAL MODELS OF MPS III

Enormous effort has gone into evaluating therapeutic protocols in animal models of the various LSD. For the sake of this review only therapies evaluated in the MPS III animal models will be discussed.

A preliminary ERT trial was performed in the caprine MPS IIID model (Downs-Kelly et al., 2000). Recombinant caprine glucosamine-6-sulphatase was administered intravenously to one MPS IIID goat kid at two-, three-, and four-weeks of age at a dose rate of 1 mg/kg goat body weight. At the completion of the study hepatic uronic acid levels in the treated MPS IIID kid were approximately 90% lower than those in the untreated MPS IIID control; the composition of the residual hepatic HS-GAG was identical to that in the untreated animal. The uronic acid content and the composition of uncatabolised HS-GAG fractions in the brain of the ERT-treated MPS IIID kid were similar to those from an untreated MPS IIID animal. There was a decrease in lysosomal storage vacuoles in hepatic cells of the treated MPS IIID kid, however the enzyme infusions had no effect on CNS lesions.

Similarly, a short-term ERT trial was performed in the mouse model of MPS IIIB (Yu et al., 2000). Macrophage-targeted recombinant human α -N-acetylglucosaminidase was made in *Lec1* mutant CHO line. This cell line does not synthesise complex oligosaccharides on glycoproteins; the α -N-acetylglucosaminidase therefore has terminal mannose residues on all six carbohydrate chains, which would be recognised by the mannose receptor of macrophages. This enzyme was intravenously administered to three-month old MPS IIIB mice. Mice were injected five-times at two-day intervals and sacrificed four-hours after the last injection. Enzyme was taken-up by liver and spleen but was minimal in the thymus, lung, kidney, heart and brain. Immunohistochemistry and electron microscopy showed that only macrophages were involved in enzyme uptake and correction in the liver and spleen, however the storage of GAG was reduced to almost normal levels.

In vitro studies have shown that rAAV vectors containing the human α -N-acetylglucosaminidase cDNA mediated efficient expression of recombinant α -N-acetylglucosaminidase in human MPS IIIB fibroblasts and mouse MPS IIIB somatic and brain primary cell cultures, and degraded the accumulated GAG (Fu et al., 2002). A direct microinjection of vectors resulted in long-term expression of recombinant α -N-acetylglucosaminidase in many brain regions of the adult MPS IIIB mouse, with the main target cell being neurons (Fu et al., 2002).

1.13 THE BLOOD BRAIN BARRIER

A major obstacle in treating MPS III and other MPS in which the CNS is affected is transferring the enzyme across the restrictive BBB into the brain from peripheral circulation. Paul Ehrlich (1885) conducted the first experiments that indicated the existence of a barrier between brain and non-neuronal organs and tissues. Upon intravenous injection of acidic dye substances into rats the CNS remained unstained.

The BBB has a number of important features. It is a membranous barrier, highly resistant to solute-free diffusion and segregates brain interstitial fluid from the circulating blood. This barrier has two endothelial cell plasma membranes in series, forming the luminal and antiluminal membrane of the brain capillary epithelium that are separated by about 0.3 μ m of endothelial cytosol (Saunders et al., 1999a). Endothelial cells line cerebral vessels and are joined by continuous tight junctions that seal off the intercellular space effectively, restricting the entry of protein into the brain. A similar situation exists in the choroid plexus, except that it is epithelial cells that are joined by tight junctions rather than endothelial cells. Other important and unique features of cerebral endothelial cells, compared with those in most other tissues, include the apparent lack of intracellular fenestrations and pinocytotic vesicles, effectively removing the trans-cellular route for free solute movement into the brain (Davson and Segal, 1996). There is also a net negative charge on the luminal surface of the endothelium (Kroll and Neuwelt, 1998).

The key factor that determines the penetration of a molecule into the CNS is lipid solubility and molecular size. Highly lipid-soluble molecules are thought to dissolve in the lipid structure of cell membranes and can therefore transverse cell membranes. Several studies have shown an association between the octanol/water partition coefficient (used as an index of lipid solubility) and penetration into the brain (Brodie et al., 1960; Rapoport et al., 1979; Levin, 1980). The relationship between penetration or permeability and lipid solubility breaks down at a molecular weight above approximately 700 Da (Pardridge, 1993).

It is unclear when BBB development begins in humans. It has been reported that the cellular mechanisms necessary for certain features of the internal environment of the brain appear at different stages throughout brain development. Experimental evidence suggests that tight junctions in cerebral vessels and in the choroid plexus of the immature brain are formed very early in development, possibly, as early as vessels begin to invade the embryonic brain and spinal cord and as soon as the choroid plexuses begin to differentiate (Mollgard et al., 1976; Shultze and Firth, 1992; Kniesel et al., 1996). Evidence from a variety of species including fetal sheep, fetal rat and neonatal rat suggest that the immature brain is more permeable than the adult brain to low molecular compounds, such as sucrose and insulin (Dziegielewska et al., 1979; Ferguson and Woodbury, 1969; Habgood et al., 1993). Due to the sequential development of different barrier mechanisms it is difficult to predict whether or not a particular molecule will enter the developing brain more than in the adult (Saunders et al., 1999b).

1.13.1 HOW DRUGS GET INTO THE BRAIN

With the exception of lipid-soluble small molecules with a molecular mass less than 700 Da, nearly all existing pharmaceuticals will not be transported through the BBB *in vivo* in pharmacologically significant amounts. The design of future CNS drugs should include, at the earliest stages of CNS drug development, special requirements that ensure BBB transport. Identifying the chemical features that are important for BBB permeability and

controlling the entry of drugs into the brain are major areas of current interest in the search for and development of new CNS-targeted drugs.

A number of experimental methods aimed at delivering drugs into the CNS include BBB modification, pro-drugs and direct intrathecal injections, which are discussed in more detail below.

1.13.1.1 BBB MODIFICATION

The natural role of the BBB is to protect the CNS from exposure to infective or harmful agents and metabolic degradation products, and to maintain the homeostatic environment of the brain. Therefore, treatments that irreversibly modify the protective properties of the BBB are likely to be harmful and unacceptable. Modification of the BBB is hazardous due to the fact that the overall permeability of the barrier is increased and this implies a certain risk. The effect of intra-arterial infusion of concentrated solutions on the BBB was demonstrated by Rapoport et al (1979; 1980; 1986), the transient opening of the BBB led to the belief that therapeutic agents could be delivered to the brain using this mechanism.

For BBB disruption to be viable and have any significant clinical role in humans it must be transient and reversible. Osmotic shock is the most common way to reversibly open the BBB (Neuwelt and Hill, 1983; Mykleburst et al., 1994). The intracarotid infusion of hypertonic solutions is the most proven method available for reversible BBB disruption. This procedure results in an osmotic shift of water out of endothelial cells and into the blood, causing the blood volume to expand and cerebral endothelial cells to shrink, in turn causing a separation of tight junctions (Brightman et al., 1973; Rapoport et al., 1980). Hypertonic solutions used to disrupt the BBB include mannitol, arabinose, lactamide, saline, urea and several radiographic contrast agents. The most accepted hypertonic solution is mannitol (Brightman et al, 1973) which has been commonly used for administration to patients in both pre-clinical and clinical studies. The procedure is reversible, with re-closure occurring approximately two-hours after injection (Rapoport et al., 1980).

In rats, the human hexosaminidase A (enzyme deficient in Tay Sachs disease) was delivered in an enzymatically active state across the BBB following osmotic BBB modification by the administration of mannitol (Neuwelt et al., 1984). Subcellular fractionation studies showed that most of the human enzyme delivered across the barrier was functionally active and appeared to be inside a subcellular organelle.

1.13.1.2 PRO DRUGS

Another strategy for poly-peptide delivery across the BBB is to couple the non-transportable polypeptide pharmaceutical to a BBB transport vector, making a pro-drug. A BBB transport vector is a polypeptide that undergoes receptor-mediated or absorptive-mediated transcytosis through the BBB via the endogenous peptides, such as insulin or transferrin. After delivery the pro-moiety is enzymatically cleaved and the active compound is released.

A delivery system has been developed that exploits one of the endogenous transport systems present at the BBB (Friden, 1994). This delivery system utilises antibodies against the transferrin receptor as a carrier molecule for drug delivery to the brain. The compound to be targeted to the brain is linked to the carrier antibody either chemically or through genetic engineering to create a fusion protein. It is advantageous to use the transferrin receptor as a portal of entry into the brain since the brain capillary endothelial cells have a high density of this receptor on their membrane surface. A High level of transferrin receptors is unusual for cells in a non-proliferative state and may be explained by the fact that neuronal cells require relatively high amounts of adenosine triphosphate (Friden, 1994). Another reason for exploiting the transferrin receptor to gain entry to the brain was evidence showing that compounds that bind to this receptor undergo transcytosis across the BBB.

Schwarze et al (1999) reported evidence that an 11 amino acid motif from HIV Tat protein has a transduction domain that allows entry of nanoparticles into cells, improving the biodistribution of recombinant reporter proteins after systemic delivery. The chimeric

protein is able to cross the BBB when partially denatured (Schwarze et al., 1999). Fusion proteins of human GUSB and the 11 amino acid protein transduction motif from HIV Tat was engineered in rAAV vectors (Xia et al., 2001). The Tat modification to the C terminus did not inhibit enzyme activity. HeLa cells infected with the construct in the presence or absence of M6P revealed that the Tat motif allowed for M6P independent uptake; native GUSB is taken up via the M6P pathway. When these viral constructs were injected intravenously or directly into the brain of mice there was an increase in the distribution of GUSB secreted from transduced cells (Xia et al., 2001).

The carrier protein melanotransferrin (P97) represents an approach aimed at directing enzyme therapeutics across the BBB. P97 is a glycosylated protein that possesses a high level of sequence homology with human serum transferrin, human lactoferrin and chicken transferrin (Brown et al., 1982; Rose et al., 1986). No cellular receptor for P97 has been identified. P97 was shown to be present in capillary endothelium in normal human brain (Rothenberger et al., 1996). In contrast, in patients with Alzheimer's diseases, P97 was shown to localise to microglia cells associated with senile plaques (Jefferies et al., 1996; Yamada et al., 1999). P97 was proposed as being a potential biochemical marker for Alzheimer's disease after the finding that serum levels of P97 are increased five- and six-fold over normal in these patients (Kennard et al., 1996; Kim et al., 2001). Furthermore these results suggest that P97 may cross the BBB to a greater extent than do other proteins present in the serum. This hypothesis was recently investigated by experiments aimed at evaluating the uptake of P97 in brain following its administration in animals (Demeule et al., 2002). P97 was found to highly accumulate in the mouse brain following intravenous injection and *in situ* brain perfusion.

1.13.1.3 DIRECT INJECTION INTO THE CNS

Direct injection into the CNS could be a useful way of introducing long-acting, slow-releasing preparations such as implanted devices (Harbaugh et al., 1988) or genetically modified cells that are able to produce drugs *in situ*, secreting them directly into the CNS.

Direct injection of viral vectors into the brain has been investigated in MPS animal models, and shown to be effective in reversing storage lesions in neural cells surrounding the site of injections. RAAV containing the human GUSB gene was stereotactically, intracranially injected into the striatum of adult MPS VII mice (Bosch et al., 2000a). Six-weeks after injection GUSB activity was evident and comparable to that of heterozygous mice in the injected hemisphere. A complete reversion of lysosomal storage lesions was evident in the injected regions, as well as in most neurons located in surrounding areas in the non-injected hemisphere (Bosch et al., 2000a). In a follow-up experiment in 10-week old MPS VII mice, this time using a HIV-based vector containing the human GUSB gene, mice were given either a single intrastriatal injection or multiple injections in both cerebral hemispheres and in the cerebellum (Bosch et al., 2000a). Clearance of lysosomal storage was observed far beyond injection sites. In mice receiving multiple injections of the vector, complete correction or significant reduction of the pathology was observed in every section (comprehensive serial sections of the brain were analysed), suggesting disease regression in the entire brain (Bosch et al., 2000b).

1.14 SECONDARY STORAGE OF GANGLIOSIDES

The sialic acid-containing glycosphingolipids known as gangliosides are found in the plasma membrane of neurons and are thought to play a role in numerous important functions including modulation of ion fluxes (Hilbush and Levine, 1992), protein phosphorylation (Wu and Ledeen, 1994) and neurotransmitter receptor actions. Gangliosides themselves also serve as receptors for a variety of exogenous ligands (Goodman and Walkley, 1996).

Gangliosides are normally degraded by hydrolases in the lysosome. GAG fragments, particularly HS, inhibit this degradative process (Liour et al., 2001). Studies of brain tissue from storage diseases in humans and animals have revealed that some types of neurons, in particular cortical pyramidal cells, display a unique response to the disease process: they sprout new dendrites (Goodman and Walkley, 1996). These neuritic processes emerge firstly from the axon hillock or from a greatly expanded hillock region known as a meganuerite. The new dendritic membrane sometimes contains spines and receives synaptic input resembling those on normal dendrites, including prominent asymmetrical synapses. This phenomenon of ectopic dendritogenesis has not been observed in other neurological diseases and certainly is not evident in all storage disorders (Walkley, 1998).

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In addition to storing GAG, some MPS diseases also store gangliosides. In both human and caprine MPS-IIID (Jones et al., 1998), and humans and dogs affected by MPS-IIIA (Fischer et al., 1998), the primary storage of HS in the CNS is accompanied by abnormal storage of G_{M2} and G_{M3} gangliosides. Elevated amounts of G_{M2} and G_{M3} gangliosides were detected in the neurons of the cerebral cortex of MPS IIIA mice (Bhaumik et al., 1999).

The murine model of MPS IIIA will be useful in studies attempting to understand the relationship between the primary enzyme deficiency, secondarily-induced biochemical abnormalities and neuronal dysfunction leading to clinical neurological disease.

1.15 SUMMARY

The MPS are a family of heritable disorders caused by a deficiency of lysosomal enzymes needed to degrade GAG. The undegraded or partially degraded GAG are stored in lysosomes. One of the MPS, MPS IIIA or Sanfilippo A syndrome, results from a deficiency of the lysosomal enzyme NS, which is required for the degradation of HS. Sanfilippo syndrome is characterised by severe CNS degeneration, but has minimal somatic involvement.

Therapies for MPS IIIA and other related storage disorders are being intensely investigated, with the main focus on ERT, BMT, substrate deprivation, and gene and cell replacement therapies. Before any treatment strategy can be implemented clinically, it must first undergo extensive evaluation. It is thus advantageous to have animal models for these disorders in which therapy strategies can be tested. The recent discovery of a naturally occurring MPS IIIA mouse will enable the investigation and optimisation of therapeutic approaches to this disorder. The CNS is a major site of pathology in MPS IIIA patients, thus therapy protocols must include a way of targeting enzyme across the restrictive BBB and into the brain. The mouse model will provide the opportunity to test novel therapeutic approaches to treat all four MPS III sub-types.

1.16 AIMS OF THE PROJECT

The overall objective of this project was to evaluate the efficacy of ERT with recombinant human NS (rhNS) in the murine model of MPS IIIA. A number of specific objectives were proposed to achieve this:

- 1) To establish the MPS IIIA mouse colony at the WCH, Adelaide, and to characterise the progression of pathology in these mice.**

This included the development of a genotype assay necessary for efficient management of the colony.

- 2) To characterise the mutation giving rise to MPS IIIA in the mice and also characterisation of murine NS.**

This included expression of wild type and mutant murine NS CHO-K1 cells and characterisation.

- 3) To establish a neurological test (the Morris water maze) that was able to discriminate between MPS IIIA affected and normal mice.**

- 4) Evaluate rhNS distribution in MPS IIIA mice.**

- 5) Evaluate efficacy of ERT in MPS IIIA mice.**

An understanding of the efficacy of ERT in the MPS IIIA mice will provide information on the ability of ERT to prevent or modify the CNS pathology associated with this disease.

CHAPTER 2.0

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ELECTROPHORESIS

Acrylamide	Bio-Rad, USA
Ammonium Persulphate	Bio-Rad, USA
N,N,N ¹ ,N ¹ -tetramethyl- Ethylenediamine (TEMED)	Bio-Rad, USA
Pre-poured gels	Gradipore
Agarose, DNA grade	Boehringer, Mannheim, Germany
Ethidium Bromide	Geneworks, Australia
Bromophenol Blue	BDH Chemicals Ltd. England
PUC19 HpaII size markers	Geneworks, Australia
C-14 Methylated markers	Life Technologies, Gibco BRL
Coomassie Brilliant Blue R	Sigma Chemical Co., USA
Pre-stained markers	Life Technologies, Gibco BRL

2.1.2 ENZYMES AND ANTIBODIES

Purified rmNS	Briony Gliddon, WCH
Purified rhNS	Peter Clements, Liz Melville and Briony Gliddon, WCH
Restriction enzymes	New England Biolabs
Proteinase K	Sigma Chemical Co., USA
Taq DNA polymerase	Roche diagnostics., USA
Polyclonal α -rhNS (12 μ g/ml)	Kelly Perkins, WCH
Monoclonal α -rhNS (23B2)	Kelly Perkins, WCH

But strange

Sheep anti-mouse Europium labelled
Sheep anti-mouse 1gG HRP conjugate

Silenus, Australia
Silenus, Australia

2.1.3 RADIOCHEMICALS

EXPRESS[³⁵S] protein labelling mix

DuPont NEN Research Products,
USA

Na₂³⁵SO₄ (2mCi/ml)

DuPont NEN Research Products,
USA

2.1.4 BUFFERS, SOLUTIONS AND SUBSTRATES

10 x Loading buffer

50% (v/v) glycerol, 1% (w/v)
SDS, 100mM EDTA, pH 8.0
0.1%(w/v) Bromophenol blue

TAE

40mM Tris-acetate, 2mM EDTA,
pH 8.3

Solubilisation buffer

1% (w/v) Na Cholate, 0.1% (w/v)
SDS, 0.5% (v/v) TritonX-100 – in
PBS

SDS-PAGE electrophoresis buffer

25mM Tris-HCl, pH 7.5,
0.1mM EDTA

SDS-PAGE gel loading buffer

1% (w/v) SDS, 4M urea, 80mM
Tris-HCl, 0.192 M glycine, 0.1%
(w/v) Bromophenol blue

Phosphate buffered saline (PBS)

137 mM NaCl, 2.7 mM KCl,
4.3 mM Na₂HPO₄, 1.4 mM
KH₂PO₄, pH 7.2

Tritium labelled heparin tetrasaccharide
Substrate for sulphamidase

Viv Muller, WCH

Delfia Assay Buffer

9g/L (w/v) NaCl, 5g/l (w/v) BSA
0.5g/L (w/v) NaAz, 0.5g/L (w/v)
ByG, 0.1g/L (w/v) Tween 40,
6.06g/L (w/v) Tris Base,
7.85mg/L (w/v) Diethylene
triamine penta acetic acid

abbrev's

Delfia Wash Buffer

225g/L (w/v) NaCl, 15.2g/L
Tris Base, 0.5g/L (w/v)
Thimerosal, 1.25g/L (w/v)
Tween 20

Delfia Enhancement Solution

1g/L (w/v) TritonX-100,
6.8mmol/L (v/v) KHPthalate,
100mmol/L (v/v) CH₃COOH,
50µmol/L TOPO, 15mmol/L
β-NTA

Abbrev's

2.1.5 ANTIBIOTICS

G418 sulphate (Neomycin) Geneticin
Penicillin/Streptomycin sulphate

GIBCO-BRL

CSL Ltd, Australia

2.1.6 CELL LINES

CHO-K1

ATCC, CRL 9618

2.1.7 TISSUE CULTURE

All disposable tissue-culture flasks, cell factories, plates and pipettes were supplied from Corning. All other materials used for tissue culture were obtained from the following suppliers:

CSL Limited, Australia

Dulbecco's Phosphate Buffered
Saline (PBS) without Calcium
and Magnesium
Fetal calf serum (FCS)

Fungizone
Trypsin/Versene solution (0.1%
w/v)

GIBCO-BRL

Dulbecco's Modified Eagle
Medium (DMEM) (powder)
Ham's F12 media (powder)
DMEM without cysteine
Methionine, L-glutamine ,
(100 ml aliquots)

2.1.8 MISCELLANEOUS MATERIALS, CHEMICALS AND KITS

Mannose-6-phosphate

Sigma Chemical Co., USA

X
Horse Raddish Peroxidase
Substrate Kit

Bio-Rad, USA

Bio-Rad Protein Assay Dye
Reagent concentrate

Bio-Rad, USA

Bovine serum albumin

Sigma Chemical Co, USA

Dimethylsulphoxide (DMSO)

Sigma Chemical Co, USA

SDS

Sigma Chemical Co, USA

Pansorbin cells

Calbiochem

Affi-Gel 10

Bio-Rad, USA

DEAE-Sephacel	Whatman, UK
CM-Sepharose™ Fast Flow	Amersham, Pharmacia
Deoxyribonucleoside triphosphates (dNTPs)	Boehringer Mannheim
Ovalbumin	Sigma Chemical Co.
Poly-prep chromatography columns	Bio-Rad, USA
Polyvinylchloride plates (96 well)	Costar (Cambridge, MA, USA)
Immulon 4 removawell® strips and holder	Dynex technologies,inc, USA
Delfia enhancement solution	Wallac
Tuberculin syringes	Becton Dickinson (Franklin Lakes, NJ, USA)
Needles	Becton Dickinson (Franklin Lakes, NJ, USA)
Skim milk powder	Diploma, 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. Kilsyth, Australia; Boehringer Mannheim, Mannheim, Germany; Sigma Chemical Co. St Louis, USA, and Anal®, BDH Chemicals Australia, Pty. Ltd, Melbourne, Australia.

2.2 METHODS

2.2.1 RESTRICTION ENZYME DIGESTION

All restriction enzyme digestions were carried out utilising the conditions specified for each individual enzyme as recommended by the manufacturer.

2.2.2 AGAROSE GEL ELECTROPHORESIS OF DNA

Electrophoresis of DNA for analytical purposes was done by combining DNA with 0.25 volumes of dye loading buffer and using gel containing 1%-5% (w/v) agarose in 1 X TAE buffer. After staining the gel in a solution of 10 µg/ml ethidium bromide, the DNA was visualised under UV light, and a positive photograph taken using polaroid land film 667 (Polaroid Corp., Cambridge, Massachusetts, USA).

2.2.3 TNT TRANSCRIPTION/TRANSLATION SYSTEM

One microgram of wild-type and mutant pcDN3.1 constructs were added to the TNT[®] Coupled Reticulocyte Lysate System (Promega) mixture with or without microsomes according to the manufacturer's specifications. Reactions were analysed via SDS/PAGE according to the method of Laemmli (1970) and visualised via autoradiography. *used?*

2.2.4 ELECTROPORATION OF CHO-K1 CELLS

Plasmids (25 µg) were electroporated into CHO-K1 cells as previously described (Anson *et al.*, 1992). Briefly, CHO-K1 cells were grown to confluence in Ham's F12 *X* supplemented with 10% (v/v) FCS at 37°C in a 5% CO₂ atmosphere. Cells were *X* harvested by treatment with trypsin-versene, washed once with PBS and then resuspended in PBS at 1.2×10^7 viable cells/ml. One ml of resuspended cells were placed into disposable electroporation chambers (Gibco-BRL) containing 25 µg of plasmid DNA and then equilibrated to 0°C. The cells were electroporated using a BRL Cell Porator and a pulse of 275 V/330 mF. The cells were grown in non-selective medium for 24 hrs and then sub-cultured 1:5 into medium containing 0.75 mg/ml Geneticin (G418).

2.2.5 PREPARATION OF CELL LYSATES AND DETERMINATION OF NS ACTIVITY

Preparation of cell lysates: CHO-K1 cells were harvested with 10% (v/v) trypsin-versene (Gibco-BRL) in PBS buffer, and washed with PBS. Cell lysates were prepared by seven cycles of freeze-thaw in 0.5M NaCl, 20 mM Tris-HCl, pH 7. Growth medium and cell lysates were clarified by centrifugation. Total cellular protein was quantified using the Bio-Rad protein assay. Once prepared cell lysates were kept on ice at all times and frozen at -20°C or -80°C immediately after use.

Total NS determination using tritium labelled heparin tetrasaccharide: NS activity was determined using the heparin derived tritiated tetrasaccharide substrate as described by Hopwood and Elliott (1982). A 5 µl aliquot of either cell lysate or conditioned growth medium or a 1:100 – 1:1000 dilution of purified enzyme was combined with 5 µl buffer (0.2M NaAc pH 5.0) and 1 µl of substrate (410 pmol/µl). H₂O was added to the reaction to a final volume of 12 µl. Reactions were incubated overnight at 37°C for cell lysate and conditioned medium, or for 20 – 45 min for purified protein. The reaction was terminated by the addition of 100 µl of 10 mM NH₄OH. The reaction was transferred to HPLC vials and analysed using the HPLC (Hewlett Packard Series 1100 with Packard 150 TR Flow Scintillation Analyser). Assays were product exceeded 25% were repeated.

Rates were determined using the equation below:

$$\left[\frac{[\text{Area Product}]}{[\text{Area Product} + \text{Substrate}]} \times \frac{100}{1} \right] - \left[\frac{[\text{Area Product}]}{[\text{Area Product} + \text{Substrate of blank}]} \right]$$

= Net % Conversion.

Handwritten notes: "x 100 may be" with an arrow pointing to the 100/1 term, and "strange" with an arrow pointing to the second bracketed term.

Rate (pmol/min/mg protein)

$$= \text{Net \% conversion} \times \frac{\text{pmol substrate per incubation}}{\text{Time (min)}} \times \frac{1}{\text{mg protein/incubation}}$$

2.2.6 IMMUNOQUANTIFICATION OF NS.

Polyclonal antibody, prepared in a rabbit against rhNS and precipitated with octanoic acid dissolved in PBS and then concentrated to 12 µg/ml (Perkins et al., 1999). α rhNS polyclonal antibody was diluted in 0.1M NaHCO₃ to a final concentration of 5 µg/ml and added to individual wells of (n) numbers of strips, and incubated at 4°C overnight (100 µl/well). Each well was then aspirated and washed 2 X with Delfia Wash Buffer (Wallac) using programme number 7 (2 [aspirate + wash] + aspirate) on the Delfia Plate Washer (Wallac). Standards and samples were serially diluted in Assay Buffer (Wallac), with 0 to 5 ng of purified rhNS used as a standard. Fifty µl of standard or sample was added to the wells in duplicates; the last two wells left blank. 160 µg/µl monoclonal α rhNS-23.B2 (Perkins et al., 1999) was also purified on an IgG column and diluted in Assay Buffer to a final concentration of 200 ng/ml and 50 µl was added to each well. The plate was mixed on a Plate Shaker (Milenia, Micromix 4, Biomediq) for 15 min at room temperature and then incubated overnight at 4°C. Plates were allowed to return to room temperature, after which wells were aspirated and washed six-times with Delfia Wash Buffer using programme number 6 (6[aspirate + wash] + aspirate). One hundred µl of an affinity purified europium (EU³⁺) conjugated sheep anti-mouse IgG (Silenus) second antibody diluted 1:1000 (v/v) in Assay Buffer was added to each well. The plate was mixed on the plate shaker for 1 hour on setting 3 at room temperature. Wells were aspirated and washed six-times with Delfia Wash Buffer using programme number 6 on plate washer. Two hundred µl of Enhancement Solution (Wallac) was added to each well and the plate was mixed on the plate shaker for 10 minutes on setting 5. Fluorescence was read on the Delfia Plate Reader (Wallac, 1234 Delfia Research Fluorometer). All results were extrapolated through a standard curve. A BCA protein assay (Pierce Biotechnology) was performed on all samples according to manufacturer's specifications. Results were expressed as ng NS/mg total protein.

2.2.7 PULSE-CHASE LABELING

G418 resistant mass cultures of CHO-K1 cell lines expressing wild-type mNS and D31N mutant mNS were grown to confluency in 75cm² tissue culture flasks. The cells were pre-

incubated for 30 minutes in 5 ml DMEM containing cysteine/methionine and supplemented with 10% (v/v) FCS, PS. Medium was removed and replaced with DMEM cyteine/methionine supplemented with 10% (v/v) FCS, PS containing 100 μ Ci EXPRE³⁵S³⁵S protein labelling mix, and incubated at 37°C for 30 minutes. Labelling medium was removed and cells were washed with serum-free medium to remove excess free label. Cells were chased with 10 ml of Ham's F12 medium supplemented with 10% (v/v) FCS. After 0, 0.5, 2, 4, 8 and 24 hr, the chased medium was collected and clarified by centrifugation (1500g, 3 min). At each time point, cells from one flask in each group were harvested with trypsin, washed in PBS and resuspended in 1 ml solubilisation buffer (PBS containing 1% (w/v) NaCholate, 0.1% (w/v) SDS, 0.5% (v/v) Triton X100), and used for immunoprecipitation.

2.2.8 IMMUNOPRECIPITATION

Growth medium and solubilised CHO-K1 cell lysates were immunoabsorbed using a rabbit polyclonal antibody raised against rhNS (1:1,500,000 titre). Pansorbin cells (Calbiochem; 1.5 ml) were equilibrated by five washes in 1 ml solubilisation buffer by microcentrifugation/resuspension. Each sample (5-10ml volume) was pre-cleared by the addition of 75 μ l equilibrated Pansorbin cells. After a 24-hr incubation at 4°C, the Pansorbin cells were removed by centrifugation at 6000 rpm for 10 min. Rabbit α rhNS wass added to each surpernatant (1 μ l/ μ l) and incubated at 4°C for 24 hr. Pansorbin cells (75 ml) were added to each sample, incubated at 4°C for a further 24 hr and then sedimented by centrifugation. The pellets, which contained the NS-antibody-protein-A complexes, were washed four-times in solubilisation buffer, once in water and then resuspended in sample buffer (1% (w/v) SDS, 4M urea, 80 mM Tris-HCl, pH 6.8, 0.1% (W/V) Bromophenol Blue). Samples were boiled for 5 min, centrifuged at 12,000g for 1 min to pellet Pansorbin cells and the supernatant was analysed via SDS-polyacrylamide gel electrophoresis and autoradiography.

2.2.9 ISOLATING A HIGH EXPRESSING CLONE

G418 resistant mass cultures of CHO-K1 cells transfected with the pcDNA3.1mNS vector were harvested using trypsin, washed two-times using PBS and resuspended in PBS. This cell resuspension was serially diluted in Ham's F12 medium supplemented with 10% (v/v) FCS, 1% (v/v) PS, and 0.75 mg/ml (w/v) G418 into 100 ml dishes. Plates were incubated at 37°C in 5% CO₂ until single colonies were formed. Formation of single colonies was determined by an independent group of cells forming clumps. These colonies were isolated from the plates using cloning rings. Individual colonies were then harvested using trypsin and serially diluted into a 96- well dish containing Ham's F12 medium supplemented with 10% (v/v) FCS, 1% (v/v) PS and 0.75 mg/ml (w/v) G418. Plates were incubated at 37°C in 5% CO₂ until single colonies per well were observed. Wells containing only single colonies were harvested using trypsin, washed two-times with PBS, expanded into a 24-well dish and incubated at 37°C until confluent. Confluent cells were conditioned with Ham's F12 medium for 78 hr, medium was harvested and conditioned, by centrifugation (1500g, 3 min) and assayed for NS activity using the radiolabelled tetrassaccharide substrate. The well expressing the highest level of NS activity was expanded up to T25 cm² flasks and was ultimately used for the production of mg amounts of rmNS.

2.2.10 PRODCUTION OF LARGE AMOUNTS OF CONDITIONED MEDIUM FOR PURIFICATION OF RECOMBINANT HUMAN NS

The Braunbiostat B Bioreactor (2L vessel) was initially seeded with 20 T75 flasks containing the CHO-K1/rhNS-26 cell line (Bielicki et al., 1998) (approximately 10 mg NS/L culture medium) in COON'S medium supplemented with 2-5% FCS. Inside the vessel, the cells grow on cytoline beads and the medium is initially supplemented with low percentage FCS, which is gradually reduced to 0%. One-litre of medium is collected daily and stored at 4°C. Only media containing 0% FCS is used for purification.

2.2.11 PREPARATION OF COLUMNS FOR PROTEIN PURIFICATION

Two 100 ml volume columns were used for purification of rhNS (and rmNS). One containing CM-Sepharose prepared according to manufacturer's specifications, and the other containing DEAE-Sephacel also prepared according to manufacturer's specifications. Columns were attached to retort stands and kept at 4°C at all times. At the completion of purification, columns were regenerated for future use.

2.2.12 PURIFICATION OF rhNS

RhNS was purified by a two-column procedure (Bielicki et al., 1998) from conditioned medium, clarified by centrifugation (2000g; 3 min). Approximately 3-4 L of the medium was concentrated on a hollow fibre concentrator at room temperature until a final volume of approximately 100 ml was achieved. The concentrated medium was dialysed against 50 mM sodium acetate, pH 5.0 (buffer A) overnight at 4°C. The dialysed medium was centrifuged (2000 g, 2 min) before application to a 100 ml CM-Sepharose column equilibrated in buffer A. After loading, the column was washed with buffer A containing 50 mM NaCl and the rhNS was then eluted using gravity feed with buffer A containing increasing concentrations of NaCl ranging from 80 mM to 150 mM with a final elution of the column with 1M NaCl in buffer A. Fifty ml fractions were assayed using the radiolabelled tetrasaccharide mixture for NS activity; those containing enzyme were pooled and concentrated in an Amicon Ultrafiltration stirred-cell and dialysed overnight against 20 mM Tris, pH 7.0 (buffer B) at 4°C. The dialysed medium was centrifuged (2000 g, 2 min) before application to a 100ml DEAE Sephacel column equilibrated in buffer B. After loading, the column was washed with buffer B, rhNS was then eluted by gravity feed with buffer B containing 100mM NaCl with a final elution of the column with 1M NaCl in buffer B. Fifty ml fractions were assayed for NS activity using the radiolabelled tetrasaccharide substrate. Fractions containing NS activity were analysed by SDS/PAGE by the method of Laemmli (1970) to check for purity. All fractions of pure protein were filter sterilised and stored at 4°C; fractions were always assayed for activity before being used.

2.2.13 KINETIC ANALYSIS

Kinetic data (K_m and V_{max}) for purified mNS using the radiolabelled tetrasaccharide substrate mixture at concentrations ranging from (5-80 mM) were obtained from Lineweaver-Burk plots. Blank values for each substrate concentration were deducted from values obtained in the presence of enzyme. The results from 12 substrate concentrations were used to generate Lineweaver-Burk plots. K_m was determined as the negative reciprocal of the x-intercept and V_{max} as the reciprocal of the y-intercept. (ref)

2.2.14 CORRECTION OF HUMAN MPS IIIA FIBROBLASTS

Cultured skin fibroblasts from a clinically severe MPS IIIA patient (SF 4636), which had undetectable levels of NS activity, and from a normal individual were grown to confluency in T25 cm² tissue culture flasks in DMEM containing 10% (v/v) FCS and PS. To deplete SO_4^{2-} pools before labelling, the above medium was removed, the cells were washed twice with PBS and incubated with 4 ml/flask Ham's F12 medium containing 10% (v/v) FCS only. After two-hr the medium was replaced with fresh Ham's F12 containing 10% (v/v) FCS and 10 μ Ci/ml $Na_2^{35}SO_4$. After labelling for 24 hr the cells were rinsed with PBS and the medium replaced with mNS in the form of conditioned medium from the mNS-4 cell line at a concentration of 70 nmol/min/ml in the presence or absence of M6P (5 mM final concentration). Cells were harvested three-days later by trypsin treatment and washed twice with PBS by centrifugation (1500 g for 3 min) per resuspension. Cell pellets were finally resuspended in 200 μ l of 20 mM Tris/HCl/0.25M NaCl, pH 7.0. The cells were lysed by six cycles of freeze/thaw and the resulting cell lysates clarified by microcentrifugation (13,000g; 5 min; 4°C). Cell lysates were then assayed for NS activity, protein content and radioactivity (³⁵S c.p.m). units

2.2.15 ANIMAL MAINTENANCE

The colony was originally established in 2000 from four heterozygotes and four homozygotes obtained from Dr. P. Stanley at the Albert Einstein Institute for Medical Research, New York. Following importation and quarantine, the mice were housed in the Animal Care Facility at the Women's and Children's Hospital, Adelaide. The colony was

attended to by trained animal care staff who fed, cleaned and administered routine medications to the mice when needed. Cages were cleaned weekly. Mice were fed Mouse Cubes (Ridley AgriProducts, 21% minimum crude protein, 2% minimum crude fat, 15% maximum crude fibre, 1% maximum added salt, 2,220 IU/Kg vitamin A and 400 IU/Kg vitamin D3) ad libitum and water was provided ad libitum.

2.2.16 DISEASE PREVENTION

Mice were treated annually with IVOMEK (Active constituent: Ivermectin 0.8 g/L, dilution 30 ml/4L water; Merial, Australia) supplemented in their water for 12-weeks. IVOMEK is a broad spectrum antiparasitic drug, and was routinely administered by trained animal carers on all animals housed in the animal care facility.

2.2.17 COLONY BREEDING MANAGEMENT

Mice were bred as needed. Heterozygote crosses were performed to maintain relative numbers of normal mice. MPS IIIA homozygote crosses were performed with mice before 20-weeks of age to obtain large numbers of MPS IIIA mice. Generally, one male was placed with one female, and the male was allowed to remain in the cage with the female for one-week after which he would be removed and caged separately. Females were then checked regularly two-weeks post pairing, and daily three-weeks post pairing, for any sign of pups. The birth date of mouse pups was taken as being the date on which the pups were first observed. Pups were toe-tagged on day, six- to eight of life and were weaned at three-weeks of age. Female and male mice were caged separately.

2.2.18 GENOTYPING MPS IIIA MICE

2.2.18.1 TOE CLIPPING

A sample of toe tissue corresponding to identification number (Figure 2.1) was cut from mice at six- to eight days of age using sharp, sterile scissors which were swabbed with 70% ethanol between each mouse. All of the pups in the cage were removed from the mum and placed under soft towels on a heating pad. Following toe excision the toe tissue was placed in an Eppendorf and kept cold on dry ice for transportation to the laboratory.

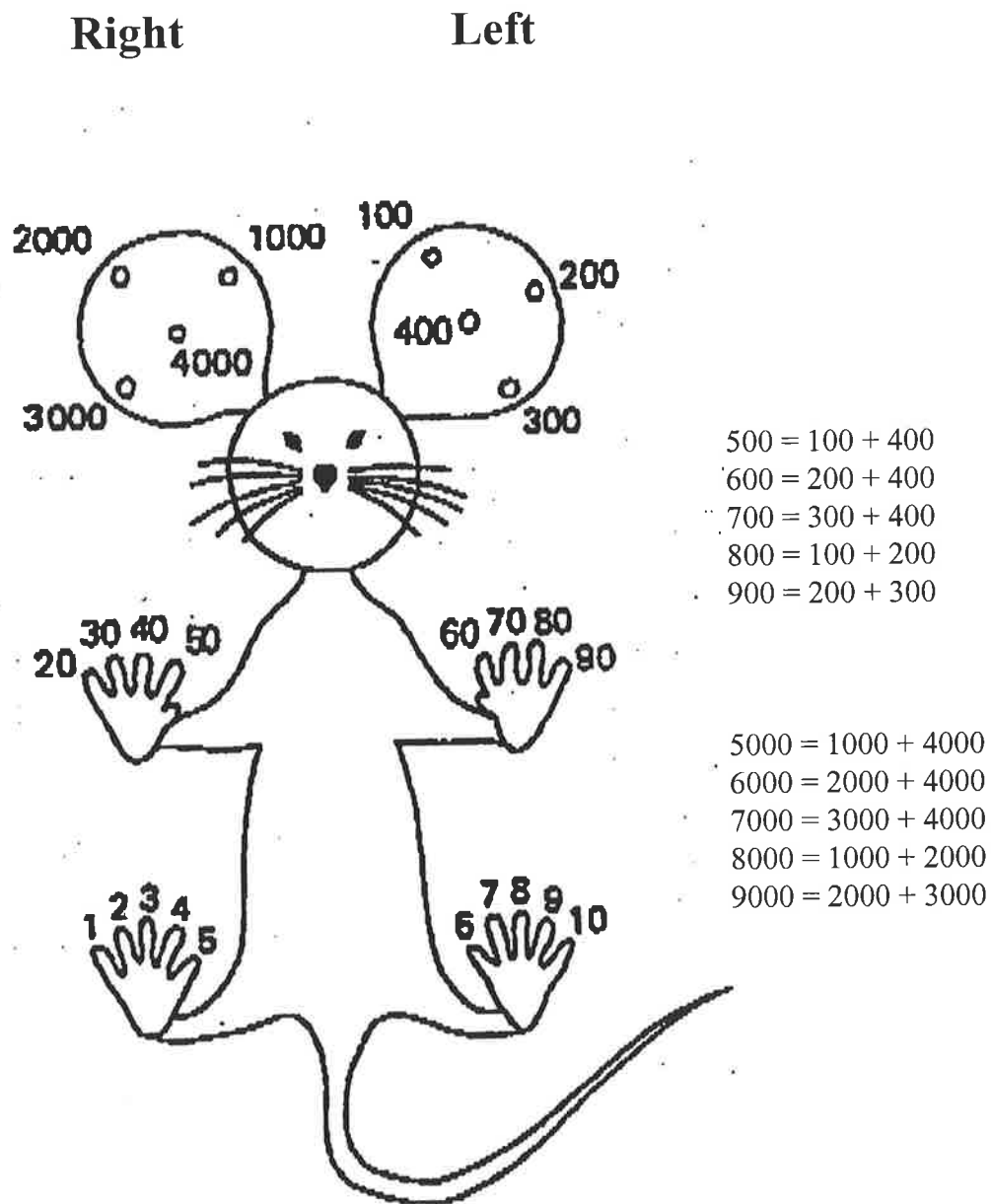


Figure 2.1: Toe-tagging mice.

Mice were identified by assigning a number to the mouse. Toes corresponding to this number were clipped following the diagram above.

X At the completion of the procedure all pups were rolled in bedding material and returned to the mum simultaneously, to avoid her rejecting them. Pups were monitored for the next two- to three-days. No infections or death have resulted from this procedure in the colony thus far.

2.2.18.2 GENOMIC DNA PREPARATION

The toe tissue was kept cold on ice. Fifty ml of toe lysis buffer (50 mM Tris/HCl pH, 8.0, 2 mM NaCl, 1 mM EDTA, 0.5% (v/v) Tween 20, 0.5% (v/v) Triton X100 and 0.4 mg/ml (w/v) proteinase K – added fresh from frozen stock) was added to each toe tissue and incubated overnight at 37°C, heated to 95-100°C for 10 min to inactivate the proteinase K, vortexed, and spun in a microfuge for 2 min at 13,000g to pellet debris (Nadon and Draeger, 1996).

2.2.18.3 PCR AND RESTRICTION DIGEST ANALYSIS

Two µl of genomic DNA from mouse toe tissue was added to 5 µl 10 X PCR buffer (Boehringer Mannheim) and mixed with 100 ng of oligonucleotides 5'MSI2 (5' NNT CTG TCT TCC TCA GCG 3') and 3'MS4 (5' GAT AAG GCT GTG GCG GGA CAG GG 3'), 5 mM dNTP mix and 2.5 units Taq polymerase (Boehringer Mannheim). H₂O was added to the reaction to a final volume of 50 µl. The mutation region was amplified following 3 min at 94°C, 35 cycles of 45 sec at 94°C, 45 sec at 57°C, 40 sec at 72°C and 4 min final extension at 72°C. PCR products were subjected to digestion with Aci I (New England Biolabs) at 37°C for 2 hr before electrophoresis on a 3.5% agarose gel. Aci I cuts between the GG in the sequence CAGCGG.

2.2.19 ENZYME ADMINISTRATION

Filter sterilised enzyme preparations (stored at 4°C) were transported on ice. A proportion of mice from each treatment group was weighed prior to enzyme administration. Enzyme dose was calculated according to body weight, and was diluted in PBS, pH 7.2, to give a volume of 100 µl. Injections in newborn mice were made through the superficial temporal vein on day one of life (Sands and Barker 1999). All pups in the cage were removed from

the mum and placed under soft towels on a heating pad. Individual pups were held under a 6 x magnifying glass with an attached fluorescent lamp, making the vein easier to target. A 30-gauge, 0.5-inch needle (Becton Dickinson) attached to a 1 ml tuberculin syringe (Becton Dickinson) was used for injections. The needle was inserted bevel up under the skin, parallel to the vein in the same direction as blood flow. While under the skin the needle was moved directly over the vein and advanced into the vessel, and 100 µl of the enzyme preparation was slowly injected. Gauze padding was immediately placed at the site of injection to arrest bleeding. If injections were unsuccessful the superficial temporal vein on the other side was attempted. Upon completion all pups were rolled in bedding material and returned to the mum. Pups were observed for one day following injection. No pups died whilst performing this technique.

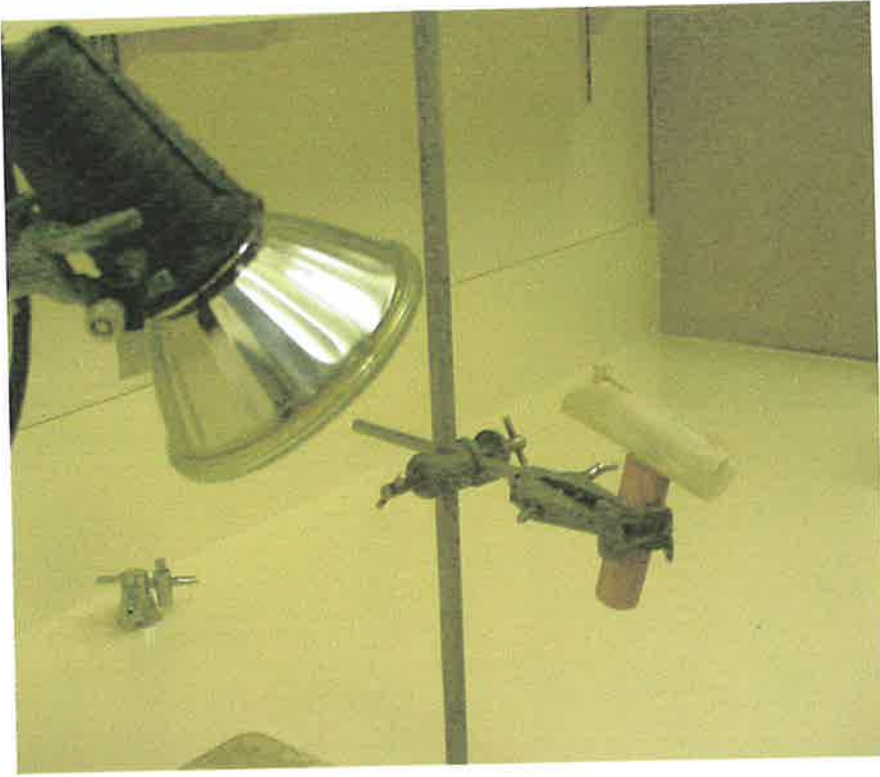
Mice two-weeks of age were injected intraperitoneally. Mice were held firmly in one hand and injected with a 30 gauge 0.5-inch needle (Becton Dickinson).

Mice three-weeks of age or older, were injected through the tail vein. Mice were coaxed into a holder attached to a retort stand (Figure 2.2). Whilst in the holder mice were warmed under a heating lamp for 3-5 min; close observation was made to ensure mice were not distressed from excessive heat. The tail was swabbed with 70% ethanol prior to enzyme administration. A 29 gauge 0.5-inch needle (Becton Dickinson) attached to a 1 ml tuberculin syringe was used in mice up to approximately 10-weeks of age, after which a 27 gauge ½ -in needle (Becton Dickinson) was used. The tail was held firm whilst the needle was being inserted into the vein at the end of the tail, and 100 µl of the enzyme preparation was slowly infused. Gauze padding was immediately placed at the site of injection to arrest bleeding. Upon completion, mice were removed from the holder and replaced to their cage. If injection was unsuccessful, the needle was reinserted further up the vein (closer to the base of the tail). Injected mice were monitored for up to 30 min following injection.

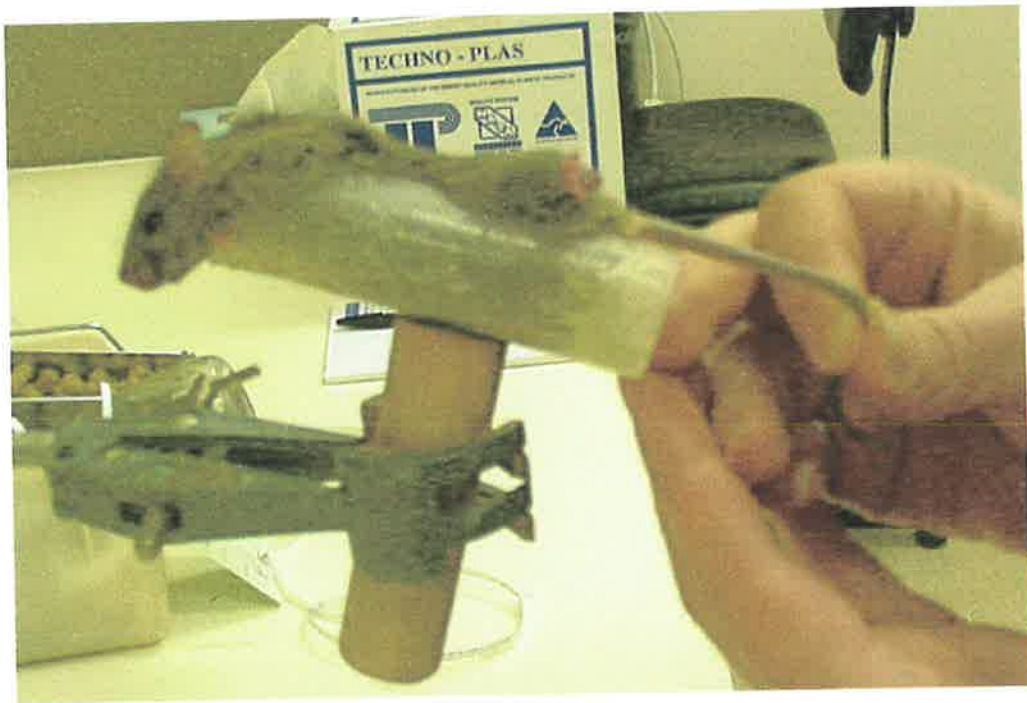
Figure 2.2: Holder for tail vein injections in adult mice.

- A) The holder used to restrain mice for tail vein injections. Essentially, a tube 8.5 cm in length with a 2.5 cm diameter base had a 7 cm wedge 5 mm wide removed. This was attached to a wooden base 8 cm long. The holder was held in place by clamping the wooden base to a retort stand at chest height (sitting down). A heating lamp was held approximately 20-to 30 cm from the mouse.
- B) The mouse was coaxed into the holder using a ruler or some other object of similar size, with front and rear paws over the sides of the holder. The tail was held firm for injections.

A)



B)



2.2.20 ANTIBODY TITRES

Blood samples (500 μ l) for determination of plasma antibody titre to rhNS were collected into EDTA tubes. Plasma was separated by microcentrifugation (13,000 g for 5 min) at room temperature. Plasma was stored at -20°C until assayed.

Polyvinylchloride plates were coated with 0.5 μ g purified rhNS per well. Plates were sealed with adhesive tape and incubated for 2 hr at 37°C , then overnight at 4°C . The plates were then washed three-times with 200 μ l 0.02M Tris/HCl buffer, pH 7.0, containing 0.25M NaCl and 1% (w/v) ovalbumin (washing/blocking solution, Buffer A). The final wash was left in the wells and the plates were incubated for at least 2-hr at room temperature to block any remaining reactive sites on the wells. To determine any reactivity against ovalbumin in the same plasma samples, uncoated plates (-rhNS) were also blocked with the wash solution.

Plasma samples were pre-absorbed overnight at 4°C with an equal volume of ovalbumin/BSA coupled Affi-Gel to remove non-specific antibodies. To couple Affi-Gel, equal volumes of Affi-Gel 10 and a solution containing 10 mg ovalbumin/ μ l and 10 mg BSA/ml were mixed for 1-2 hr at room temperature, then overnight at 4°C . Pre-absorbed plasma samples were serially diluted across the plates in Buffer A. Plates were incubated for 2 hr at room temperature and washed three-times with 200 μ l of Buffer A. One hundred μ l of sheep anti-mouse IgG (1/1000 dilution(v/v)) diluted in Buffer A was then applied to each well and plates were incubated for 1-hr at room temperature. Plates were then washed three-times with 200 μ l of buffer A, 100 μ l per well of ABTS substrate (prepared according to the manufacturer's instructions) was applied to each well and the plates were incubated for 20 min at room temperature prior to quantitating colour development on an ELISA reader (Ceres 900 Hdi, Bio Tek Instruments, Winooski, Vt, USA) at an absorbance of 414 nm.

The antibody titre was determined as being the last dilution at which the absorbance was above background levels.

2.2.21 POSTMORTEM PROCEDURE AND SAMPLE COLLECTION

Mouse pups were sacrificed by decapitation, whilst adult mice were sacrificed by cervical dislocation. Postmortem procedure involved general examination of organs. Relevant organs were removed, placed in vials and immediately placed on dry ice for transportation to the laboratory. Tissue samples were stored at -20°C , and were weighed before use.

2.2.22 MORRIS WATER MAZE

A detailed description of the MWM test can be found in Chapter 5. Briefly, mice were acclimatised to a pool (childrens' inflatable wading pool) filled 30 cm deep with water ($21-25^{\circ}\text{C}$). Testing officially began after the acclimatisation phase. Approximately 500 g of milk powder (Diploma) was added to the water and mixed to give a cloudy appearance. The acquisition phase was performed on days one- to five, immediately followed by the probe phase on day six. The re-learning phase was performed on days seven- to 10, whilst the reversal phase was performed on days 11- to 12. Latency to find the platform was measured with a lab timer. Mice were towel- dried between each trial and placed on a heating pad. During the 12 days of testing mice were treated prophylactically with Bactrim (0.8 mg/ml Sulphamethoxazole, 0.16 mg/ml Trimethoprim, Roche Pharmaceuticals).

2.2.23 MORRIS WATER MAZE STATISTICS

Statistical analysis was performed by Craig Hirte, Public Research Institute, WCH.

The time taken for each animal to locate the platform was considered as the dependent variable. A natural log transformation was applied to the time taken data. A repeated measures analysis was used to assess differences in time taken over days, between groups (ie: normal and affected) and trials (1-4). Each assessment period (acquisition, relearning and reversal) was analysed separately. The analysis was performed with SPSS v10.

2.2.24 ELECTRON MICROSCOPY

All thick and ultrathin sections were prepared by Lynn Waterhouse, Centre for Electron Microscopy and Microstructure Analysis, University of Adelaide.

Electron microscopy samples were fixed in 4% (w/v) paraformaldehyde/1.25% (w/v) glutaraldehyde in PBS, + 4% (w/v) sucrose, pH 7.2, overnight at 4°C then postfixed in 1% (v/v) osmium tetroxide for 1-hr, dehydrated through 70%, 90%, 95% and 100% X-ethanol and embedded in epoxy resin. One-micron thick sections were stained with 1% (w/v) Toluidine Blue in 1% (w/v) borax and then ultrathin sections cut and stained with 2% (w/v) uranyl acetate/1% (w/v) lead citrate and examined with a PhilipsCM 100 electron microscope.

CHAPTER 3.0

CHARACTERISATION OF THE MPS IIIA MOUSE COLONY

3.1 INTRODUCTION

A naturally occurring MPS IIIA mouse was identified in collaboration between four groups in the Departments of Cell Biology, Pathology and Neuroscience at Albert Einstein College of Medicine in New York, and the Lysosomal Diseases Research Unit at the Women's and Children's Hospital (WCH) in Adelaide. The mouse was identified in a colony of mice being used in experiments attempting to generate embryonic stem cell clones (WW6.186) that carries a targeted mutation in one allele of the acetylglucosaminyltransferase III gene (*Mgat3*).

The MPS IIIA mouse carries a novel NS mutation that has not previously been observed among human patients. A collaboration between the Departments of Cell Biology at Albert Einstein, Dipartimento di Scienze Biochimiche e Biotecnologie at Università di Perugia, and the Lysosomal Diseases Research Unit, WCH, identified the mutation in the mouse sulphamidase gene responsible for the MPS IIIA phenotype, a point mutation (G to A) at nucleotide position 91 with a corresponding amino acid change from aspartic acid to asparagine (D to N) at position 31 (Bhattacharya et al., 2001). It was postulated that this mutation would have a serious effect on enzyme function based on structural considerations (discussed further in Chapter 4.0).

Disease in MPS IIIA mice closely parallels disease observed in MPS IIIA patients. Therefore, this is a valuable model for evaluating new therapy strategies for possible future use in patients. Before evaluating ERT in MPS IIIA mice it was necessary to establish baseline data and observations of the natural course or history of disease in MPS IIIA mice from birth to maturity. The aim of this chapter is therefore to describe the

disease progression and to understand the variability between different MPS IIIA mice, and the extent of differences compared with normal mice. These observations were needed for later comparison with results of ERT studies. Heterozygotes were considered to have a wild type phenotype, therefore normal mice referred to in this chapter are either wild type or heterozygotes.

We received two MPS IIIA and two heterozygote female and male mice from the Albert Einstein Institute in January 2000. The initial aim of this project was to establish an MPS IIIA mouse colony in Adelaide, and to characterise the progression of disease in these mice.

3.2 RESULTS

3.2.1 MANAGEMENT OF THE MPS IIIA MOUSE COLONY

The identification of the mutation permitted the development of a rapid screening method to identify homozygous affected and heterozygous carriers at birth, thereby facilitating the efficient management of the colony.

Mice were individually identified by a toe tagging system (see Materials and Methods, Section 2.2.18.1). At six-to eight-days of life, mice were assigned a number representative of the toes cut; this method of tagging is safe and effective and gives the ability to tag up to 10,000 mice. Genomic DNA was isolated from the toe tissue by incubation in toe lysis buffer overnight, and this was used in a PCR reaction to genotype the mice.

A NS gene fragment of 105 bp, including a portion of intron sequence and spanning the point mutation in exon 2, was amplified, PCR products were digested with Aci I and separated on a 3.5% agarose gel. The point mutation abolishes the Aci I site. A second Aci I site is present in the 3' primer, therefore 94 and 11 bp products are obtained from mutant mice, while 78, 16 and 11 bp products are obtained from wild type mice (Figure 3.1). The 11 bp and 16 bp digest products are not observed on the gel as they are too small.

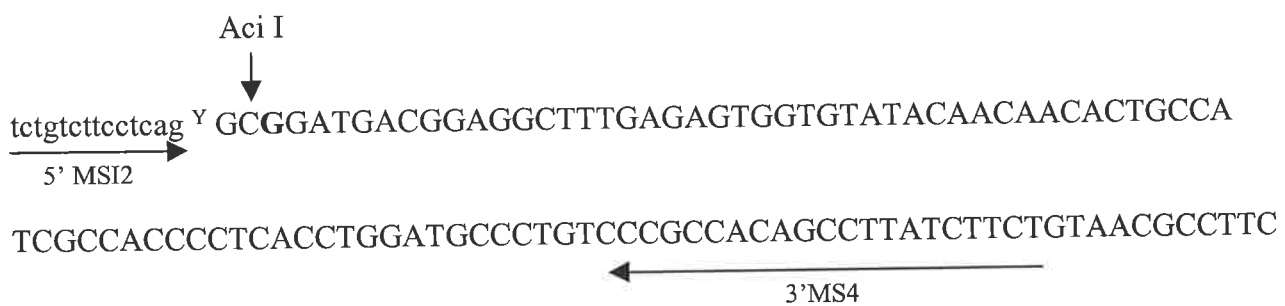
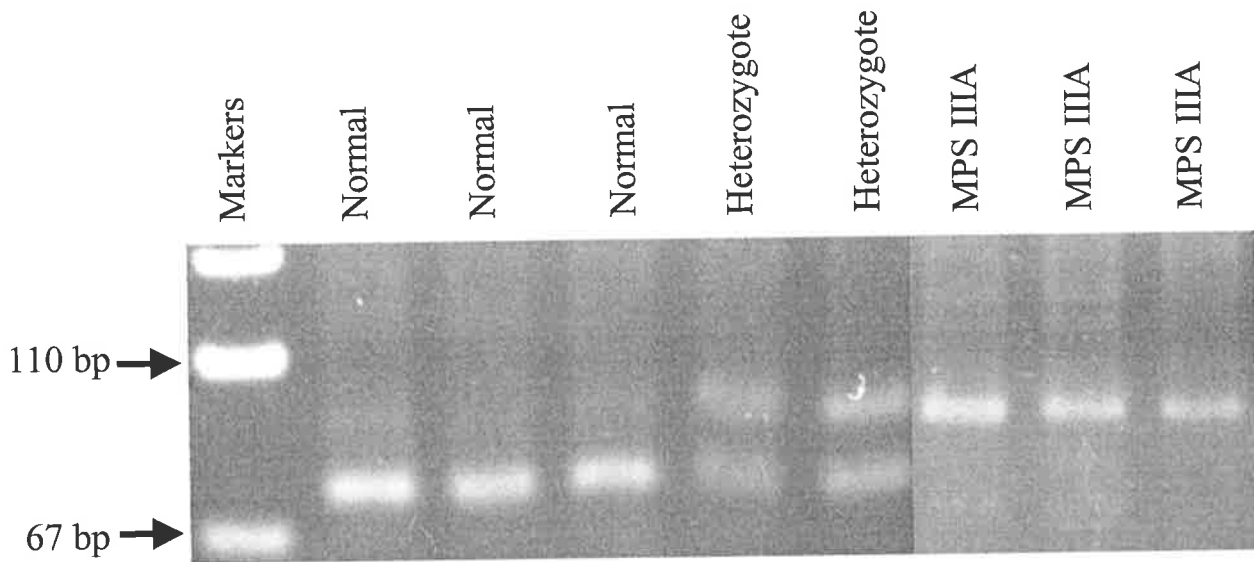
Mice were therefore genotyped and tagged by one-week of age. At three-weeks of age mice were sexed and weaned. MPS IIIA mice were caged together distinct from normal mice.

3.2.2 COLONY BREEDING DATA

MPS IIIA is inherited in an autosomal recessive manner. MPS IIIA mice are fertile and therefore it was possible to mate affected mice to obtain large numbers. However, as MPS IIIA females begin to eat their young by 20-weeks of age, they had to be mated and

Figure 3.1: Genotyping MPS IIIA mice:

The mutation region was PCR amplified, digested with Aci I restriction enzyme and electrophoresed on a 3.5% agarose gel (refer to Materials and Methods, Section 2.2.18 for detail). Genotypes are given along the top of the gel; a 94 bp product is observed for MPS IIIA, 78 bp product obtained for normal, and both the 94 bp and 78 bp product obtained in the presence of an MPS IIIA heterozygote. The molecular sizes are indicated with arrows and were calculated by comparison with pUC19/HpaII markers.



have given birth before they reached this age. This gave only a small window of time in which MPS IIIA mice could be mated, taking into consideration that mice do not become sexually mature until about eight-weeks of age and they have a three-week gestation period.

3.2.3 PHYSICAL APPEARANCE

At birth MPS IIIA mice were indistinguishable from their unaffected littermates. However, unlike unaffected controls, by three- to four-weeks of age MPS IIIA pups were very hyperactive. They would climb the tops of the cage and were very jumpy; when the cage lid was removed they would try to escape, and they were also slightly aggressive towards the handler. Paper towelling and cardboard tubes were added to the cages to assist management of the mice by providing a more enriched environment. The 'hyperactive' period lasted until the mice reached sexual maturity at eight- to 10-weeks of age. By then male MPS IIIA mice had become very aggressive toward each other. If there was more than one male in a cage fighting would occur, resulting in the loss of fur and sores on the lower back hind legs. As a result MPS IIIA male mice were caged separately. This behaviour was not observed for female MPS IIIA mice or unaffected male mice of the same age. By about six-months of age the coats of MPS IIIA mice appeared coarse and unkempt when compared to unaffected mice; a slightly hunched posture was also generally noted in MPS IIIA mice, male and female (Figure 3.2). MPS IIIA mice displayed a slightly broader and more flattened face by this age, however the degree of this phenotype was very variable among the MPS IIIA colony. MPS IIIA mice were also slightly fatter than unaffected mice weighing on average 3-to 5g heavier (Table 3.1). By six- to seven-months of age, the affected mice became less active, and would generally not move around too much in their cage, in comparison to unaffected mice. At this age 'anal prolapse' was observed in a few male MPS IIIA mice. MPS IIIA mice generally died by 10- to 12-months of age, however some died sooner at about seven-months and one lived to 14-months. Autopsy generally revealed enlarged bladders and an occasional large spleen was observed (Figure 3.3). A thicker skin was also observed, sometimes with a slightly fatty lining.

Figure 3.2: Normal and MPS IIIA mice.

The mouse on the top is a normal male, and the mouse on the bottom is an MPS IIIA male. Mice are 25-weeks old. Note the scruffy texture of the MPS IIIA coat, and also the bigger size in comparison to normal.



Table 3.1: Body weights with age of normal and MPS IIIA mice

AGE (Weeks)	Normal (grams)		MPS IIIA (grams)	
	Female (n=6)	Male (n=6)	Female (n=6)	Male (n=6)
2	4.8 ± 1.2	5.38 ± 0.8	5.01 ± 0.6	5.67 ± 0.5
8	18 ± 0.9	22 ± 1.3	22 ± 0.5	25 ± 1.1
15	25 ± 1.3	29 ± 0.3	28 ± 0.4	33 ± 1.2
20	27 ± 0.6	31 ± 1.2	32 ± 1.1	36 ± 1.3

Body weights are given in grams plus or minus the standard deviation.

Figure 3.3: Terminal illness in MPS IIIA mice.

Distended bladder observed upon autopsy of MPS IIIA mice.



3.2.4 OUTBREEDING THE MPS IIIA MOUSE COLONY INTO THE C57BL/6J STRAIN

The MPS IIIA mice received in January 2000, were of a mixed genetic background including predominantly 129SvJ and CD1 with some C57Bl/6 and SJL strain contributions. One of our long-term aims is to outbreed the colony into a stable genetic background, the C57Bl/6J strain. Having the colony in one background would make traits between the mice more uniform; behavioural studies, which form an integral role when assessing therapeutic strategies (discussed later in Chapter 5.0) are also best compared in mice with the same genetic constitution. The C57Bl/6J strain was chosen for a number of reasons: first and foremost other murine models of LSD including the MPS VII (Birkenmeier et al., 1989), MPS I (Clarke et al., 1997) MPS IIIB (Li et al., 1999), MPS II (Lamsa et al., 2002) and MPS IVA (Tomatsu et al., 2002a) mice are all in the C57BL/6J strain. This would enable direct comparisons between the MPS IIIA mice and other LSD mouse models to be made. Also, C57BL is the most widely used of all inbred strains, in fact C57BL/6 accounts for over 14% of occasions (<http://www.molgen.gla.ac.uk/>) on which an inbred strain is used. This means that it is a well characterised strain and a significant amount of information is available on it.

3.3 DISCUSSION

A colony of MPS IIIA mice has been established in Adelaide. The colony is efficiently maintained by a simple toe tagging system used to identify mice, and the toe tissue used in a PCR reaction to genotype the mice. There appears to be three distinct stages in the progression of disease in the mice; the first, at three-weeks of age, is characterised by hyperactivity; the second at nine- to 12-weeks of age is characterised by aggression, only observed in the male MPS IIIA mice. The third stage, observed at 20- to 25-weeks of age, is characterised by lethargy, and the development of a 'scruffy' coat, hunched posture and flattened, broader face. Two breeding programs are ongoing: the first is to maintain the original colony containing the mice in a heterogenous background, and the second is to outbreed the mice into the C57BL/6J strain. It is anticipated that some of the work performed in this thesis will be repeated in the outbred strain for comparison.

Following characterisation of disease progression of MPS IIIA on the mice, molecular characterisation of the mutation giving rise to the disorder was assessed. These experiments are presented and discussed in the next chapter.

CHAPTER 4.0

EXPRESSION AND CHARACTERISATION OF THE MUTANT AND WILD TYPE mNS

4.1 INTRODUCTION

W.D. USE A point mutation (G to A) at nucleotide position 91 is responsible for MPS IIIA in the mice (Bhattacharya et al., 2001). This corresponds to an amino acid change from aspartic acid to asparagine (D to N) at position 31, as the only difference between mutant and parent cDNAs. It was postulated that this mutation would have a serious effect on enzyme function based on structural considerations.

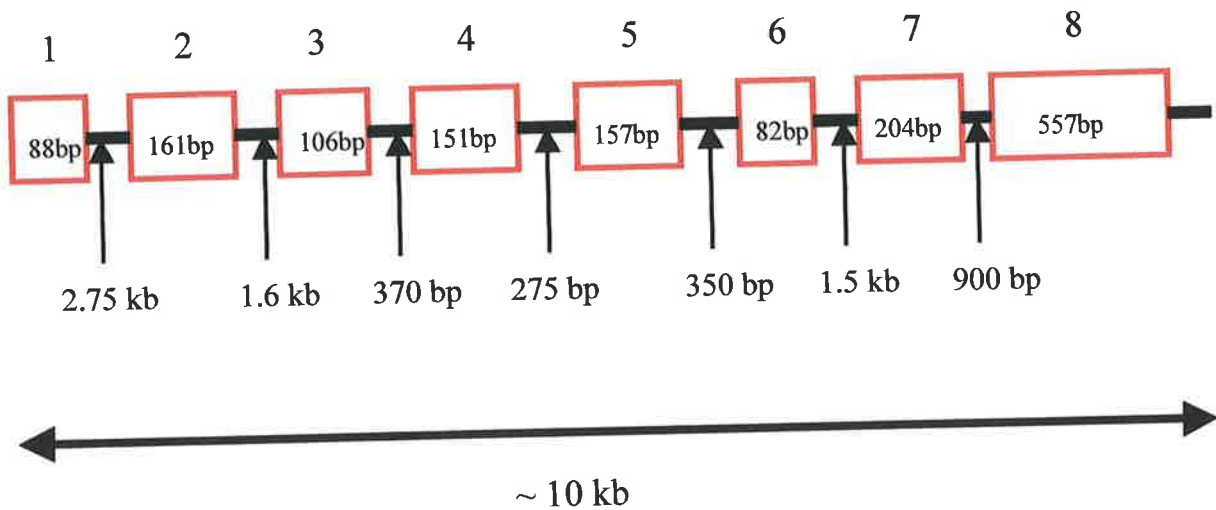
There is a high degree of homology among sulphatases (Tomatsu et al., 1991; Franco et al., 1995; Parenti et al., 1997; Hopwood and Ballabio, 2001). The aspartic acid residue, D31, in combination with D32, is proposed to be involved in coordination of a divalent metal ion needed for the catalytic event in the family of sulphatases that include NS (Waldow et al., 1999; Hopwood and Ballabio, 2001). Based on the crystal structure of N-acetylgalactosamine 4-sulphatase, two other amino acids (D273 and N274) are also likely to be involved in coordination of the divalent metal ion (Bond et al., 1997). Other amino acids proposed to be critical for catalysis in N-acetylgalactosamine-4-sulphatase based on the crystal structure (Bond et al., 1997) are conserved, both in nature and relative position, in the primary sequence of NS. Thus D53, D54, C91, P93, S94, R95, K145, H147, D300, N301 and K318 in N-acetylgalactosamine-4-sulphatase correspond to D31, D32, C70, P72, S73, R74, K123, H125, D273, N274 and K303 in human sulphamidase. Except for K303Q change between human and mouse NS all of these amino acids are conserved between the species (Bhattacharya et al., 2001).

not cited OK

Comparisons of the deduced amino acid sequences of human and mouse NS showed 88% identity and 93% similarity (Costanzi et al., 2000). The exon-intron structure of the gene has been resolved with the mouse 10 kb gene divided in to eight exons (Figure 4.1). The mouse NS gene has been localised to the distal end of chromosome 11, in a region that is homologous with a segment of human chromosome 17, containing the orthologous human gene (Costanzi et al., 2000).

The two major aims of experiments described in this chapter were, firstly, to characterise murine NS (mNS) and the D31N mutation that gives rise to MPS IIIA in the mice, by looking at expression levels and processing or maturation pathways, and, secondly, to purify and characterise recombinant murine NS (rmNS).

Figure 4.1: Structure of the mouse sulphamidase gene*



* From Costanzi et al., 2000.

The mouse sulphamidase gene extends for approximately 10 kb and has 8 exons, represented by red boxes. Introns are denoted by black bars between introns, sizes are indicated. For comparison to the human sulphamidase gene, refer to Figure 1.3.

4.2 RESULTS

4.2.1 *IN VITRO* TRANSCRIPTION/TRANSLATION OF pcDNA3.1 EXPRESSION VECTORS.

We obtained the 1.6 kb coding sequences for the wild type murine NS (mNS) and the D31N mutant NS (D31NmNS) from Dr Pamela Stanley (Bhattacharya et al., 2001). The cDNAs were cloned into the pcDNA3.1(-) expression vector at the NotI/HindIII sites. Before the plasmids were used in characterisation studies, an *in vitro* transcription/translation assay was performed on the plasmids to ensure correct orientation and the production of protein. Constructs were incubated with or without microsomes, which glycosylate the protein *in vitro*. A band was observed at 50-55 kDa in the absence of microsomes for both the mutant D31N and wild type constructs; upon the addition of microsomes a 62 kDa band was observed for both constructs. Glucosamine 6-sulphatase was included as a positive control (Figure 4.2).

labelled protein?

4.2.2 EXPRESSION OF THE mNS cDNA IN CHO-K1 CELLS

The wild type and mutant mNS pcDNA3.1 expression constructs were electroporated into CHO-K1 cells. G418-resistant mass cultures were assayed for NS activity using a radiolabelled tetrasaccharide (see Materials and Methods, Section 2.2.5). CHO-K1 cells electroporated with the control vector pcDNA3.1 (CHOpcDNA3.1) contained very low levels of endogenous NS activity (0.5 - 2 pmol/min/mg). G418-resistant mass cultures of CHO-K1 cells electroporated with the wild-type mNS pcDNA3.1 expression vector (CHOmNS) showed NS activities towards the radiolabelled tetrasaccharide ranging from 403 pmol/min/mg to 549 pmol/min/mg total cellular protein. In contrast, G418-resistant mass cultures of CHO-K1 cells electroporated with the D31N mutant mNS pcDNA3.1 expression vector (CHO D31NmNS) displayed NS activities ranging from 3 pmol/min/mg to 8 pmol/min/mg total cellular protein. Medium harvested from CHOmNS and CHOD31NmNS cells after 24-hr contained 906 pmol/min/ml and 24 pmol/min/ml NS activity, respectively (Table 4.1). Therefore, NS expressed from mutant cDNAs had

Figure 4.2: TNT *in vitro* transcription/translation analysis of pcDNA3.1 vectors.

Wild type (WT) and D31N mutant (D31N) mNS pcDNA3.1 vectors were transcribed and translated in the presence (+) or absence (-) of microsomes and run on SDS-polyacrylamide gels. Glucosamine-6-sulphatase (Glu6-S) was added as a positive control. The molecular sizes (in kDa) are indicated by arrows and were calculated by comparison with C-14 methylated standards.

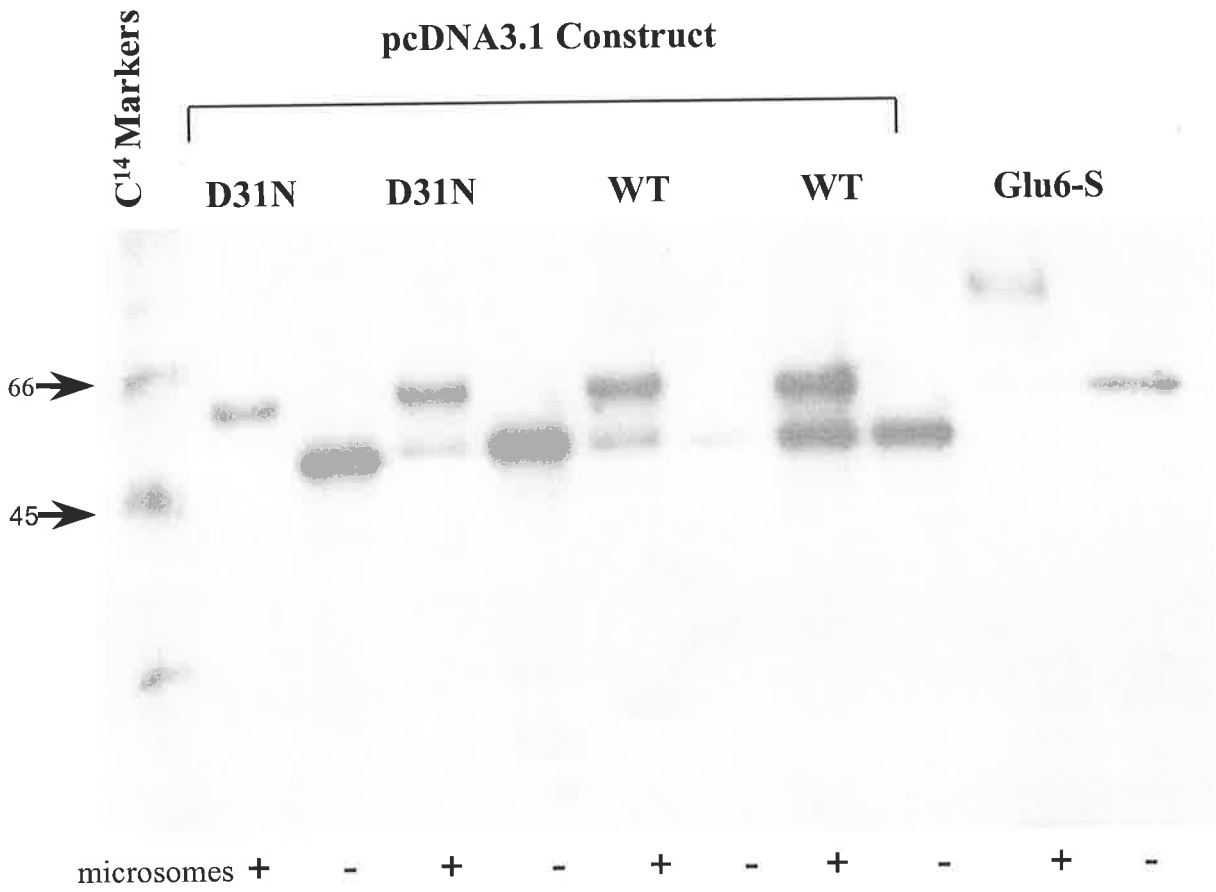
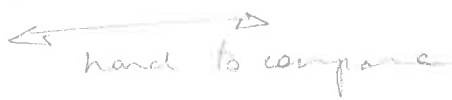


Table 4.1: Activity of wild type and mutant mNS.

	LYSATE	MEDIUM
cDNA Transfected in CHO-K1 cells	NS activity (pmol/min/mg)	NS activity (pmol/min/ml)
pcDNA3.1mNS (wild-type)	403, 549	906
pcDNA3.1 D31NmNS (mutation)	3, 8	24
pcDNA3.1 N31DmNS (corrected mutant)	408, 508	619
pcDNA3.1 (vector control)	0.5, 2	10

Co-protein?

hard to compare

Cell lysates and conditioned medium obtained from G418-resistant mass cultures of CHO-K1 cells transfected with the outlined vectors were assayed using a radiolabelled tetrasaccharide. Cell lysates were also assayed for total protein content. Duplicate results for lysate are shown.

markedly reduced NS activity (approximately 1.5%) compared to wild type cDNAs. As expected, the D31N missense mutation severely reduces NS activity. Correction of the A at nucleotide 91 to G by site-directed mutagenesis (Bhattacharya et al., 2000) restored NS activity to wild type levels (Table 4.1). *mutated*

4.2.3 MATURATION PATHWAY OF WILD TYPE AND D31N mNS IN CHO-K1 CELLS

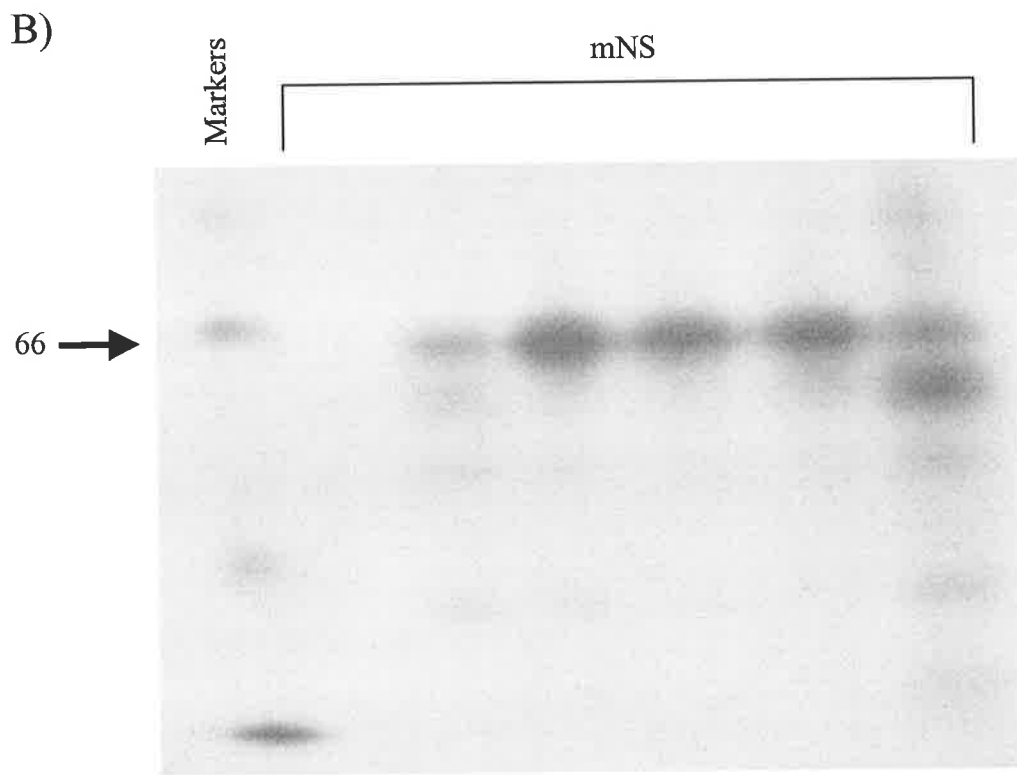
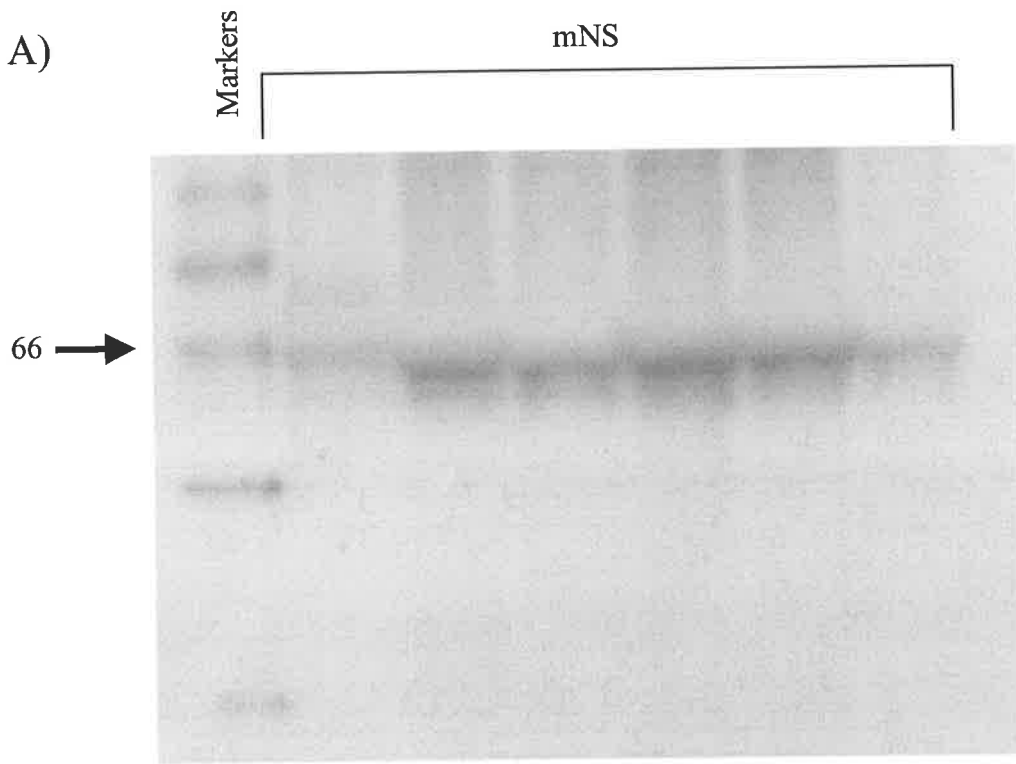
In an attempt to understand the processing pathway of wild type and mutant mNS, pulse-chase experiments were performed in stably expressing CHO cell lines containing the wild type mNS and the D31NmNS. G418-resistant mass cultures of CHOmNS and CHOD31NmNS cells were radiolabelled for 24-hr with ³⁵S-methionine. Wild type or mutant mNS present in solubilised cell lysates and culture medium was characterised by immunoprecipitation, SDS-PAGE and autoradiography. The intracellular form of ³⁵S-labelled rmNS is synthesised as a 62-65 kDa precursor molecule which is cleaved to the mature 56 kDa form within 0.5-hr and remains stable for 8-hr but shows a slight degradation one-day after synthesis (Figure 4.3A). The intracellular form of ³⁵S-labelled D31NmNS was also synthesised as a 62-65 kDa precursor molecule, which is present 0.5 hr post-labelling, and is cleaved by two-hr to 56 kDa; further cleavage *occurs before* occurs at four-hr and then *again before* again at eight-hr post-labelling (Figure 4.3C). The secretion of rmNS into the medium was detected 30-min after labelling. It is predominantly secreted as a 62-65 kDa precursor molecule and remains stable over a one-day chase period (Figure 4.3B). The secretion of D31NmNS into the medium was detected two-*hor* hor post-labelling, where it shows reduced levels of expression in comparison to wild type for up to 24-hr (Figure 4.3D).

4.2.4 ISOLATION OF A HIGH-EXPRESSING CHOmNS CELL LINE

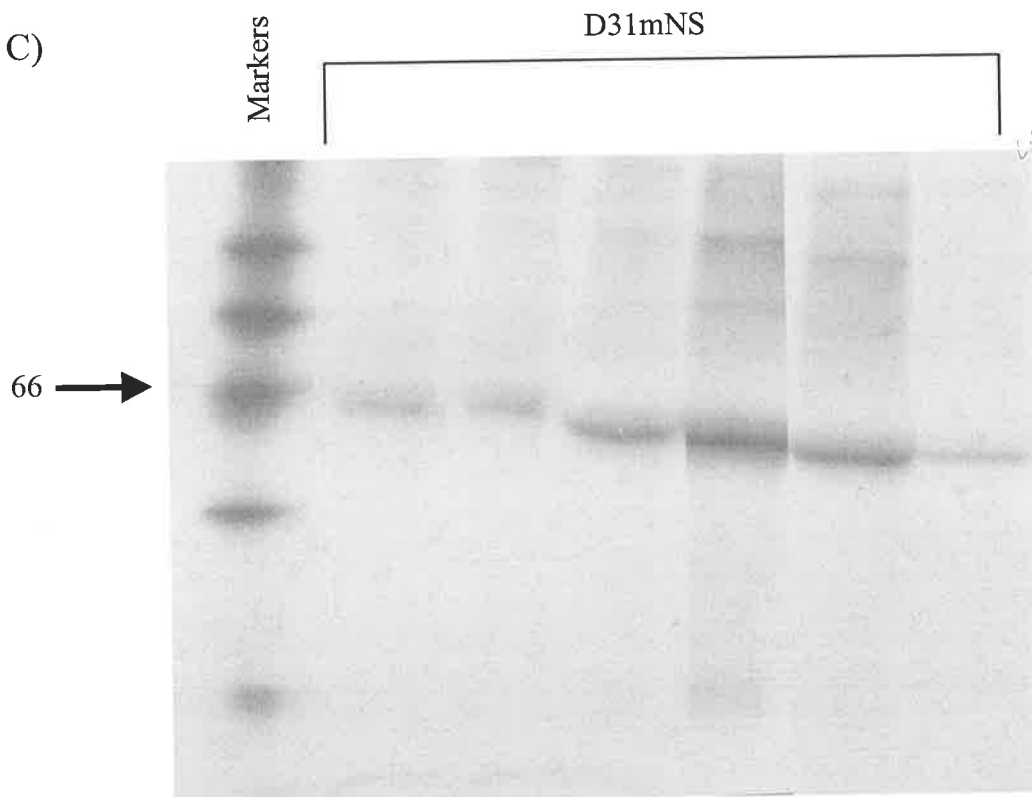
Twenty-two clones were isolated from a mass culture of G418-resistant cells resulting from electroporation of CHO-K1 with pcDNA3.1mNS by limiting dilution in microtitre

Figure 4.3: Pulse-chase labelling of wild type (opposite page) and D31N mutant (next page) mNS in CHO-K1 cells.

Wild type mNS and D31NmNS were grown to confluence in 75 cm² tissue-culture flasks and metabolically labelled with ³⁵S-methionine for 30-min and then chased for different times, from 0 to 24 hr. The cell extract (A & C) and growth media (B & D) from each chase period were then immunoprecipitated and run on SDS-polyacrylamide gels. The molecular sizes (in kDa) are indicated with arrows and were calculated by comparison with C-14 methylated standards.



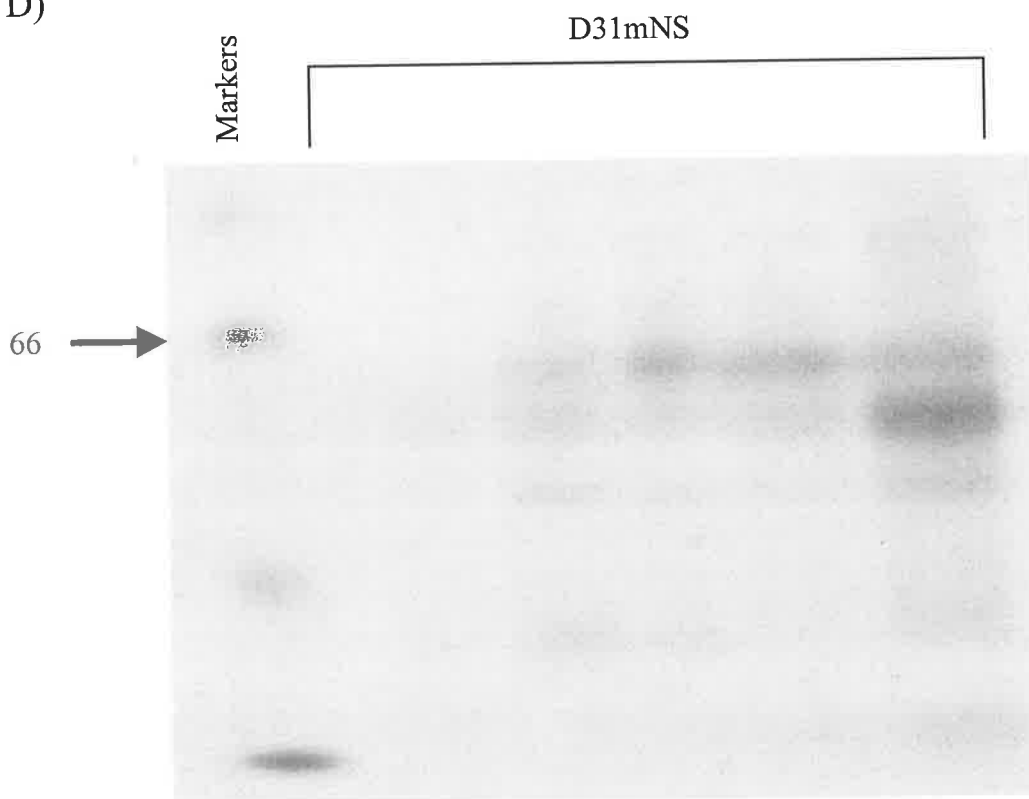
C)



Chase time points:
(hours)

0 0.5 2 4 8 24

D)



Chase time points:
(hours)

0 0.5 2 4 8 24

plates. All 22 clones were assayed directly for total NS activity secreted into the medium using the radiolabelled tetrasaccharide substrate. Total NS activity in medium conditioned for 24-hr ranged from background (1.2 pmol/min/ml) to 2,425 pmol/min/ml (Table 4.2). The four highest expressing clones designated CHOmNS2, CHOmNS3, CHOmNS4 and CHOmNS14 were expanded and assayed for NS activity. These clones accumulated from 2.6 mg to 4 mg mNS per litre in culture medium conditioned for two-days over confluent cells, as calculated from specific activities. The highest expressing clone, CHOmNS-4, was recloned by limiting dilution to ensure clonality. This clone was expanded and used for the large-scale production and characterisation of rmNS. This clone accumulated 4 mg rmNS per litre in culture medium conditioned for two-days over confluent cells.

Sig
2/19/92

4.2.5 PURIFICATION OF rmNS

CHOmNS-4 was expanded for large-scale production of rmNS. Production of two- to four litres of conditioned medium for purification was done in cell factories with a surface area of 1,264 cm². Cells harvested from 10-12 T75 flasks were seeded into the cell factories in COON'S DMEM medium. Serum-free conditioned medium was harvested every three-days for about eight-weeks when the factory was no longer viable.

Under the static culturing conditions of CHO-K1 cells used in this study, cellular overgrowth became evident one- to two-weeks post-confluency. This led to formation of cellular clumps that eventually peeled off the culturing surface. Although some recolonisation was observed, this generally led to poor cellular coverage of the culture vessel, hence decreased production of rmNS. The chemical compound DMSO has been shown to restore cell-contact growth inhibition (Borys and Papoutsakis, 1992; Fiore and Degrassi 1999; Berg et al., 2001) such that confluent cells become arrested in the G1 phase of the cell cycle. Therefore, at each medium change COON'S DMEM was supplemented with 1-2% DMEM and also 1-2% FCS for two-days. This enabled the cells to be maintained for longer. The addition of FCS in the culture medium was not favourable as the medium was to be used in the purification of rmNS. The absence of

Table 4.2: NS expressing single colonies.

NS activity of 22 clones isolated from CHO-K1 cells over-expressing mNS. The four highest expressing colonies designated in red were expanded and assayed for NS activity.

CHO-K1 clone	NS activity pmol/min/ml
mNS-1	1025
mNS-2	1585
mNS-3	1119
mNS-4	2425
mNS-5	92
mNS-6	148
mNS-7	851
mNS-8	926
mNS-9	279
mNS-10	614
mNS-11	204
mNS-12	139
mNS-13	185
mNS-14	1025
mNS-15	814
mNS-16	399
mNS-17	328
mNS-18	740
mNS-19	639
mNS-20	465
mNS-21	262
mNS-22	124

sg
figs

serum in the culture medium also reduces the cost of production, but more importantly it reduces the total protein concentration of the medium, which eases the purification of the secreted protein. Therefore, after the two- days this medium was changed and discarded and the cells were once again grown in serum-free medium.

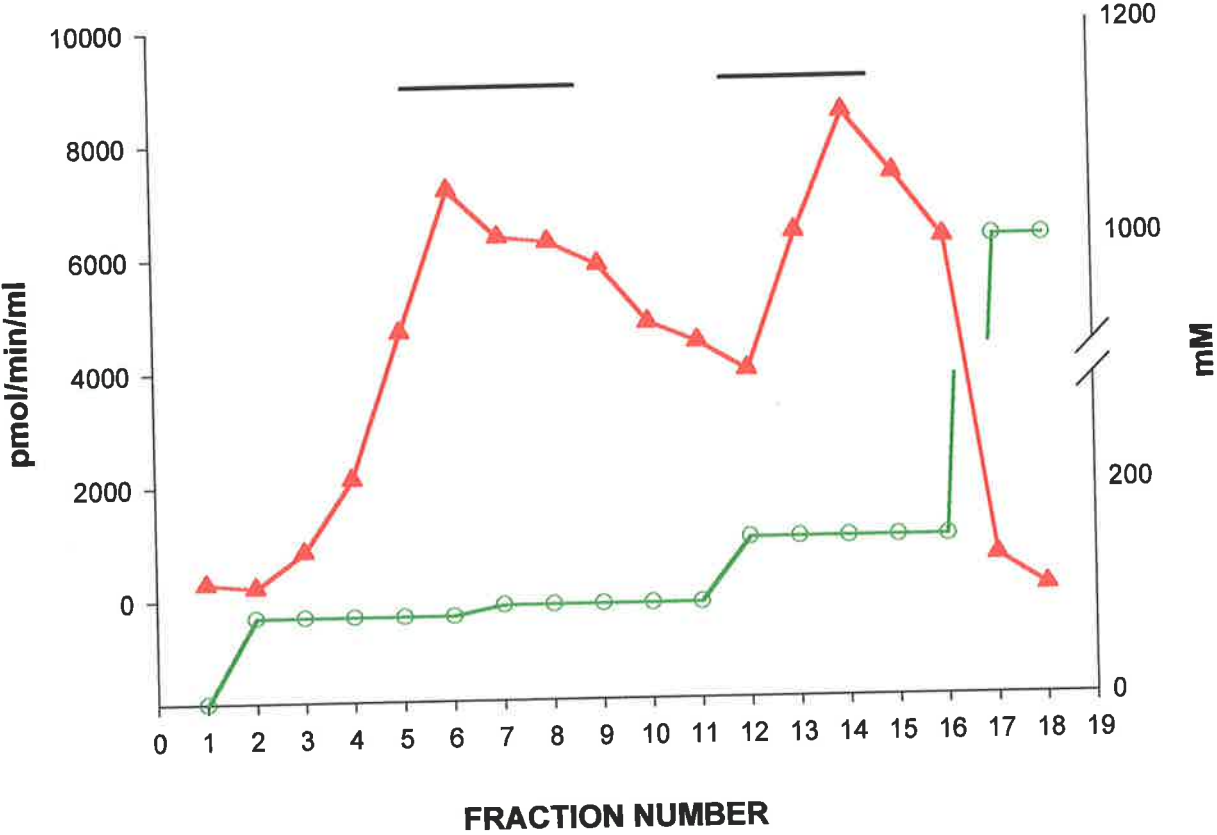
Four-litres of serum-free conditioned medium was concentrated through a hollow fibre concentrator to give a final volume of 100 ml. A similar method to the two step ion-exchange procedure reported for rhNS (Bielicki et al., 1998) was employed to purify rmNS. After concentration the conditioned medium was dialysed to remove inhibitors of NS. NS activity after dialysis doubled. The concentrated medium was then applied to a cation-exchanger, CM-Sepharose. The rmNS binds to the Sepharose at pH 4.0, which contrasts with the hNS which binds to the CM-Sepharose at pH 5.0. This reflects the differences in the number of charged residues each NS molecule contains. Sequence analysis predicts mNS to have an isoelectric point of 6.42 and contain 174 charged amino acid groups (103 positively charged amino acids, and 71 negatively charged amino acids). In contrast, hNS has a predicted isoelectric point of 7.71 and contains only 109 charged amino acid groups (59 positively charged amino acids and 50 negatively charged amino acids).

NS eluted from the column with increasing NaCl concentrations ranging from 80-150 mM NaCl. NS activity was found to be eluted in a broad peak between 90 and 150 mM NaCl (Figure 4.4A). These eluates were pooled and concentrated in an Amicon ultrafiltration stirred cell. The dialysed concentrate was applied to an anion exchanger, DEAE-Sephacel; the rmNS binds to the exchanger at pH 7.0. Recombinant mNS was eluted with increasing NaCl concentrations (Figure 4.4B). The eluates were once again assayed for NS activity; each NS-activity-containing fraction was pooled and concentrated, and analysed by SDS/PAGE. One major polypeptide was detected at 62-64 kDa. (Figure 4.5).

Figure 4.4: Purification of rmNS.

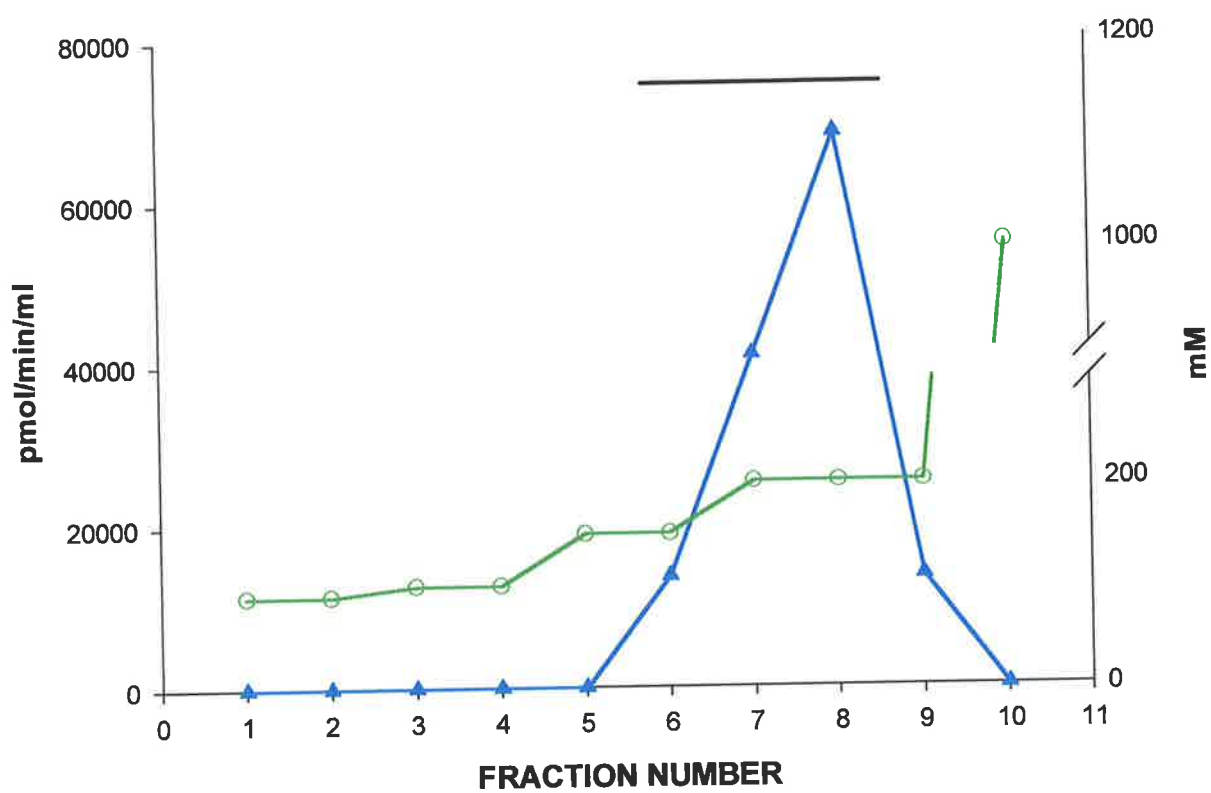
Concentrated, conditioned medium obtained from the CHO-K1mNS-4 cell line was dialysed and applied firstly to a CM-Sepharose column (A, opposite page); 18 fractions were collected and assayed for NS activity. Fractions were pooled as indicated by the horizontal bar. These pooled fractions were concentrated, dialysed and applied to a DEAE-Sephacel column (B, next page); 10 fractions were collected and assayed for NS activity. Fractions were pooled as indicated by the horizontal bar. NS activity is represented by triangles, and NaCl concentration by open circles.

CM-SEPHAROSE CHROMATOGRAPHY



—▲— NS ACTIVITY
—○— NaCl CONCENTRATION

DEAE-SEPHACEL CHROMATOGRAPHY



▲ NS ACTIVITY

○ NaCl CONCENTRATION

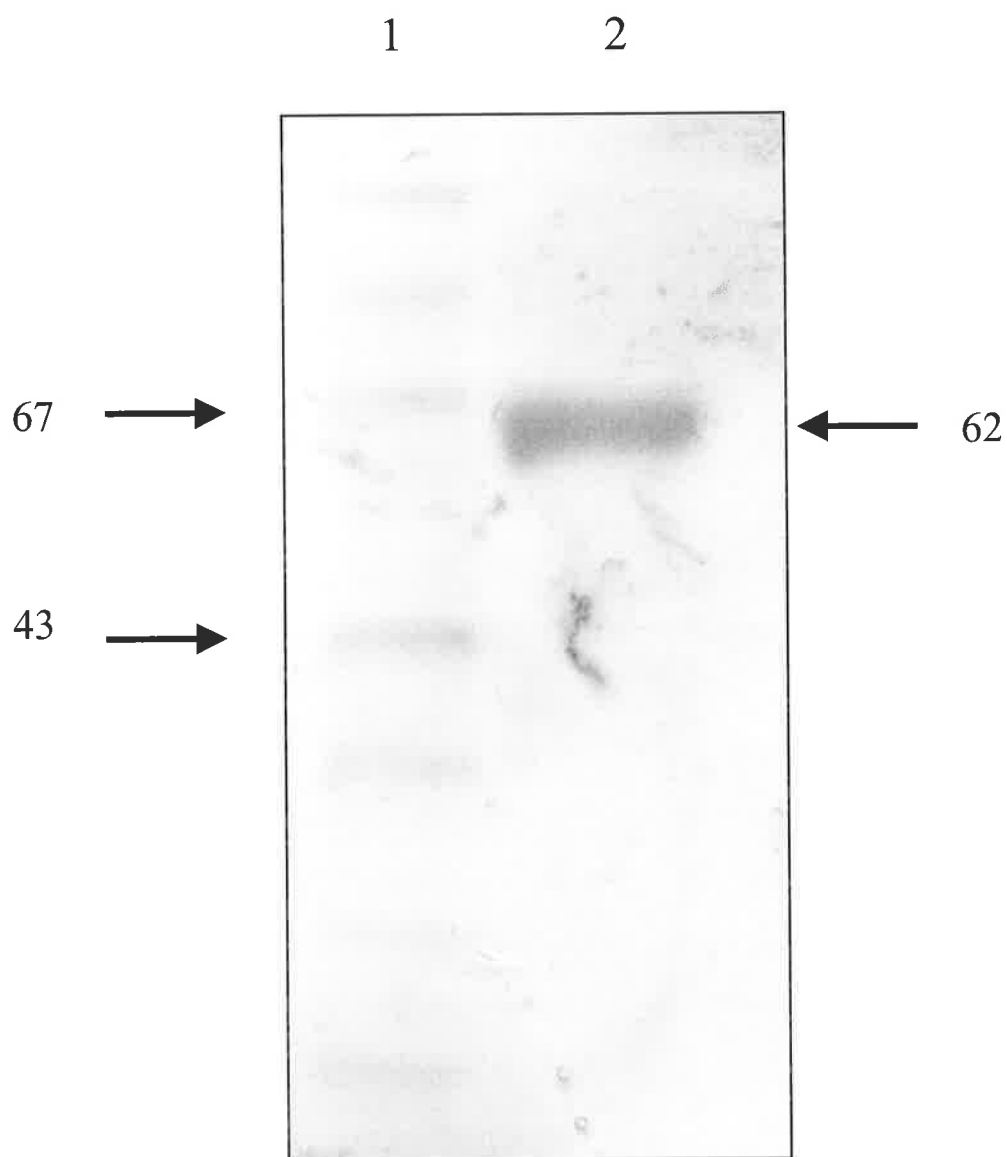


Figure 4.5: SDS/PAGE of rmNS

Lanes 1 and 2 are molecular mass standards and purified rmNS respectively, electrophoresed under reducing conditions with dithiothreitol (30mM). The gel was stained with Coomassie Brilliant blue R. Molecular mass standards in kDa and the rmNS with molecular mass indicated by arrows.

4.2.6 KINETIC PROPERTIES OF rmNS

Purified rmNS was used to determine kinetic parameters (K_m , V_{max}). Assays to produce data for Lineweaver-Burk plots were carried out using the radiolabelled tetrasaccharide substrate (concentration range 5-80 μM). The results from 12 substrate concentrations were used to generate Lineweaver-Burk plots. K_m was determined as the negative reciprocal of the x intercept and V_{max} as the reciprocal of the y intercept.

Kinetic data for the purified rmNS gave K_m of 8.4 μM and V_{max} of 800 nmol/min/mg. These values are similar to kinetic data of rhNS purified from CHO cells (Bielicki et al., 1998), and NS purified from human liver (Freeman and Hopwood, 1986) with regard to its K_m and V_{max} (Table 4.3).

4.2.7 CORRECTION OF HEPARAN SULPHATE STORAGE IN MPS IIIA SKIN FIBROBLASTS WITH rmNS.

The storage of undegraded GAG in MPS IIIA skin fibroblasts was analysed by measuring the accumulation of $^{35}\text{SO}_4$ -labelled material in cells metabolically labelled with $\text{Na}_2^{35}\text{SO}_4$.

MPS IIIA fibroblasts have undetectable levels of NS activity and store approximately 10- to 20-times more ^{35}S -labelled storage products in the form of undegraded HS fragments than normal control fibroblasts. CHOmNS4 conditioned medium was used as a source of rmNS in the correction studies. On the addition of rmNS at 70 nmol/min/ml, the level of NS activity is restored to normal and, concomitantly, storage is reduced to levels comparable with that observed in control cells. M6P added to cells receiving 350 nmol/min of rmNS inhibits the uptake of NS activity demonstrating that endocytosis of rmNS is mediated by the M6P receptor pathway (Table 4.4).

But why is storage low?

Table 4.3: Comparison of the kinetic properties of rmNS with hNS.

	Km (μ M)	Vmax (nmol/min/mg)
rmNS	8.4	800
Human liver NS*	10.3	928
rhNS [^]	12.25	934

uncertainty?

Kinetic properties were determined using the radiolabelled tetrasaccharide substrate.
For details, see Materials and Methods Section 2.2.13

* From Freeman and Hopwood, 1986

[^] From Bielicki et al., 1998

Table 4.4: Correction of the MPS IIIA defect by rmNS.

	NS Activity (pmol/min/mg)	S³⁵ radioactivity (c.p.m)
Normal SF	531, 439	89, 63
MPS IIIA SF	none detected	809, 610
MPS IIIA SF + rmNS	441, 276 <i>no storage</i>	180, 92 <i>no storage?</i>
MPS IIIA SF + rmNS + M6P	102, 83	75, 60

Normal and MPS IIIA (SF 4636) skin fibroblasts (SF) were labelled with Na₂³⁵SO₄. Fibroblasts were incubated with 70 nmol/min/ml rmNS in the presence or absence of 5mM M6P as described in Materials and Methods, Section 2.2.14. Cell lysates were assayed for NS activity and total cell protein. Duplicate results are shown.

4.3 DISCUSSION

The MPS IIIA mouse carries a novel NS mutation that has not previously been characterised among humans with MPS IIIA. Cloning of the D31NmNS mutant cDNA and the wild type mNS cDNA into the pcDNA3.1 expression vector has permitted characterisation and expression analysis. As predicted, the position of the mutation in the active site of the enzyme has a profound effect on enzyme activity. When expressed in CHO-K1 cells the D31N mutant enzyme displayed only 1.5% of wild type mNS activity. The maturation pathway of wild type mNS was similar to that observed for rhNS (Perkins et al., 1999). Intracellular D31NmNS displayed a different maturation sequence to wild type mNS. Both NS molecules were synthesised as 62 kDa precursor molecules; wild type mNS was rapidly cleaved to the mature 56 kDa form and remained stable over the 24-hr chase period. However, increased degradation of the mutant enzyme was observed over the 24-hr chase period, suggesting decreased stability for D31NmNS.

For characterisation studies of rmNS a CHO clone stably secreting mNS was generated by transfecting cells with the pcDNA3.1 expression vector containing the mNS cDNA sequence. The expression levels obtained from this clone was approximately 4 mg NS per litre of culture medium, which had been supplemented with DMSO, a chemical compound known to induce growth arrest in CHO cells (Fiore and Degross 1999). This growth arrest involves an up-regulation of the expression of the endogenous p27 and bcl-2 genes (Fussenger et al., 1998). The p27 is a growth regulating gene inducing cell-growth arrest in the G1 phase of the cell cycle, whereas bcl-2 works as a 'survival gene' reducing the apoptosis that is normally associated with growth arrest. Recombinant mNS was purified from conditioned medium by a two-step ion-exchange procedure. After dialysis the concentrated medium was applied to a cation-exchanger, CM-Sepharose. The rmNS was found to bind to the Sepharose at pH 4.0. NS activity was found to be eluted in a broad peak between 90 and 150 mM NaCl. Once concentrated these eluates were applied to an anion exchanger, DEAE Sephacel; the rmNS bound to the exchanger at pH 7.0. SDS/PAGE analysis revealed one major polypeptide at 62 kDa. The kinetic

properties of rmNS were similar to human enzyme purified from CHO-K1 cells and that purified from liver.

Similar to what has been reported for a large number of lysosomal enzymes expressed in CHO-K1 cells, the CHO-produced NS could be taken up by enzyme-deficient skin fibroblasts. This uptake involved the M6P receptor. A near normalisation of the amounts of ³⁵S-sulphated oligosaccharides was shown in fibroblasts incubated with 70 nmol/min/ml of enzyme, proving that the rmNS produced in CHO-K1 cells can correct the metabolic defect in MPS IIIA cells. The addition of rmNS and M6P led to a four-fold decrease in levels of NS activity, over that achieved with the addition of rmNS in the absence of M6P. However, this decrease in NS activity did not lead to an increase in stored ³⁵S-sulphated oligosaccharides. Therefore, the amount of detectable levels of NS in these cells (approximately 100 pmol/min/mg) compared with none detected for MPS IIIA skin fibroblasts alone, was enough to correct the storage defect associated with these cells.

In conclusion, the experiments outlined in this chapter have provided valuable information about the molecular nature of the D31N mutation, and also the characteristics of wild-type mNS. The establishment of a protocol for production and purification of mNS will permit for species-specific ERT in MPS IIIA mice. Also, the finding that rmNS has similar properties to rhNS lead us to predict that MPS IIIA mice might be tolerant to ERT by the administration of rhNS. This was convenient, since large amounts of purified rhNS were already available in the Department of Chemical Pathology. An ERT trial was therefore performed in MPS IIIA mice using this enzyme (Chapter 6.0). However, before therapy trials could be assessed it was necessary to develop a neurological test to discriminate between MPS IIIA and normal mice. The results of these experiments are discussed in the next chapter.

CHAPTER 5.0

DEVELOPMENT OF THE MORRIS WATER MAZE, A LEARNING AND MEMORY TEST

5.1 INTRODUCTION

The major site of pathology in the MPS IIIA mice is the CNS; for this reason any therapy protocols for this disease must address the problem of being able to target sufficient amounts of enzyme to the CNS in order to reverse the cognitive deficits associated with the disease. This thesis describes enzyme replacement as a means of therapy in MPS IIIA mice. Preliminary trials to evaluate ERT will be discussed in the next chapter. However, before therapy trials were initiated it was necessary to establish a test to quantitatively assess cognitive function in mice. Such a test must be able to show that therapies under evaluation were successful, or otherwise, in preventing the neurological deterioration characteristic of the MPS IIIA mice.

A number of maze procedures have been established to study cognitive function in animals, including radial arm mazes, T and Y mazes, and passive avoidance paradigms. Professor Richard Morris (1984) devised the Morris Water Maze (MWM) test as a behavioural task that assesses spatial learning abilities in rodents. Spatial learning refers to an animal's capacity to learn the location of a reward, such as the escape platform in the MWM. Briefly, mice are placed in a pool of water from which they can escape via a submerged platform hidden from view of the mouse; the test is conducted over 12 consecutive days in which the mice are trained to learn and remember the location of the platform. The MWM has two major advantages over other mazes: firstly the mouse wants to escape and, secondly, there are no local cues in water, for example Scent cues present in other mazes (Hodges, 1996).

The MWM has been successfully used in a mouse model for MPS VII, and was shown to generate data to discriminate between treated and untreated MPS VII mice enzyme and normal mice (O'Connor et al., 1998).

Tests to measure cognitive function have not previously been performed on the MPS IIIA mice. Thus, the two major aims of experiments described in this chapter were (1) to evaluate the efficacy of the MWM as a suitable test of cognitive function in normal mice versus MPS IIIA mice, and (2) to discover the age at which the MPS IIIA mice become neurologically impaired when compared to normal mice.

To meet these aims four MPS IIIA mice and four normal mice, age-matched and sex-matched underwent the MWM at seven-weeks, 15-weeks, 20-weeks and 28-weeks of age.

A description of the equipment needed to perform the MWM test, and the testing strategy is outlined in detail below.

5.2 SPECIFIC METHODS

5.2.1 EQUIPMENT

The pool

The MWM consists of a round wading pool, 143 cm in diameter and 40 cm high. The pool is filled 30 cm deep with water made opaque by the addition of skim milk powder (Figure 5.1A). The pool is arbitrarily divided into four quadrants, designated northwest (NW), northeast (NE), southwest (SW) and southeast (SE). The temperature of the water influences the motivation of the mice in the test. If the water is too cold the mice do not want to swim. However, if the water is too warm the mice want to remain in the pool and are less likely to search for the platform. A water temperature of between 21 to 25⁰C was used.

Figure 5.1: Maze Equipment.

A) The pool

A childrens' inflatable wading pool, 143 cm in was used. The pool was arbitrarily divided into four quadrants and filled approximately 30 cm deep with water at a temperature of 21-25°C; the water was made cloudy by the addition of skim milk powder. The pool was filled at the beginning of each day and drained upon completion.

B) The platform

The platform was made from clear perspex (Building and Maintenance Services, WCH). It was 29 cm tall with a circular base and top, both 10 cm in diameter. Sand was glued to the top and painted white.

A)



B)



The platform

The escape platform, made from clear plexiglass, was 10 cm in diameter and 29 cm high. To make the mice feel secure on the platform and to prevent them from sliding on a slippery surface, sand was glued to the top surface. The top surface was then painted white so that it was invisible in the water (Figure 5.1B). The platform was placed 10 cm from the wall of the pool and was submerged by 1 cm of water.

Cues

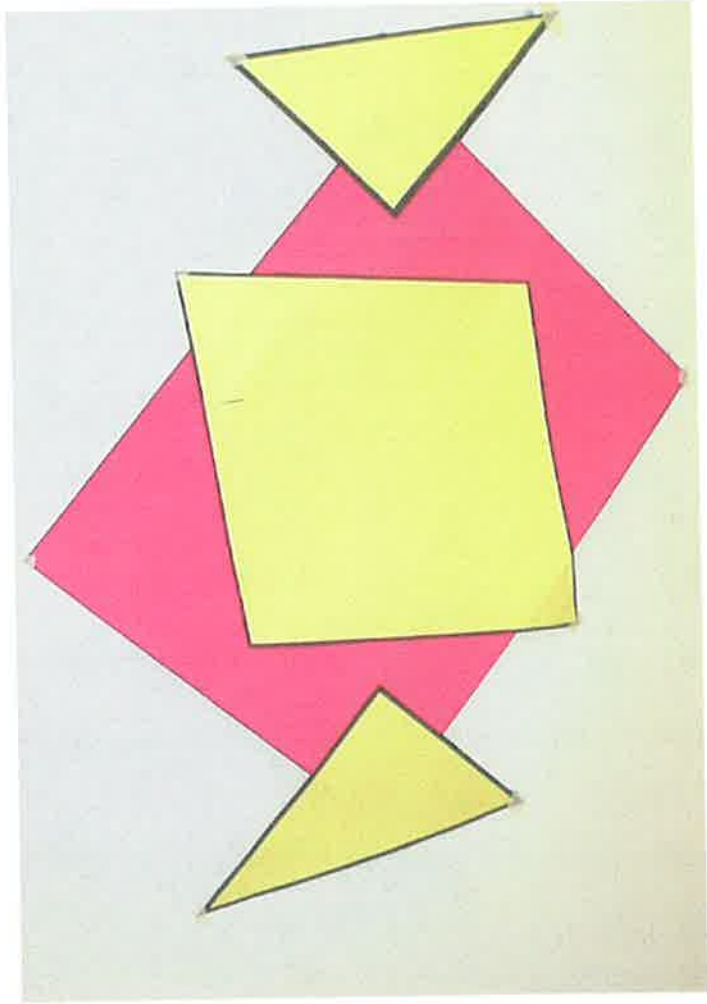
Mice solve the task of locating the platform by association; Cues are therefore an important part of the test. Theoretically, anything in the room can act as a cue to the mouse. The room utilised for the MWM test was 3.0 m x 2.5 m; it had bare white walls, a sink in the far right corner with a bin below, and a rectangular stainless steel bench along the eastern wall and, obviously, the handler. Additional external cues were made which consisted of two posters as shown in (Figure 5.2). It was important to ensure that the cues were visible from inside the pool, but not too close to the poolside.

Time and intervals of swimming

Mice were gently lowered into the water with their head pointing towards the poolside to minimise bias; the tail end was lowered in first so their head did not go under water. Mice underwent four trials on each day of testing; each trial was initiated from a different cardinal point. In the preliminary MWM experiments, where mice were tested at seven-weeks, 15-weeks, 20-weeks and 28-weeks, mice were allowed to search for 120-seconds, if the platform was not found in this time, they were guided to and allowed to remain on the platform for 10-seconds. In this case the time was recorded as the maximum time of 120-seconds (even though the platform was not found). If the mouse found the platform within this time, they were also allowed to remain on the platform for 10-seconds. After each trial the mouse was placed on a warming pad and towel-dried. A 60- to 80- second interval was allowed between mice in the trials.

Figure 5.2: Visual cues.

Aside from the features of the testing room, additional visual cues were made, consisting of simple abstract posters, which were placed on the walls and remained in the same position throughout testing procedures.



5.2.2 TESTING

Acclimatisation

All mice were acclimatised to the pool on the initial day of the test; they were handled in and out of their cages so they were comfortable with the handler and the room before swimming started. The mice were allowed to swim in the pool, which at this stage contained no milk powder or escape platform.

Acquisition phase

The acquisition phase is the initial phase of the testing and lasts for five-days. The platform is placed in the northwest (NW) quadrant and the time taken, for the mouse introduced to the pool from one of the four cardinal points to find the platform is measured. This phase requires the mice to learn the position of the platform and remember it on subsequent trials.

Probe phase

The probe phase is performed on day six after the acquisition phase. The platform is removed from the pool and the time the mouse spends in each quadrant is measured. This eliminates a 'lucky bump' from the test; a mouse that remembers the location of the platform from the acquisition phase will spend most of its time in this quadrant (NW) searching for it. This suggests that the mouse has solved the task of learning and remembering the location of the platform rather than hitting into it by chance.

Re-learning phase

The relearning phase is performed on days seven- to 10 of the test. The platform is returned to the NW quadrant and the time taken to find the platform is measured. This phase of the test requires the mouse to remember the initial location of the platform.

Reversal phase

The reversal phase is performed on days 11-12 of the test. The platform is relocated to the opposite quadrant, the southeast (SE) quadrant. The time taken to find the platform is measured. The reversal phase tests the ability of the mouse to learn and remember the new location of the platform.

Visible platform test

The visible platform test is performed after the MWM testing period. The platform is slightly raised above the water and milk powder is not added to the water, making the platform visible to the mice from inside the pool. The time taken to find the platform is measured. The visible platform test, tests for the effect of any physical disabilities in a mouse on its ability to reach the platform.

5.3 RESULTS

5.3.1 MWM AT SEVEN-WEEKS OF AGE

On the first day of the acquisition phase, in mice at seven-weeks of age, there was no difference in the time it took for normal or MPS IIIA mice to find the platform. Most mice in both groups (normal and MPS IIIA) were unable to locate the platform in the first two trials in the required time and so were guided to it (Figure 5.3A). By the second day the mice in both groups had decreased their search times by an average of about 10-sec for the normal mice and 20-sec for the MPS IIIA mice (Figure 5.3E). Toward the end of the testing the mice were observed to be more relaxed and at ease with the task than they were on day one. A decline in latencies continued for the remainder of the acquisition phase. In fact by the final day of the acquisition phase all mice in both groups were locating the platform within an average of 20-sec, a 50-sec reduction in search times from day one of the acquisition phase. As observed in Figure 5.3A, by day five of the acquisition phase search times for the individual trials had become relatively constant for all mice in comparison to the somewhat erratic pattern observed in the earlier days. No significant difference in performance was observed between either of the groups throughout the entire acquisition phase.

During the probe phase all of the mice tested spent most of their time in the target quadrant (NW), approximately 50-sec for normal mice and 40-sec for MPS IIIA mice (Figure 5.3B). This indicated that mice from both groups had learnt and remembered the location of the platform from the acquisition phase.

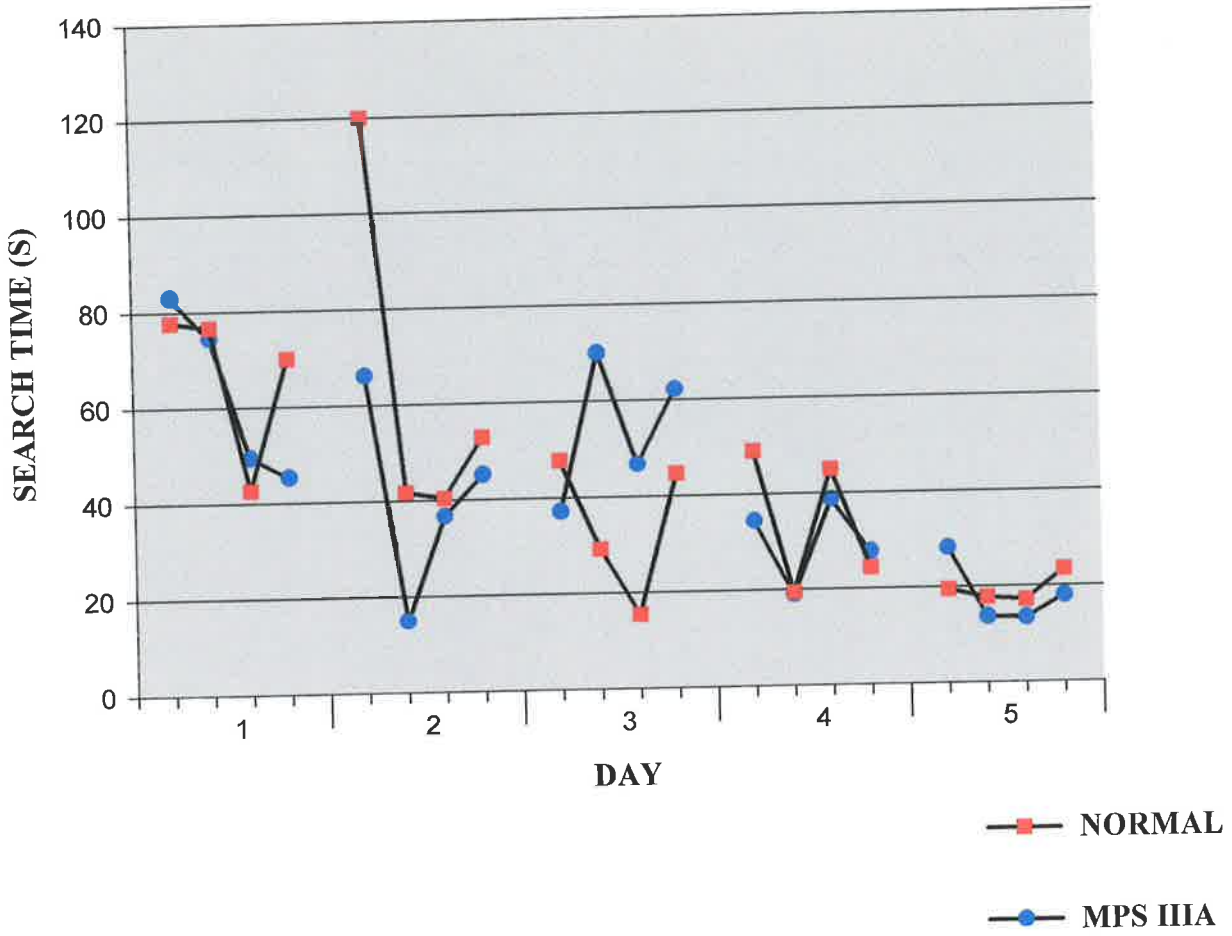
The platform was replaced to the NW quadrant during the re-learning phase and, all mice were successful in locating the platform in less than 20-sec; no hesitation was observed, and once placed in the pool the mice would head straight for the NW quadrant and onto the platform. Search times were reasonably uniform over the four trials on each day of testing, similar to that observed on the final day of the acquisition phase (Figure 5.3C). Mice in both groups had relatively decreased search times in the relearning phase when

Figure 5.3: MWM at seven-weeks of age.

- A) Mean latency per trial for both groups during the acquisition phase of the MWM test (testing days one-five). From days one-five, the platform was placed in the NW quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- B) The probe test was performed on day six immediately after the acquisition phase. The time the mice spent in the target quadrant is shown. Blue bars indicate the time spent in the target quadrant and yellow bars represent the average time spent in each of the remaining three quadrants.
- C) Mean latency per trial for both groups during the re-learning phase of the MWM test (testing days seven-10). From days seven-10, the platform was replaced in the original quadrant (NW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- D) Mean latency per trial for both groups during the reversal phase of the MWM test (testing days 11-12). From days 11-12, the platform was relocated to the SE quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- E) Mean latency per day for both groups during each phase of the MWM test. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4). Standard deviations are shown.

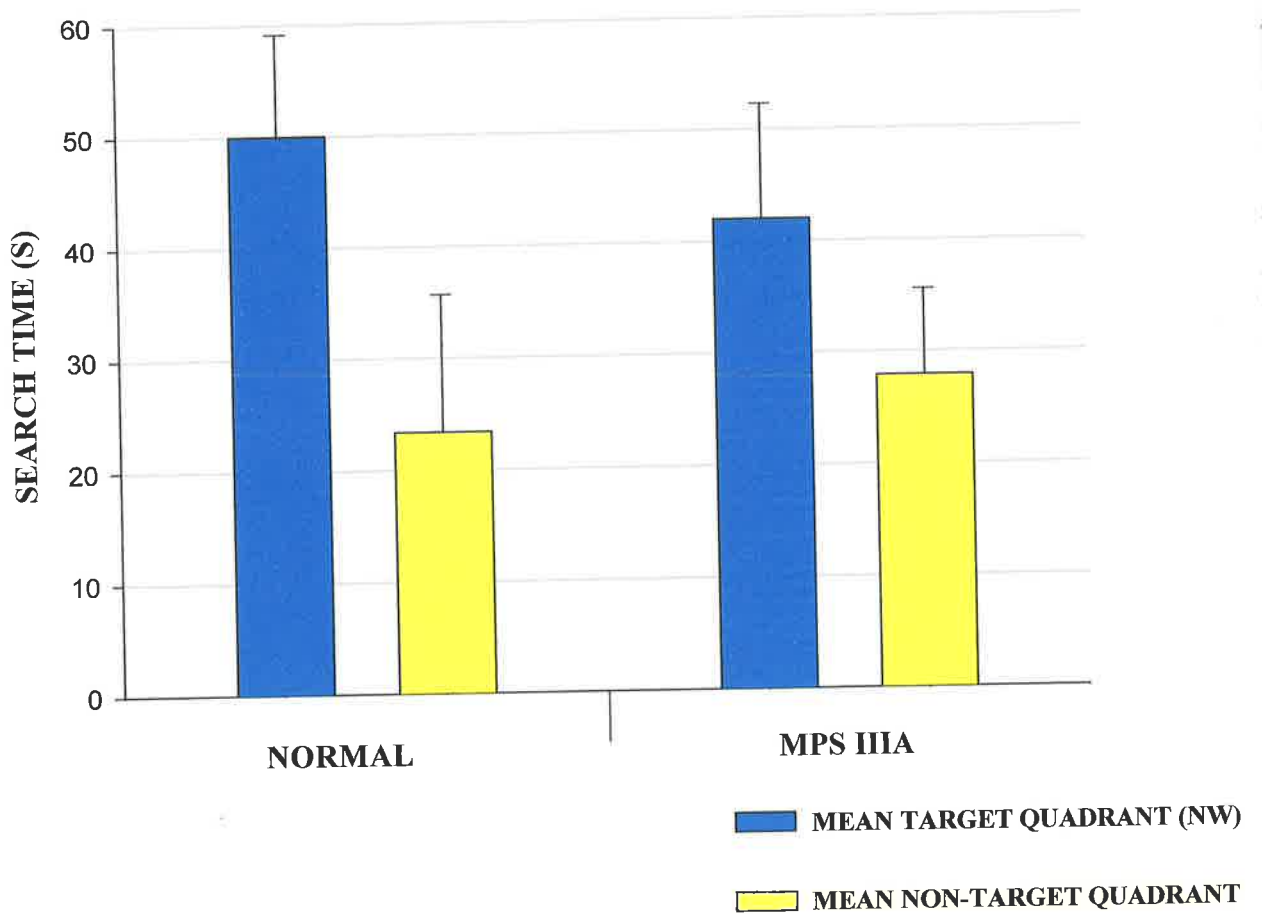
A)

ACQUISITION PHASE



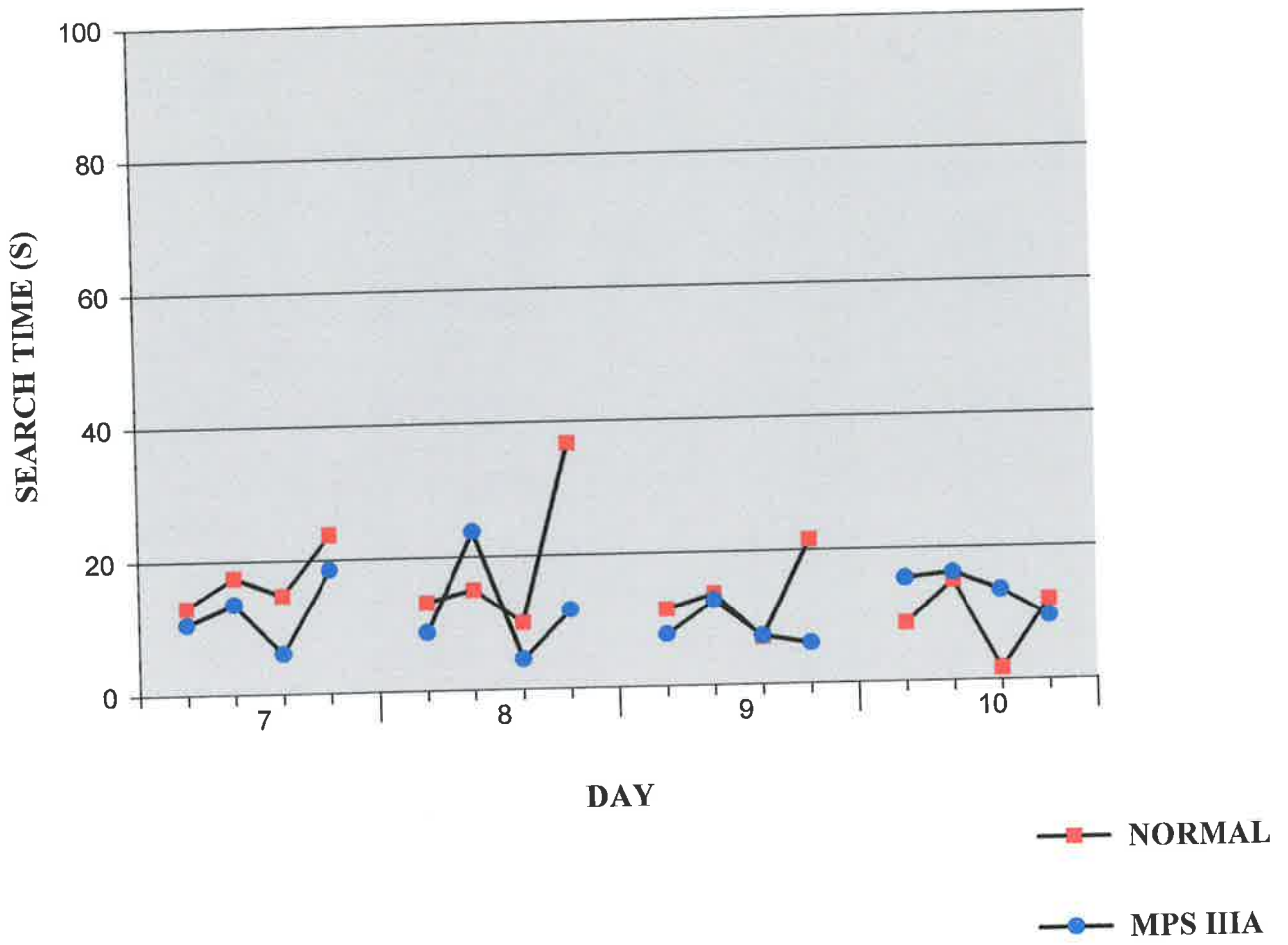
B)

PROBE PHASE



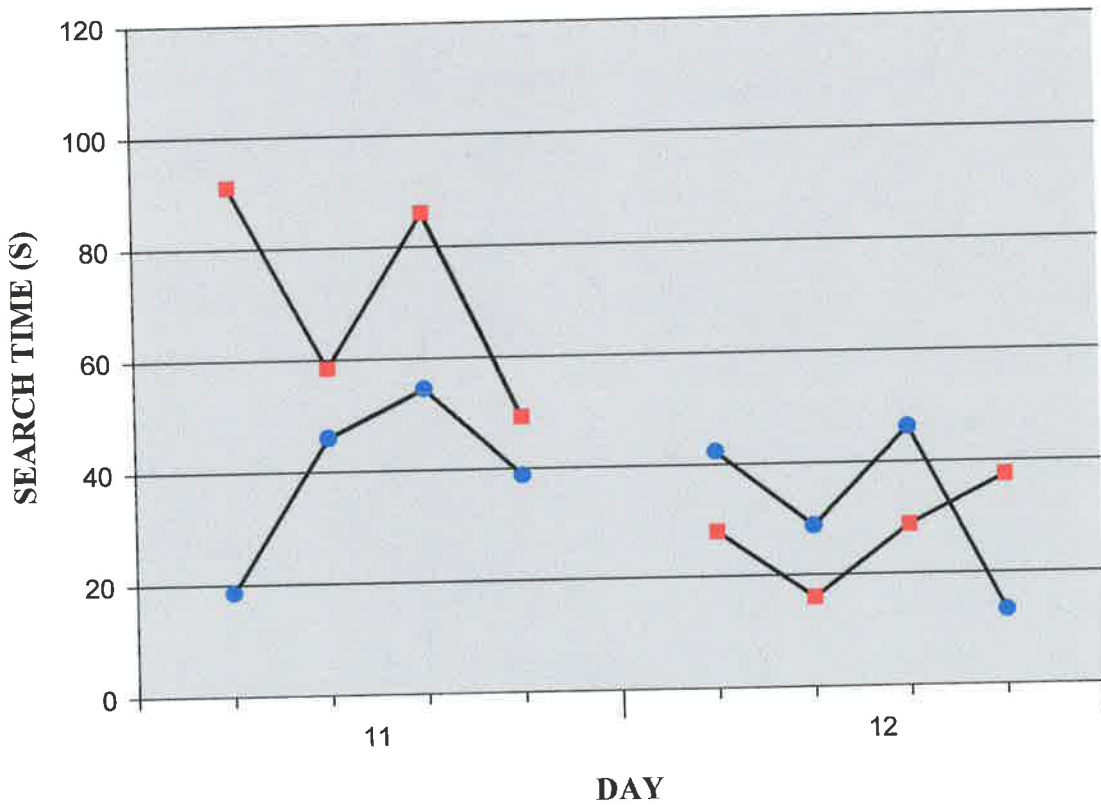
C)

RE-LEARNING PHASE



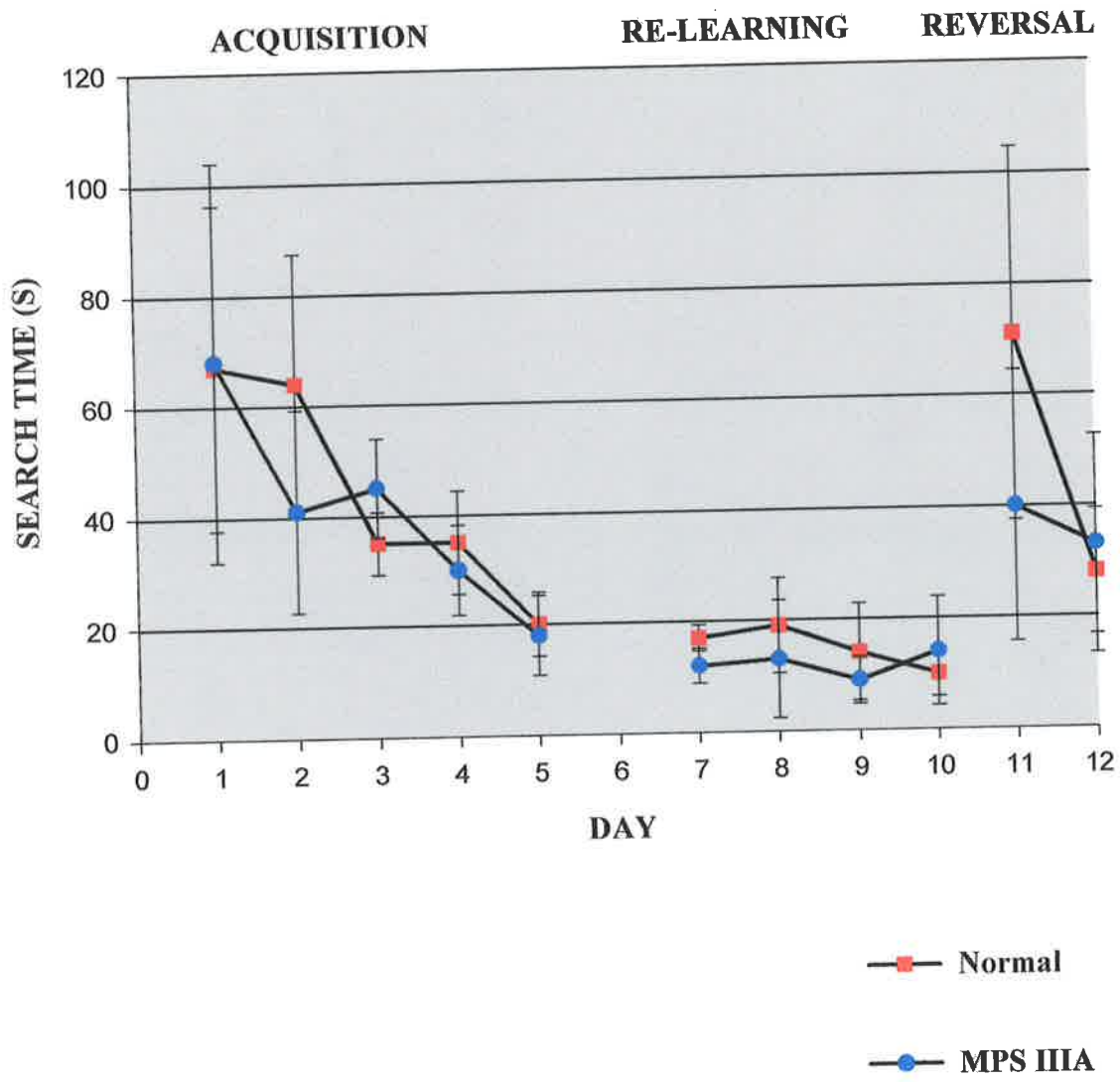
D)

REVERSAL PHASE



E)

MORRIS WATER MAZE AT SEVEN-WEEKS OF AGE



compared to the search times obtained during the acquisition phase (Figure 5.3E). Once again no significant difference in latency was observed between the normal and MPS IIIA mice.

The relocation of the platform to the SE quadrant during the final phase of MWM, the reversal phase, resulted in increased search times with mice spending most of their time in the NW quadrant searching for the platform. Search times over the four trials were erratic and reminiscent of what was observed on the first day of the acquisition phase (Figure 5.3D). Mice had decreased their search times by the second day indicating that they had learnt and remembered the new location of the escape platform and search times had once again become more constant (Figure 5.3E). Both normal and MPS IIIA mice performed this phase with equal competency.

5.3.2 MWM AT 15-WEEKS OF AGE

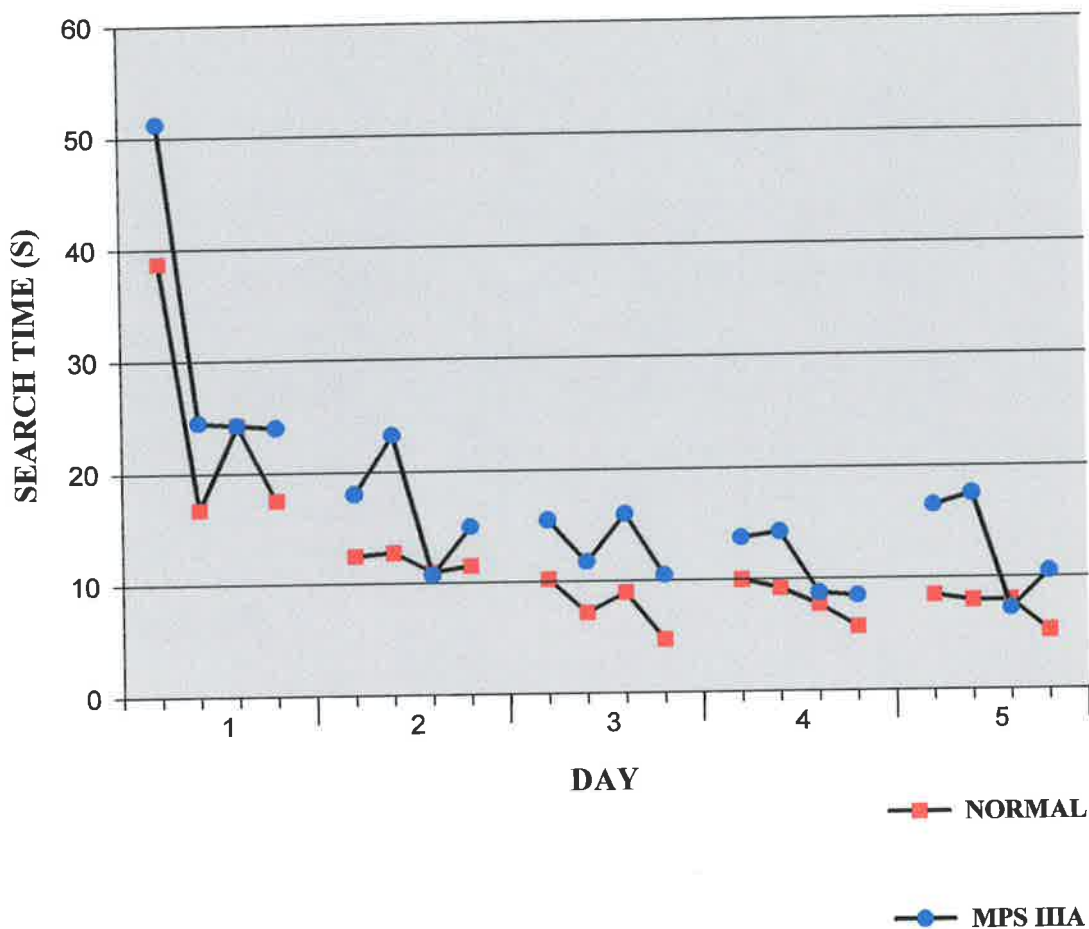
The same group of normal and MPS IIIA mice were re-tested at 15-weeks of age. It only took the normal mice an average of 25-sec to locate the platform, compared with 31-sec for the MPS IIIA mice on day one of the acquisition phase (Figure 5.4E). From the beginning of testing the mice appeared to be much more relaxed about the task at hand than they did at seven-weeks of age. The task is no longer novel to them and they have some acquired knowledge of what needs to be done. By day two of the acquisition phase the normal mice had decreased their search times to an average of about 10-sec and this performance was constant for the remainder of the phase. The MPS IIIA mice had also decreased their search times to an average of approximately 15-sec for the remaining four-days of the acquisition phase. Search times throughout individual trials were relatively constant in both groups of mice (Figure 5.4A). Overall there was not as significant a difference in search times observed between the acquisition phase and the relearning phase as was seen at seven-weeks of age (Figure 5.4E). A slight, yet significant difference was observed between the two groups, with the normal mice performing on average 5-sec quicker than the MPS IIIA mice.

Figure 5.4: MWM at 15-weeks of age.

- A) Mean latency per trial for both groups during the acquisition phase of the MWM test (testing days one-five). From days one-five, the platform was placed in the NW quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- B) The probe test was performed on day six immediately after the acquisition phase. The time the mice spent in the target quadrant is shown. Blue bars indicate the time spent in the target quadrant and yellow bars represent the average time spent in each of the remaining three quadrants.
- C) Mean latency per trial for both groups during the re-learning phase of the MWM test (testing days seven-10). From days seven-10, the platform was replaced in the original quadrant (NW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- D) Mean latency per trial for both groups during the reversal phase of the MWM test (testing days 11-12). From days 11-12, the platform was relocated to the SE quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- E) Mean latency per day for both groups during each phase of the MWM test. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4). Standard deviations are shown.

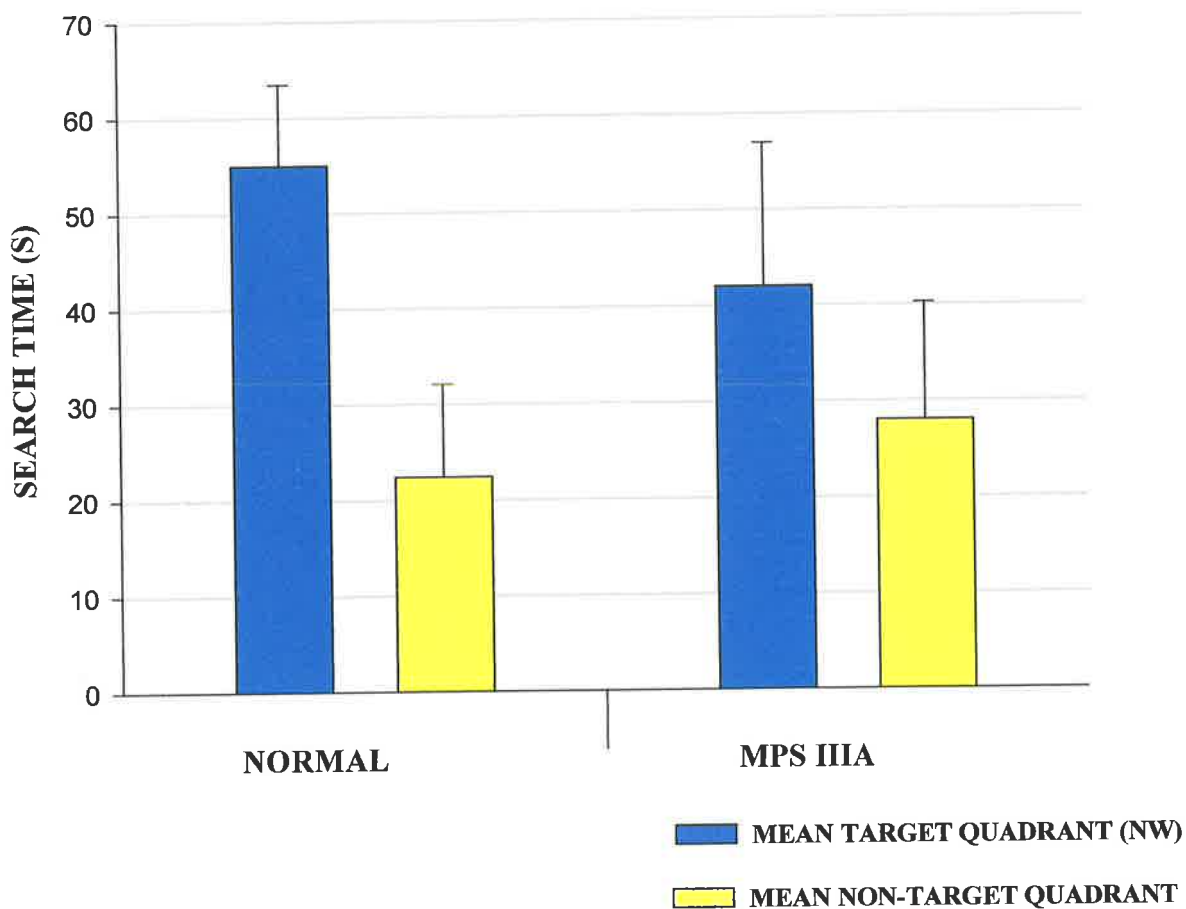
A)

ACQUISITION PHASE



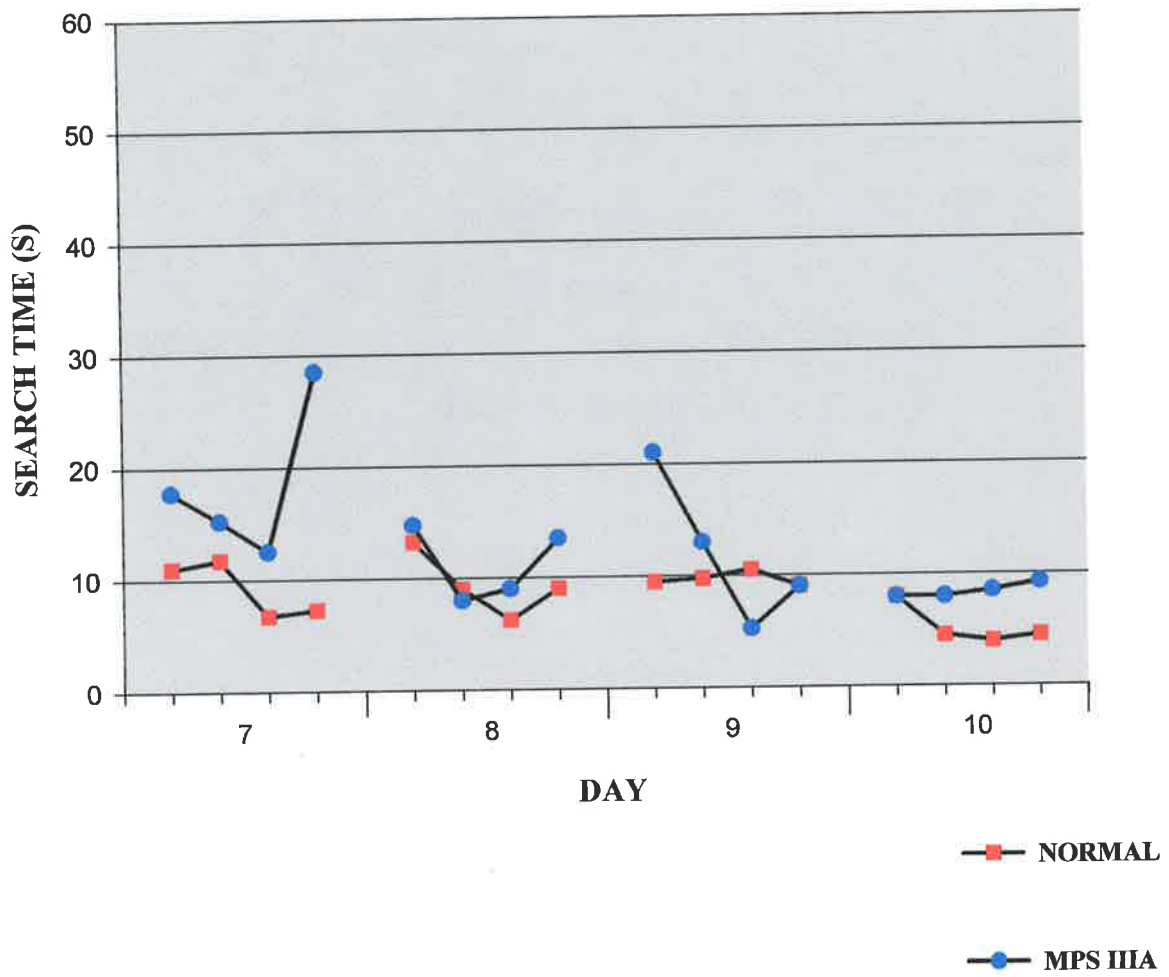
B)

PROBE PHASE



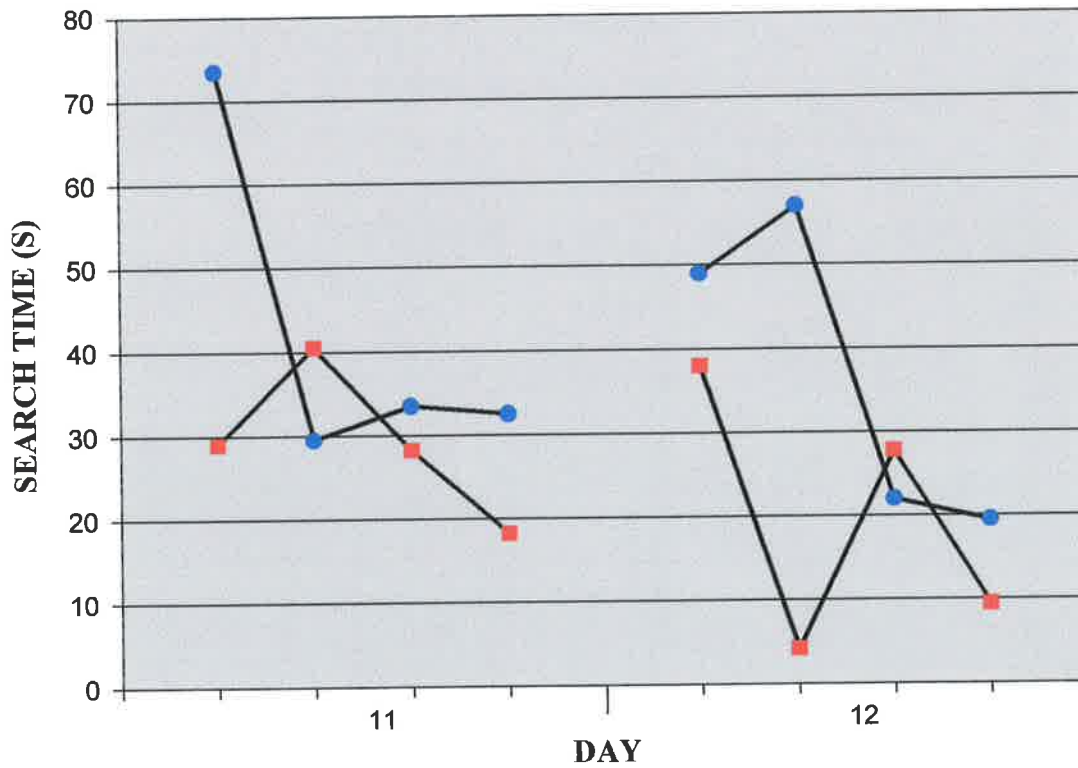
C)

RE-LEARNING PHASE



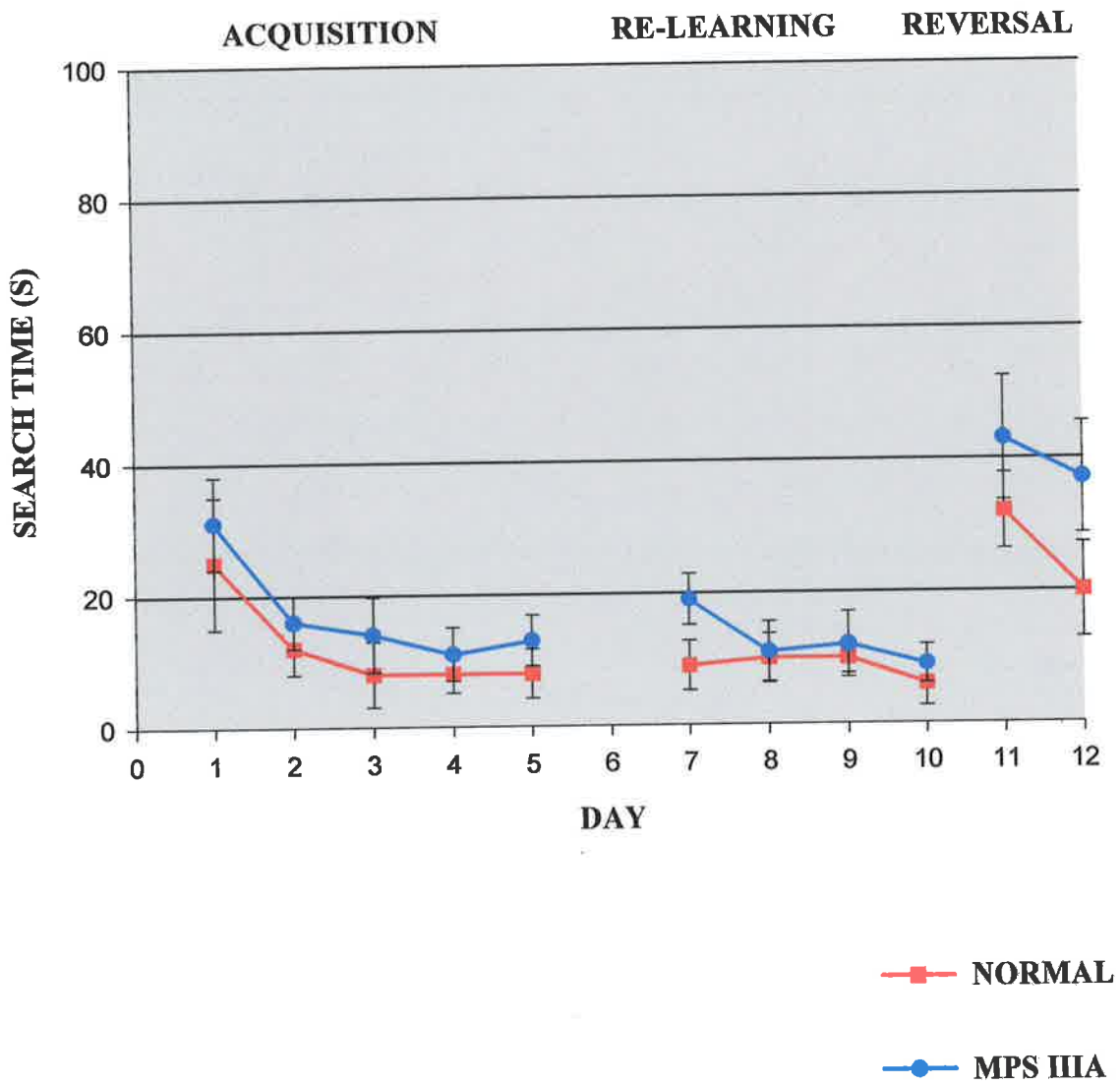
D)

REVERSAL PHASE



E)

MORRIS WATER MAZE AT 15-WEEKS OF AGE



The normal mice spent an average of 55-sec in the target quadrant during the probe phase and a mean of 22-sec in the other three quadrants (Figure 5.4B). In contrast the MPS IIIA mice spent an average of 40-sec in the NW quadrant and a mean of 28 sec in the non-target quadrants (Figure 5.4B). It would appear that the normal mice have a better knowledge of the location of the platform than the MPS IIIA mice. However, the MPS IIIA mice still display reasonable knowledge of the position of the platform.

During the four-days of the re-learning phase the normal mice were extremely competent in finding the platform; on average all mice displayed search times less than 10-sec, for each trial over the successive days. On the first day of the re-learning phase MPS IIIA mice took twice as long to find the platform when compared with the normal mice (Figure 5.4C). The MPS IIIA mice did decrease their search time for the remainder of the phase, but were overall not as efficient at finding the platform as were the normal mice (Figure 5.4E).

As observed at seven-weeks of age, the first day of the reversal phase resulted in much longer search times for both groups of mice; all mice had decreased their search times by the second day (Figures 5.4D, E).

5.3.3 MWM AT 20-WEEKS OF AGE

The same group of mice were re-tested at 20-weeks of age. A significant difference (determined by a repeated measures analysis, see Materials and Methods Section 2.2.23) in search times was observed between normal and MPS IIIA mice throughout the acquisition phase at 20-weeks of age (Figures 5.5A, E). On average it took the MPS IIIA mice approximately 20-sec longer to locate the platform than the normal mice. From observing the MPS IIIA mice, it became obvious that they were unsure of the location of the platform as they were swimming around the pool in circles. This differs from the normal mice who efficiently headed straight to the NW quadrant and hence onto the platform. This observation is emphasised in Figure 5.5A, which shows that the MPS IIIA mice have erratic search times between individual trials whereas the normal mice have

more uniform search times. Once again a decrease in search time on the first day of the acquisition phase was noticed in the normal mice from 15-weeks of age to 20-weeks of age. The MPS IIIA mice performed significantly worse on the first day of the acquisition phase at 20-weeks of age than they did at 15-weeks of age. The normal mice had once again slightly improved in their search times.

During the probe phase the normal mice spent approximately 40% of their time in the target quadrant and an average of only 15% of their time was spent in the other three quadrants. In comparison the MPS IIIA mice spent an equal amount of time in all four quadrants, not discriminating between the target and non-target quadrants (Figure 5.5B).

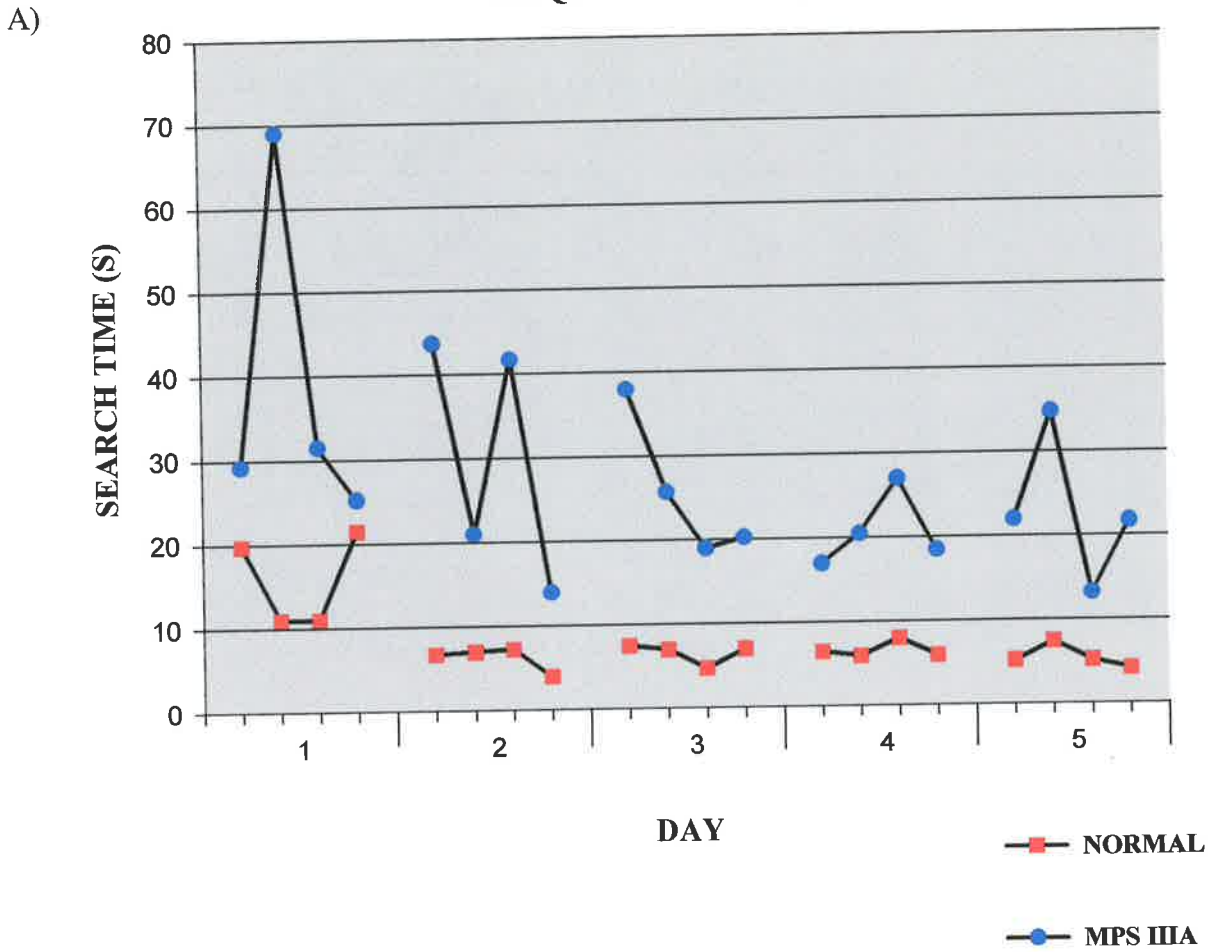
A similar trend was observed during the re-learning phase; the normal mice maintained search times under-10-sec, whilst it took the MPS IIIA mice an average of 25-sec to find the platform (Figures 5.5C, D)

An important observation was made during the reversal phase, when the platform was removed to an opposite quadrant (SE). As was displayed at seven-weeks and 15-weeks of age, the normal mice had an increased latency to find the platform on day one of this phase. It took them 50-sec to locate the platform, opposed to an average of around 10-sec in both the acquisition and re-learning phases (Figures 5.5D, E). This is because they spent the majority of search time in the NW quadrant which they had learnt was the location of the platform. However, the re-location of the platform to the opposite quadrant of the pool did not appear to affect the search times for the MPS IIIA mice. They spent an average of approximately 30- sec searching, which is comparable to the latency recorded during the acquisition and re-learning phases. By day two of the re-learning phase the normal mice had decreased their search times by 30-sec; no difference in search time was recorded between days one and two for the MPS IIIA mice.

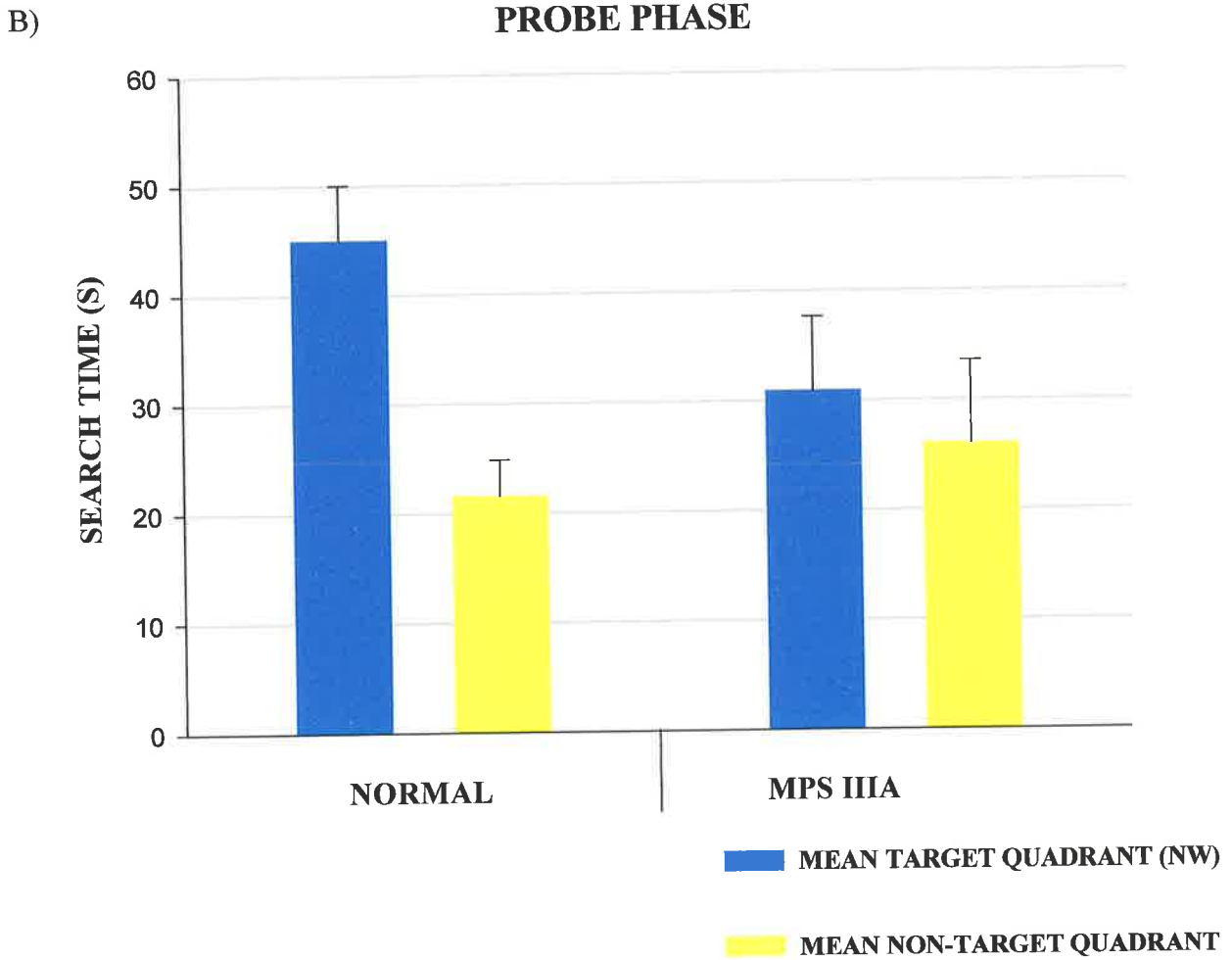
Figure 5.5: MWM at 20-weeks of age.

- A) Mean latency per trial for both groups during the acquisition phase of the MWM test (testing days one-five). From days one-five, the platform was placed in the NW quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- B) The probe test was performed on day six immediately after the acquisition phase. The time the mice spent in the target quadrant is shown. Blue bars indicate the time spent in the target quadrant and yellow bars represent the average time spent in each of the remaining three quadrants.
- C) Mean latency per trial for both groups during the re-learning phase of the MWM test (testing days seven-10). From days seven-10, the platform was replaced in the original quadrant (NW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- D) Mean latency per trial for both groups during the reversal phase of the MWM test (testing days 11-12). From days 11-12, the platform was relocated to the SE quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- E) Mean latency per day for both groups during each phase of the MWM test. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4). Standard deviations are shown. *, Significant ($P < 0.01$) difference in latency between normal and MPS IIIA mice (see Materials and Methods, Section 2.2.23 for description).

ACQUISITION PHASE

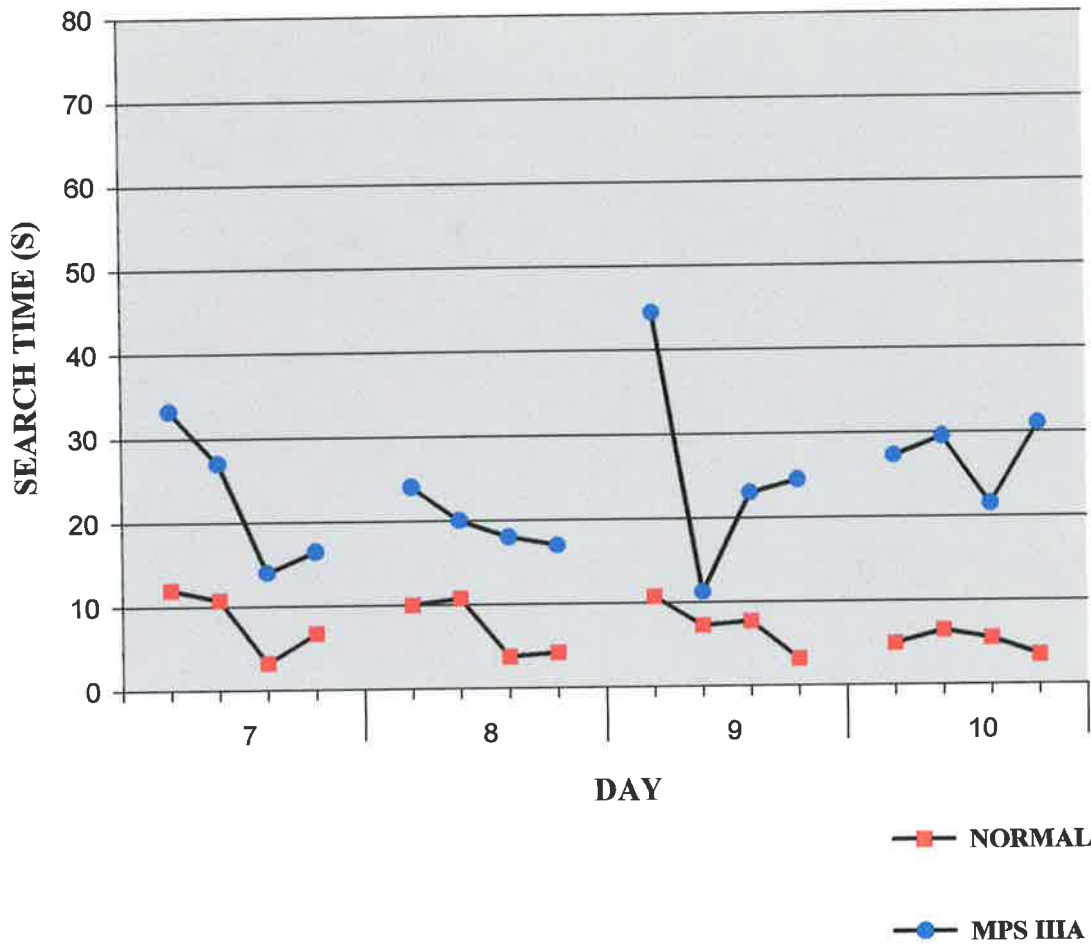


PROBE PHASE



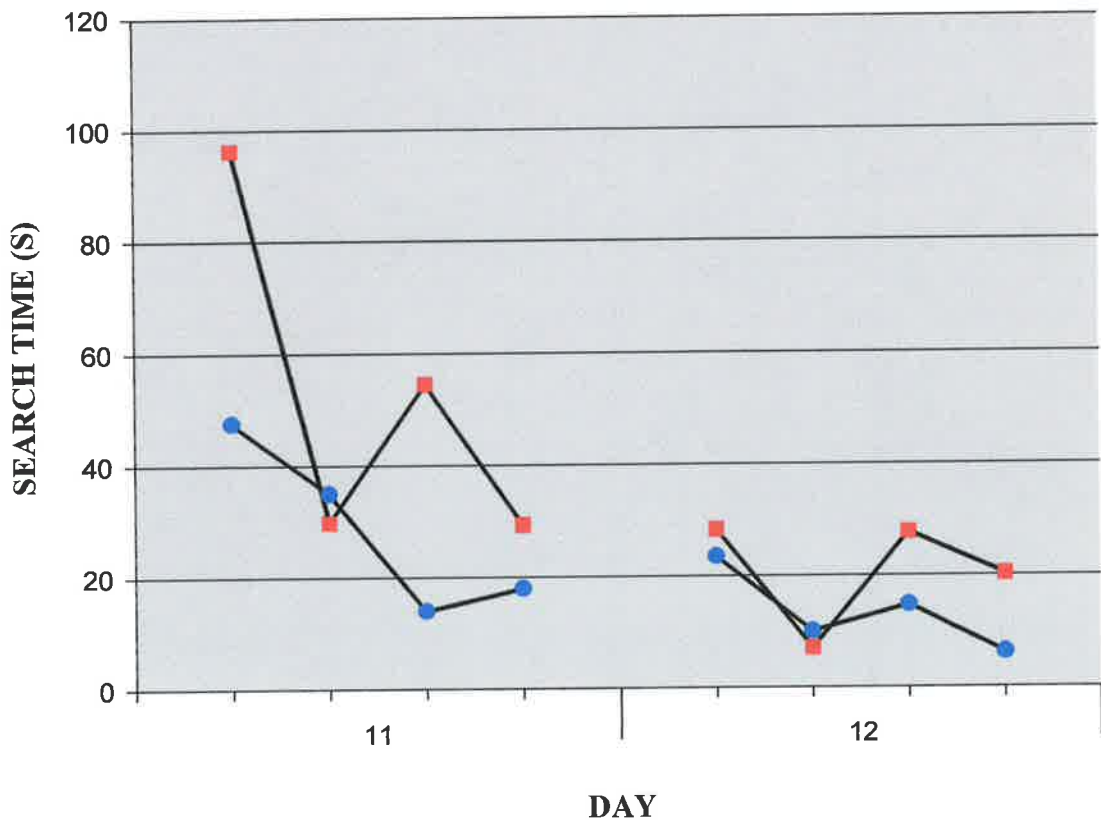
C)

RE-LEARNING PHASE



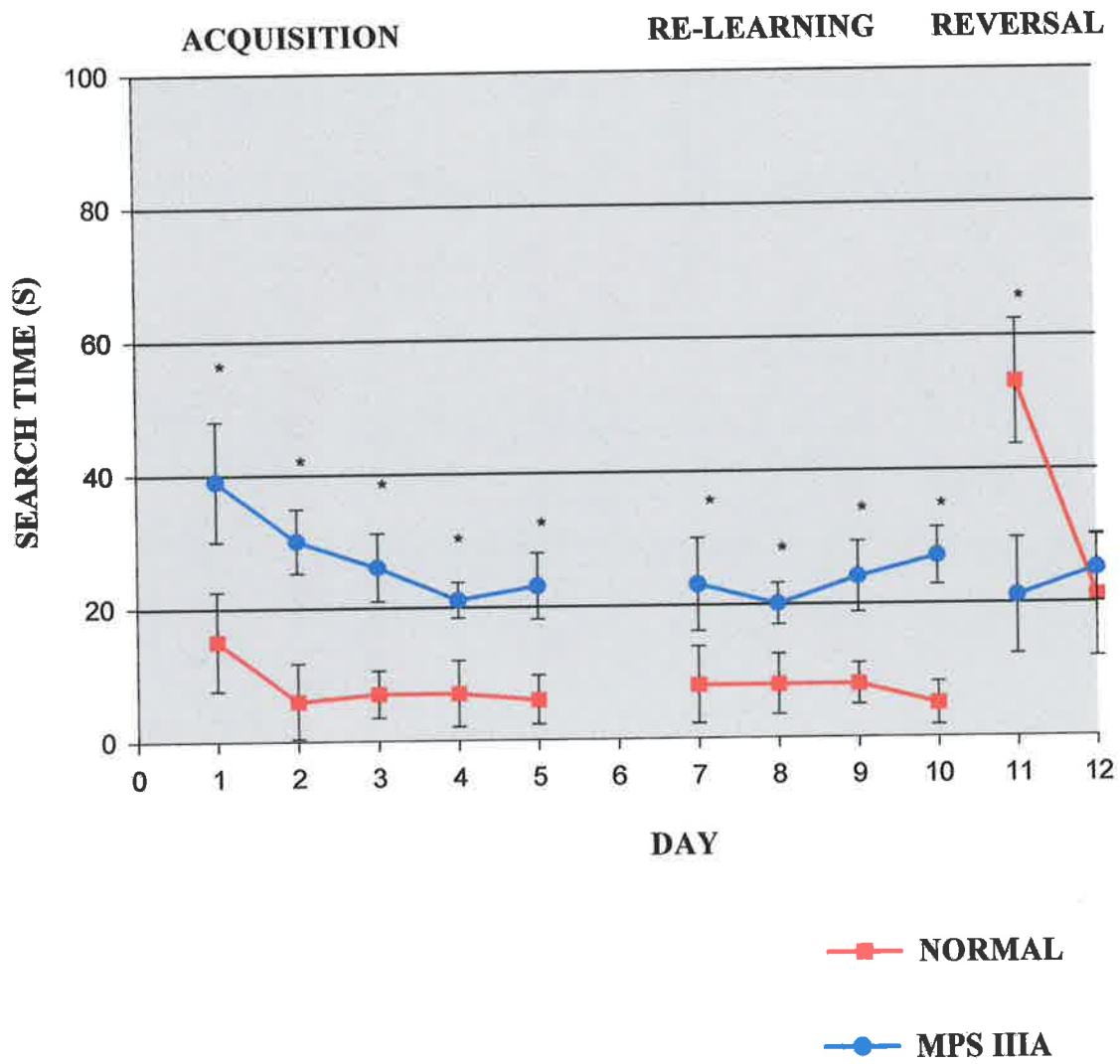
D)

REVERSAL PHASE



E)

MORRIS WATER MAZE AT 20-WEEKS OF AGE



5.3.4 MWM AT 28-WEEKS OF AGE

The same group of mice were re-tested at 28-weeks of age. The results obtained at this age are similar to those from the 20-week data. During both the acquisition and re-learning phases the normal mice located the platform in an average of six-to 12- sec. In comparison, it took the MPS IIIA mice an average of 25-to 35-sec to find the platform (Figures 5.6A, E). As was observed at 20-weeks of age, the MPS IIIA mice appeared to be randomly swimming around the pool until the platform was encountered. From Figure 5.6E no significant difference was observed between the acquisition phase and re-learning phase between the individual groups.

During the probe phase the normal mice spent the majority of thier time in the target quadrant, whereas the MPS IIIA mice spent as much time in the non-target quadrants as they did in the target quadrant (Figure 5.6B)

Once again search times recorded in the reversal phase for the MPS IIIA mice had not altered from those recorded during the acquisition and re-learning phases. The normal mice had increased search times on day one of the reversal phase, but had decreased times by the second day (Figures 5.6D, E)

5.3.5 MWM AT 20-WEEKS OF AGE ON NAIVE MICE

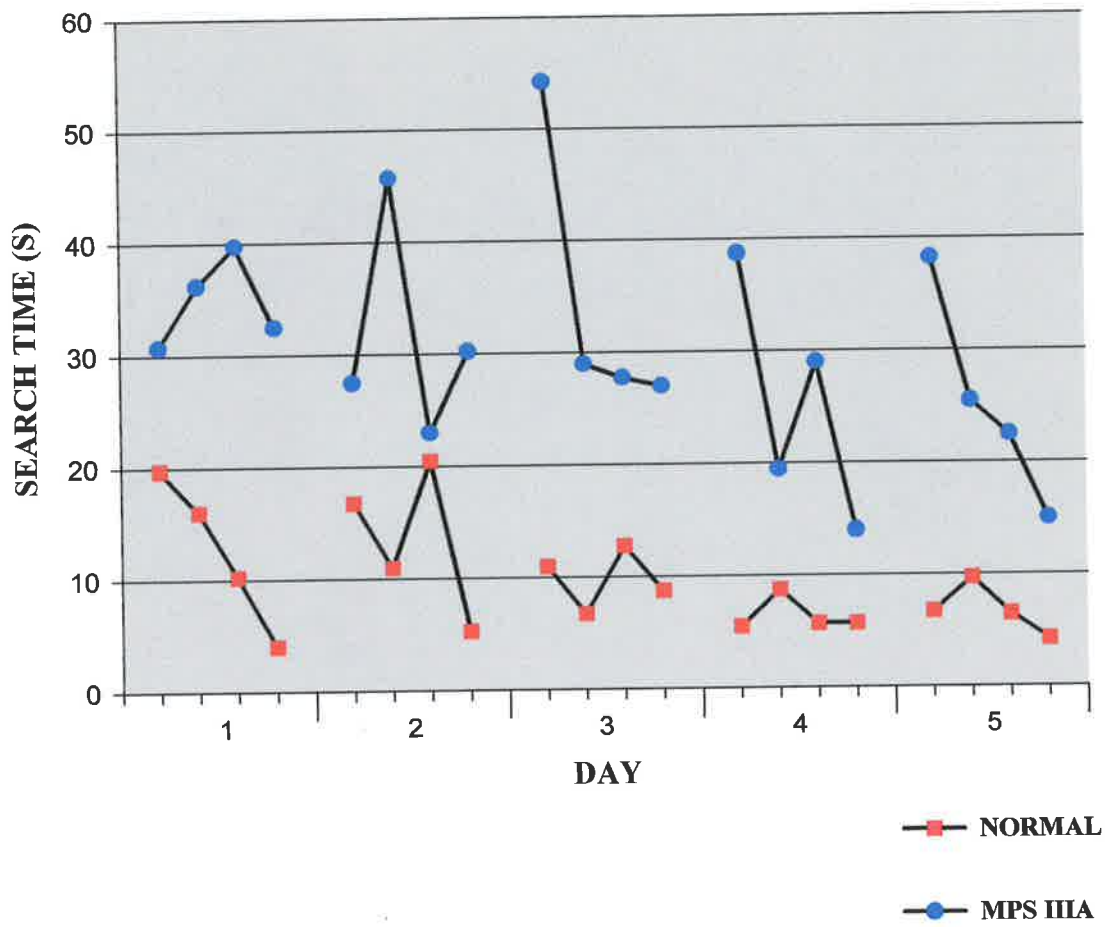
From the studies undertaken with the normal and MPS IIIA mice at the four time points it was evident that by 20-weeks of age MPS IIIA mice had lost the ability to learn and remember the location of the platform. It was also concluded from these preliminary studies that an allocated search time of 120-seconds was unnecessary, as both groups of mice were able to locate the platform in well under this time. Also, on day six of the probe phase, when mice swam four trials for the maximum 120-seconds, signs of lethargy towards the end of testing was demonstrated by the mice, indicating that this length of time was too long. A decrease in allocated search time such as 90-seconds might be more appropriate.

Figure 5.6: MWM at 28-weeks of age.

- A) Mean latency per trial for both groups during the acquisition phase of the MWM test (testing days one-five). From days one-five, the platform was placed in the NW quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- B) The probe test was performed on day six immediately after the acquisition phase. The time the mice spent in the target quadrant is shown. Blue bars indicate the time spent in the target quadrant and yellow bars represent the average time spent in each of the remaining three quadrants.
- C) Mean latency per trial for both groups during the re-learning phase of the MWM test (testing days seven-ten). From days seven-10, the platform was replaced in the original quadrant (NW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- D) Mean latency per trial for both groups during the reversal phase of the MWM test (testing days 11-12). From days 11-12, the platform was relocated to the SE quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4).
- E) Mean latency per day for both groups during each phase of the MWM test. Red lines represent normal mice (n=4) and blue lines represent MPS IIIA mice (n=4). Standard deviations are shown. *, Significant ($P < 0.01$) difference in latency between normal and MPS IIIA mice (see Materials and Methods, Section 2.2.23 for description).

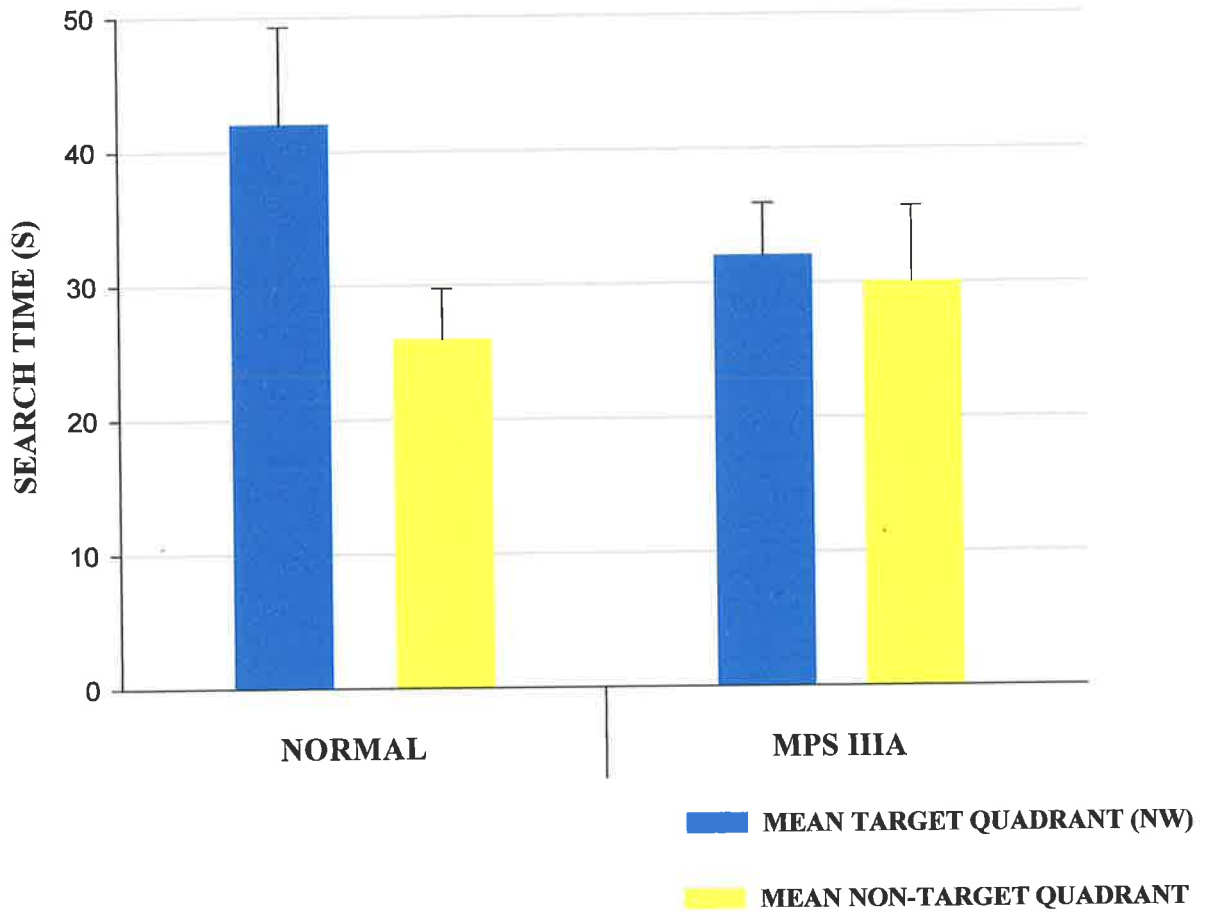
A)

ACQUISITION PHASE



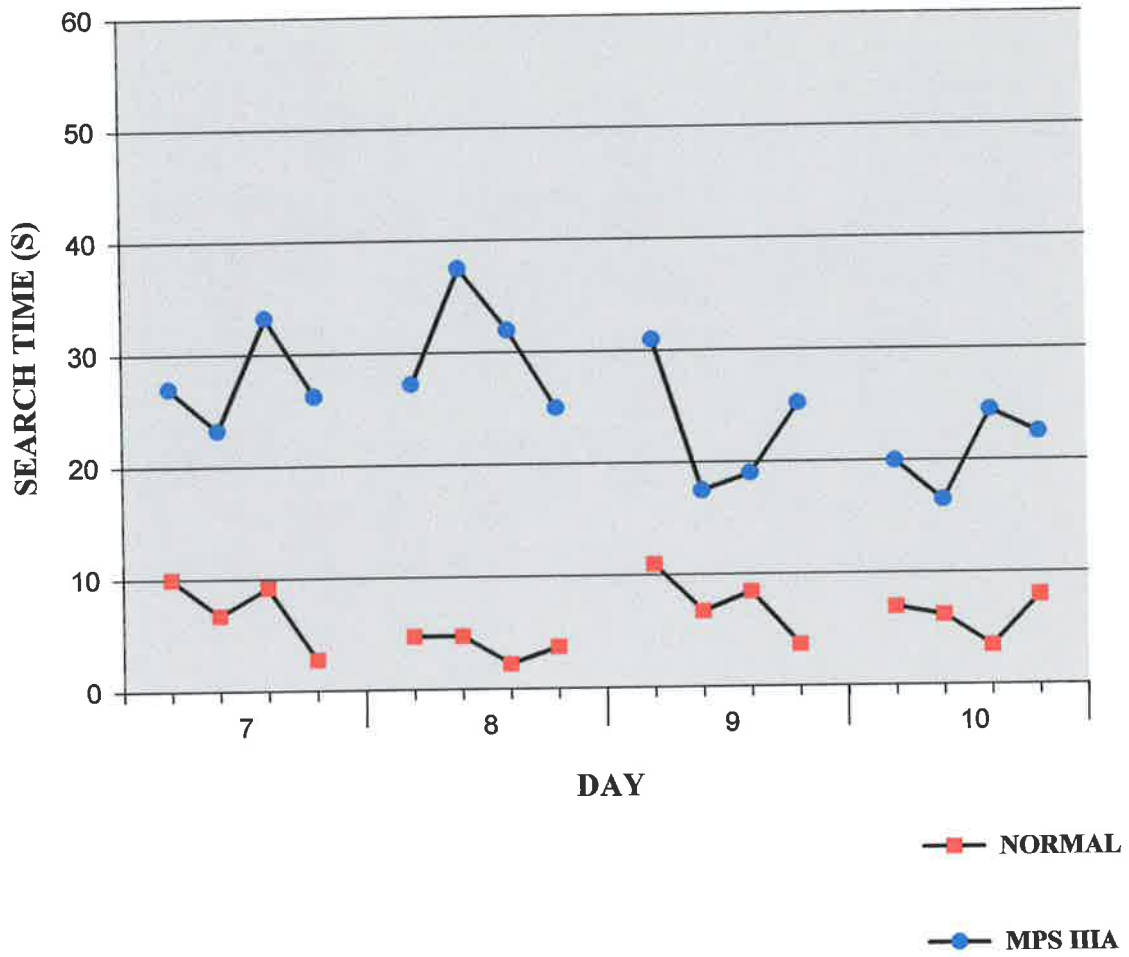
B)

PROBE PHASE



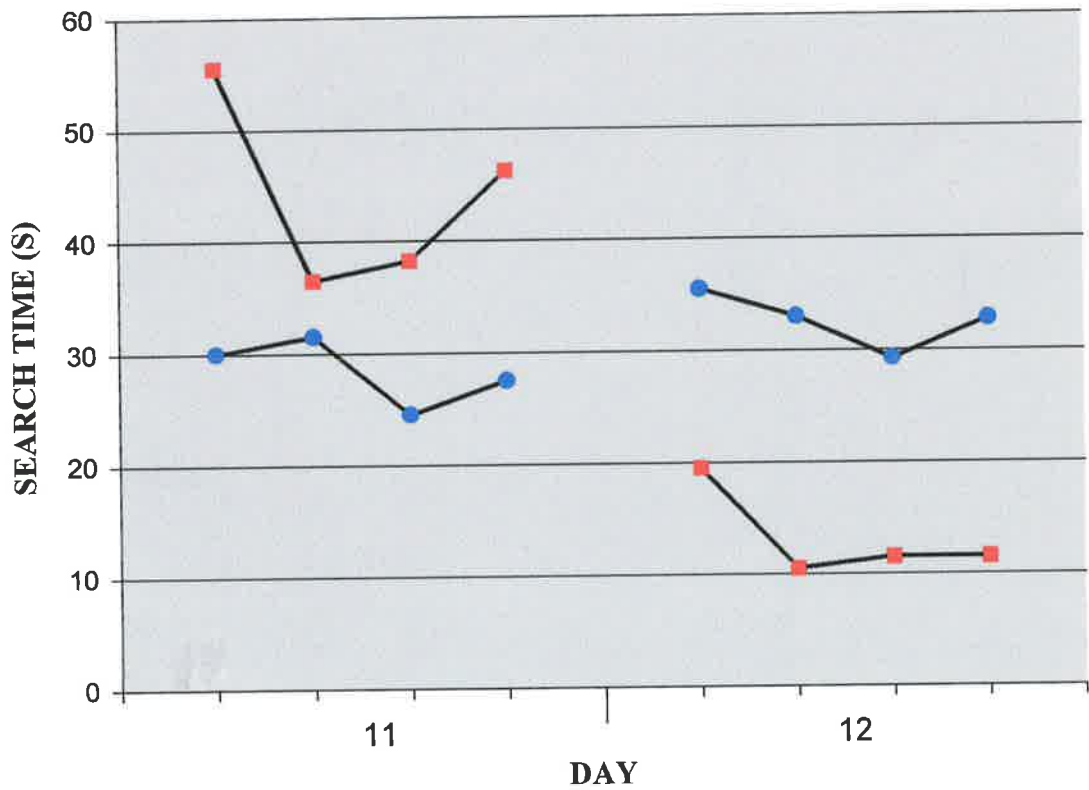
C)

RE-LEARNING PHASE



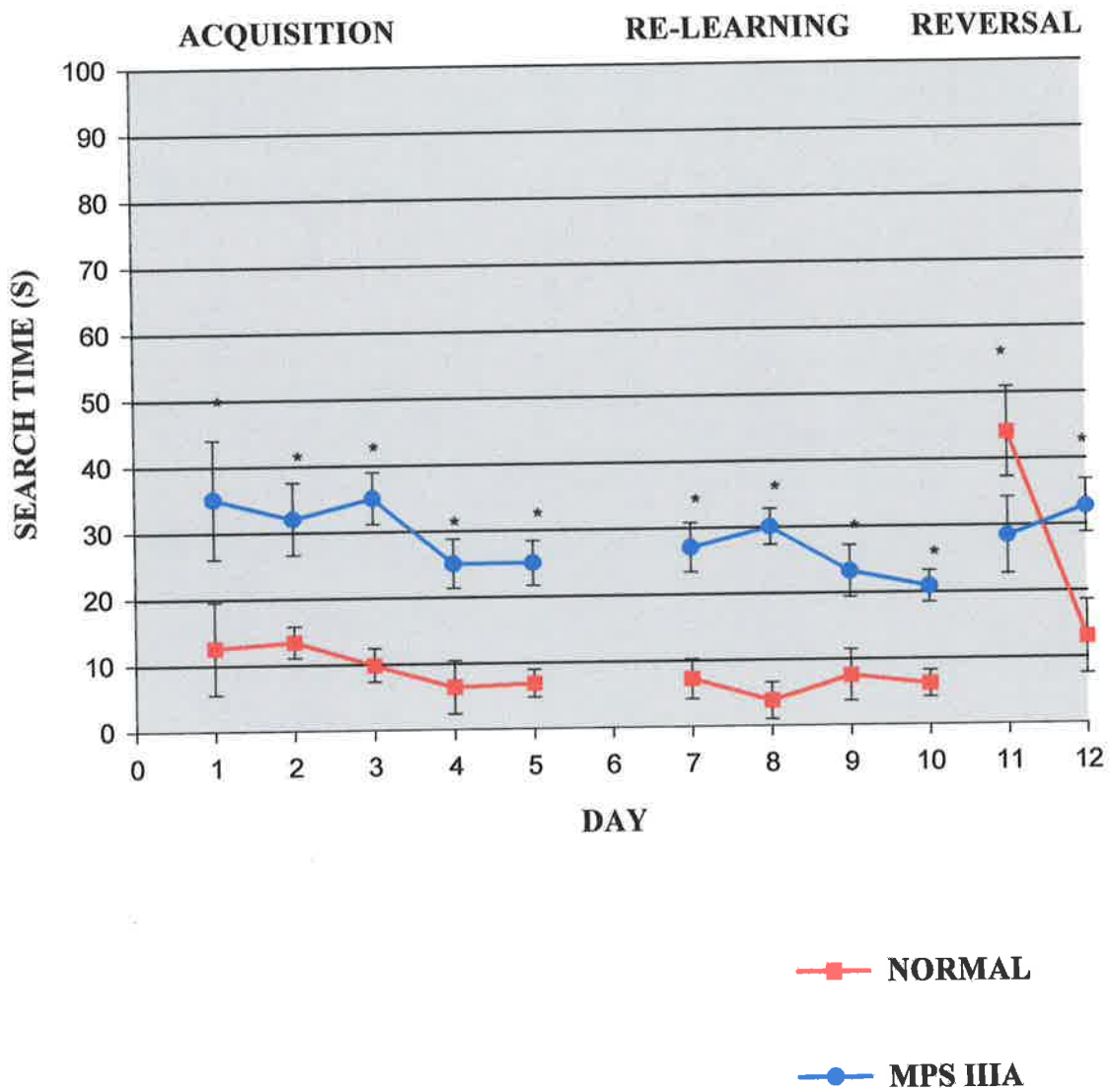
D)

REVERSAL PHASE



E)

MORRIS WATER MAZE AT 28-WEEKS OF AGE



For the MWM to be conclusive it should be performed on mice that have not previously undergone this testing, i.e. naive mice. We therefore repeated the MWM on 10 previously untested normal male mice and 10 previously untested MPS IIIA male mice, all 20-weeks of age, with an allocated search time of 90-seconds to locate the platform.

On the first day no significant difference was observed in search times between the normal and MPS IIIA mice, with both groups taking an average of approximately 80-sec to locate the platform. By day two the normal mice had decreased their search times by 20-sec and by day six they had decreased their search times by 60-sec. In contrast the MPS IIIA mice had decreased their search times by only seven-sec on day two and this remained constant on day three. By day four they had decreased their search times by 20-sec and this was decreased by a further 5-sec on day five (Figures 5.7A, E). A significant difference in search times was observed between normal and MPS IIIA mice.

As expected, the normal mice spent most of their time in the probe phase in the NW quadrant and the MPS IIIA mice spent an even amount of time in all quadrants (Figure 5.7B)

During the re-learning phase the normal mice successfully found the platform in less than 10-sec, compared with an average of around 30-sec for the MPS IIIA mice (Figure 5.7C). As opposed to the 20-week old non-naive mice a significant difference in performance between the acquisition phase and the re-learning phase for the individual groups was observed (Figure 5.7E)

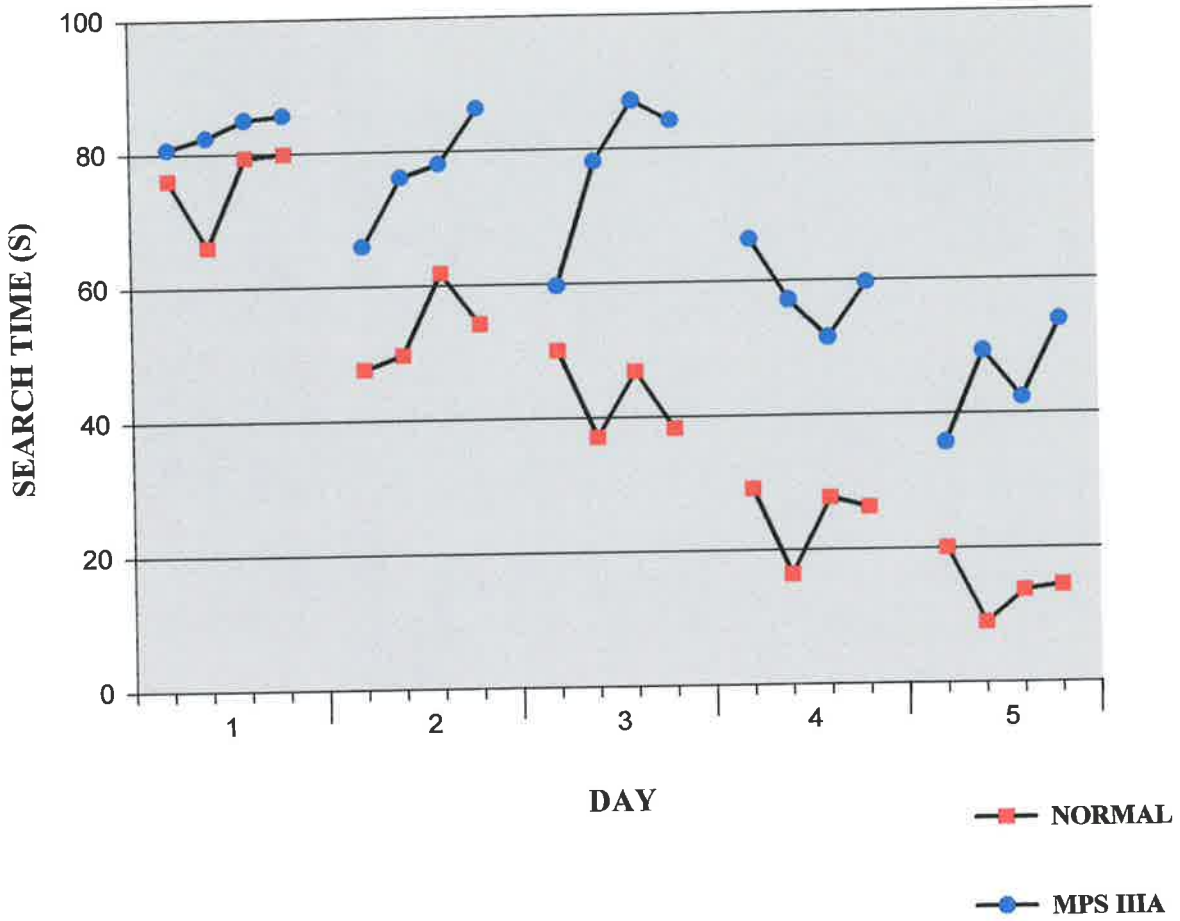
On day one of the reversal phase it took the normal mice an average of 50-sec to find the platform; they decreased their search time to 20-sec on the second day. It took the MPS IIIA mice an average of approximately 40-sec to locate the platform on both days of testing (Figures 5.7D, E)

Figure 5.7: MWM on naïve mice at 20-weeks of age.

- A) Mean latency per trial for both groups during the acquisition phase of the MWM test (testing days one-five). From days one-five, the platform was placed in the NW quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=10) and blue lines represent MPS IIIA mice (n=10).
- B) The probe test was performed on day six immediately after the acquisition phase. The time the mice spent in the target quadrant is shown. Blue bars indicate the time spent in the target quadrant and yellow bars represent the average time spent in each of the remaining three quadrants.
- C) Mean latency per trial for both groups during the re-learning phase of the MWM test (testing days seven-10). From days seven-10, the platform was replaced in the original quadrant (NW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=10) and blue lines represent MPS IIIA mice (n=10).
- D) Mean latency per trial for both groups during the reversal phase of the MWM test (testing days 11-12). From days 11-12, the platform was relocated to the SE quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice (n=10) and blue lines represent MPS IIIA mice (n=10).
- E) Mean latency per day for both groups during each phase of the MWM test. Red lines represent normal mice (n=10) and blue lines represent MPS IIIA mice (n=10). Standard deviations are shown. *, Significant ($P < 0.01$) difference in latency between normal and MPS IIIA mice (see Materials and Methods, Section 2.2.23 for description).

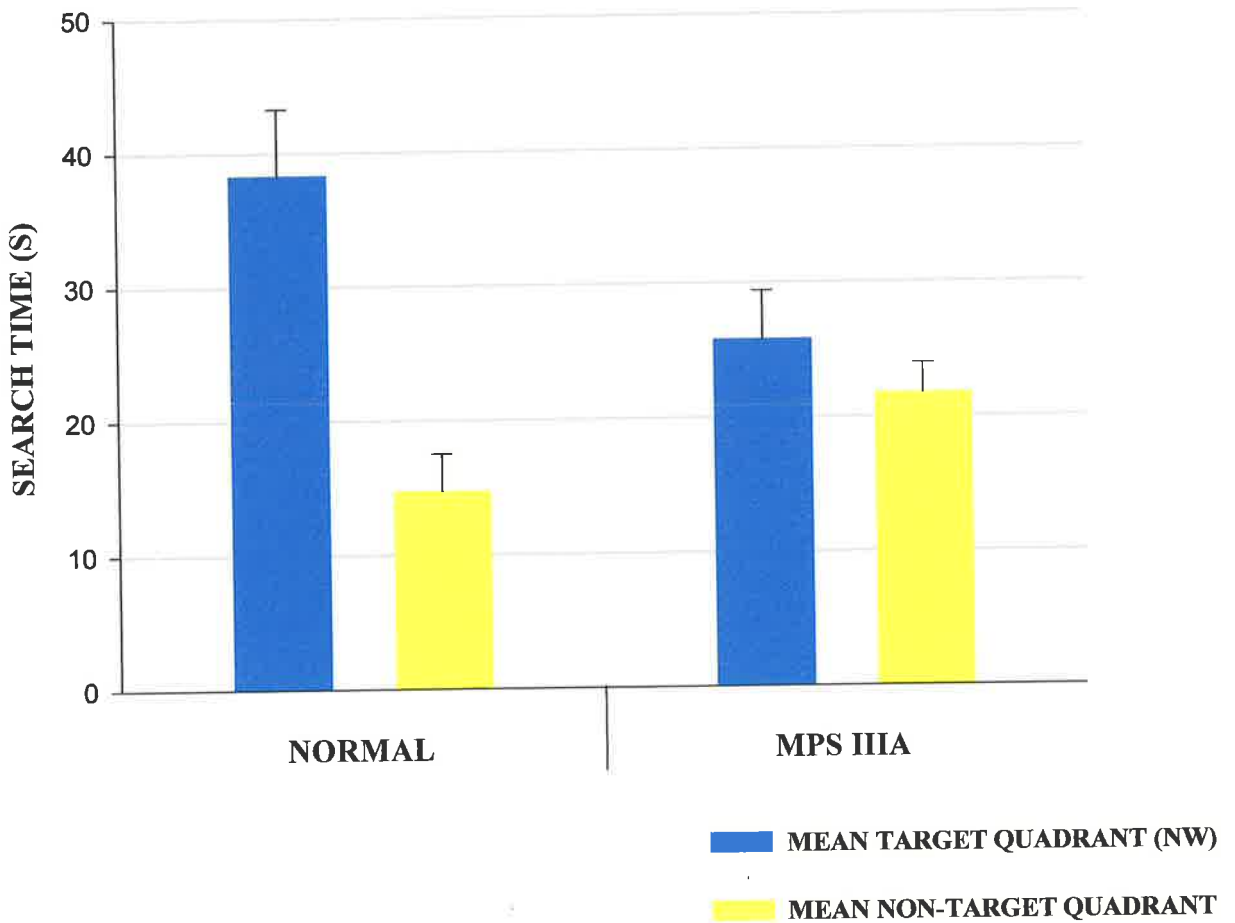
A)

ACQUISITION PHASE



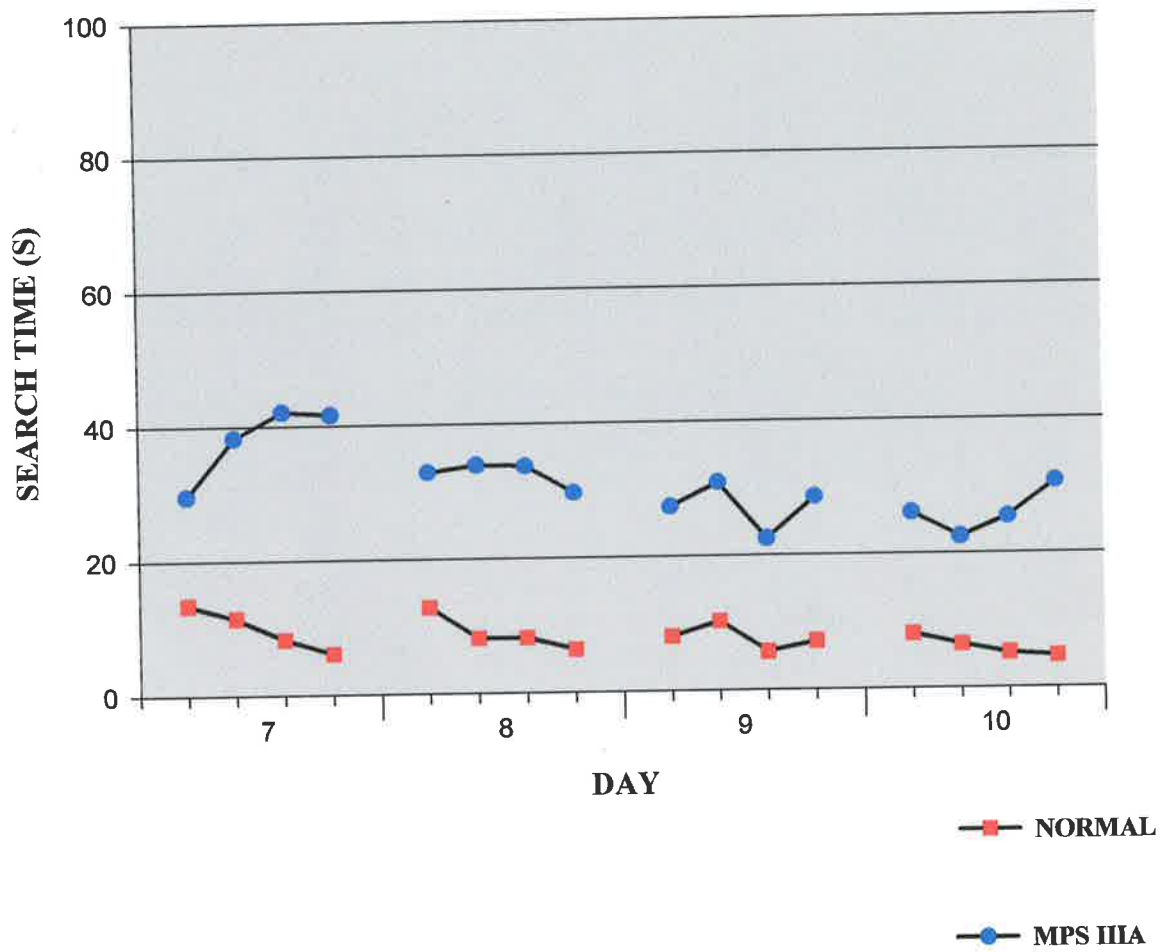
B)

PROBE PHASE



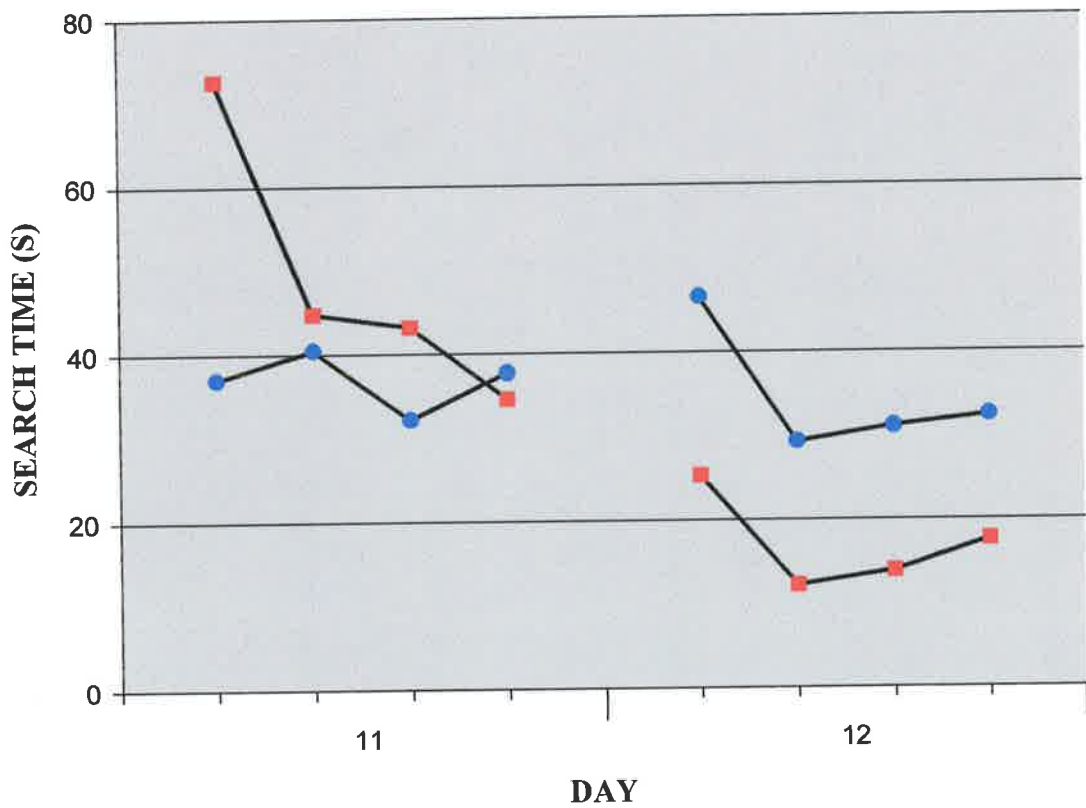
C)

RE-LEARNING PHASE



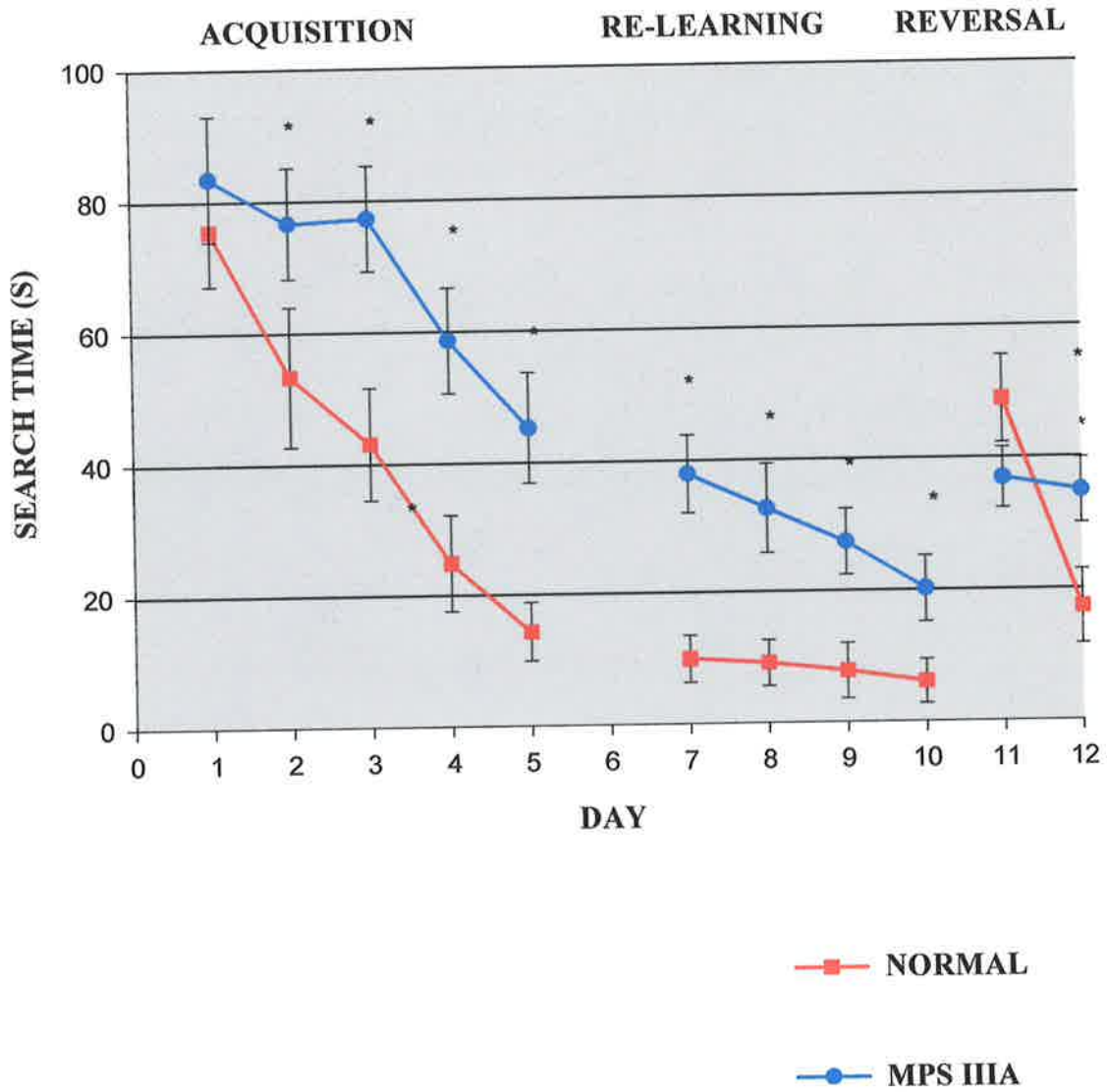
D)

REVERSAL PHASE



E)

MORRIS WATER MAZE AT 20-WEEKS OF AGE NAIVE MICE



5.3.6 VISIBLE PLATFORM TEST

The same group of 20-week old mice underwent a visible platform test. The platform was raised from the water, which contained no milk powder. The normal mice located the platform within less than 10-sec, where as the MPS IIIA mice located the platform within 15-sec. Unlike the normal mice, the MPS IIIA mice did not head directly to the quadrant containing the platform but rather they headed straight for the platform once it was in view. This important test demonstrates that the MPS IIIA mice are fully capable of swimming to the visible platform, indicating that their vision, motor coordination and motivation to escape the water are unaffected by the disease at this age.

5.4 DISCUSSION

Maze procedures offer a variety of ways of assessing learning and memory in animals and of studying the effects of neurological deterioration, such as that observed in the MPS IIIA mice. Learning and remembering the location of food resources, predators, escape routes, and immediate kin is one of the most essential forms of higher cognitive processing in mammals (Silva et al., 1998). Two of the most often studied forms of place learning are spatial learning and contextual conditioning. Spatial learning refers to an animal's capacity to learn and remember the location of a reward, while contextual conditioning relies on an animal's ability to associate specific places with aversive stimuli (Silva et al., 1998). The MWM assesses spatial learning abilities in rodents. It measures an animal's ability to develop a strategy to find a hidden platform (acquisition), remember where the platform was located (probe), re-learn where the platform was originally placed (re-learning), and finally to develop a strategy to find a hidden platform located in a new position (reversal) (O'Connor et al., 1998).

The MWM was performed on MPS IIIA and normal mice at various ages up to 28-weeks of age. Before this study it was unknown whether the MPS IIIA mice were mentally impaired from birth or if they become progressively mentally retarded with age, and, if the latter, at what age did loss of essential cognitive skills such as learning and memory occur.

The MWM performed on mice at seven-weeks of age showed no significant difference (determined by a repeated measures analysis, see Materials and Methods, Section 2.2.23) in search times between the MPS IIIA and normal mice in any phase of the testing. Mice in both groups were naive to the testing at this age and as a result their search times for the first two days approached the maximum time, suggesting that the mice were still learning the location of the platform and were probably still acclimatising to the test. However, after this time they very quickly learnt the location of the platform.

Interestingly, at the beginning of testing at 15-weeks of age the mice in both groups were able to locate the platform relatively quickly, in comparison to that observed at seven-weeks of age. One possible explanation for this is that the mice had retained memory of the task and therefore were not intimidated by the pool, water, swimming etc. So, whilst the MWM works on short-term memory, we have demonstrated a capacity of long-term memory in the mice. The MPS IIIA mice performed significantly worse than the normal mice at this age, suggesting that neurological deterioration was starting to occur.

By 20-weeks of age a significant difference in performance was observed between the MPS IIIA mice and normal mice. The MPS IIIA mice performed significantly worse than the normal mice in every phase of the testing. The MPS IIIA mice also performed worse on the first day of testing than they had at 15-weeks of age, suggesting that they had lost the capacity to remember. This was best highlighted in the reversal phase; the latency to find the platform for the MPS IIIA mice was not altered by the relocation of the platform to the opposite quadrant, suggesting that the mice had no memory of where the platform was and were just randomly swimming around until the platform was encountered. The increase in search times observed for the MPS IIIA mice was due to a decline in CNS function, rather than physical impairments due to the disease, as shown by performing a visible platform test. This test demonstrated that the MPS IIIA mice were as capable of seeing and swimming to the platform as the normal mice. For the MWM to be truly quantitative it should be performed on mice naive to the test. This became obvious from the trials at 15-weeks, 20-weeks and 28-weeks of age where no significant difference in search times was observed between the acquisition and the relearning phases due to the memory retention of the mice, particularly the normal mice. Repeating the MWM on naive mice at 20-weeks of age similarly showed a significant difference in search times between MPS IIIA and normal mice. It is also interesting to note that the MPS IIIA mice have other severe behavioural disturbances at this age; as discussed earlier the breeding of affected mice must occur before 20-weeks of age since after this age, the mum will kill and eat all of her pups.

By 28-weeks of age MPS IIIA mice have developed the characteristic hunched posture, which could affect their ability to perform in the MWM and caution must therefore be taken when analysing data from this age group. This highlights limitations to the MWM, in that it is only effective before the onset of such physiological abnormalities. This renders it futile in trying to accurately assess cognitive function beyond about 25-weeks of age. In this case other neurological tests will need to be assessed for example the repeated acquisition and performance chamber (RAPC) (Brooks et al., 2000). This test was successfully employed to assess spatial learning and memory in MPS VII mice (Brooks et al., 2002) after the onset of skeletal pathology which renders the mice incapable of performing the MWM.

Changing the allocated search time from 120-seconds to 90-seconds proved successful. A search time of 90-seconds was long enough for the mice to locate the platform. Mice were also less tired after each trial of the probe phase, with an allocated search time of 90-seconds compared with when they were swam for 120-seconds.

From these studies, we have demonstrated that the MWM is a reliable and repeatable test for assessing CNS function in MPS IIIA mice. We conclude that severe neurological deterioration of the MPS IIIA affected mice occurs by 20-weeks of age, as assessed by the MWM.

Following establishment of the MWM test, ERT trials were assessed in the MPS IIIA mice. These experiments are presented and discussed in the next chapter.

CHAPTER 6.0

ENZYME REPLACEMENT THERAPY FROM BIRTH AND FROM SIX-WEEKS OF AGE IN MPS IIIA MICE

6.1 INTRODUCTION

The close comparison of disease characteristics between MPS IIIA in the mouse and humans makes this mouse model particularly useful to study the pathophysiology of the disease and to develop novel therapeutic approaches for this and other LSD. As already outlined the major obstacle to address when designing a therapy for MPS IIIA is targeting sufficient amounts of enzyme across the restrictive BBB to the CNS, the major site of pathology in this disease. It has been proposed that in mice the tight junctions between the endothelial cells which form the barrier are incompletely formed at birth, therefore the BBB is 'leaky' to molecules passing by in circulation (Stewart and Hayakawa, 1987). Horseradish peroxidase (HRP), a commonly used vascular tracer that does not cross the intact BBB, was intraperitoneally injected into newborn mice and HRP activity in the brain was analysed four-hours later; a leakage of HRP from circulation into mouse fetal and newborn brain was demonstrated. This indicated higher vascular permeability in fetal and newborn brain than that in the adult brain. By 10-14 days of age the BBB is believed to be fully intact (Stewart and Hayakawa, 1987).

Enzyme distribution studies have been investigated in newborn MPS VII mice (Vogler et al., 1993). Newborn MPS VII mice, given a single intravenous injection of GUSB, demonstrated enzyme activity present in the liver, brain, heart and bone, all major sites of pathology in these mice (Vogler et al., 1993). ERT has also been shown to be an effective means of transiently treating MPS VII mice if initiated at birth. MPS VII mice treated weekly with GUSB from birth to five-weeks of age had an improved phenotype and increased GUSB activity in the liver, spleen, kidney and brain at six-weeks of age (Sands

et al., 1994). A noticeable reduction in storage was also observed in all of these tissues. MPS VII mice enzyme treated from birth show an improvement in behavioural performance, and, these mice performed similarly to normal mice in the MWM test (O'Connor et al., 1998).

The two major aims of experiments described in this chapter were firstly to determine the distribution and fate of rhNS in newborn and six-week old MPS IIIA mice, and, secondly, to perform a preliminary ERT trial with rhNS in newborn and six-week old MPS IIIA mice to evaluate the viability and efficacy of long-term therapy.

In an attempt to demonstrate that enzyme injected intravenously into newborn mice is able to pass through the immature BBB into the CNS, but is unable to do so once the BBB has matured in adult mice, an enzyme distribution study was performed. The experimental plan was to evaluate the distribution of 1 mg rhNS per kg of mouse body weight in the tissues of MPS IIIA mice .

ERT was performed in MPS IIIA mice from birth and from six-weeks of age and in normal control mice. Mice received weekly intravenous injections of 1 mg/kg rhNS. Age-matched MPS IIIA mice and normal mice received weekly injections of enzyme dilution buffer (PBS, pH 7.2). To evaluate efficacy of ERT, clinical appearance, behaviour, plasma anti-rhNS antibody titre, MWM and tissue histopathology were assessed.

6.2 SPECIFIC METHODS

6.2.1 DISTRIBUTION STUDY:

ENZYME PREPARATION AND TISSUE ANALYSIS

A preparation of rhNS with a specific activity of 341 nmol/min/mg was used for the enzyme distribution studies in both newborn and adult mice (See Materials and Methods, Section 2.2.12).

Organs were removed from mice and placed in individual containers and stored on dry ice for transportation. All organs were weighed. Whole organs from six-week old mice were homogenised in homogenation buffer (0.02M Tris HCl/0.05M NaCl, pH 7.2) using a tissue homogeniser (Janke and Kunkel, Ultra-turrax T25). Whole organs from newborn mice were minced in homogenation buffer, using scalpel blades. All homogenations were freeze/thawed six-times to disperse cellular membranes, and centrifuged at 13,000g for 3 min. Supernatants were kept and stored at -70°C . The presence of rhNS was detected according to the method outlined in Materials and Methods, Section 2.2.6.

6.2.2 ENZYME PRODUCTION AND PURIFICATION FOR ERT

An estimated total of 30 mg of rhNS was needed to complete the planned 20-week ERT study. At the commencement of therapy 26 mg of previously purified rhNS was available (Liz Melville and Peter Clements, Department of Chemical Pathology, WCH). It was therefore necessary to purify an additional 4 mg rhNS. As a result two separate batches of enzyme were used in the study. Batch one consisted of the 26 mg rhNS previously purified, with a specific activity of 620 nmol/min/mg and was used for the first 17 weekly injections. The second batch consisted of 4 mg rhNS with a specific activity of 581 nmol/min/mg, and was used for the last three-weeks of injections. Both batches of enzyme were purified in exactly the same manner, using a two-step ion exchange procedure (see Materials and Methods, Section 2.2.12). Throughout the 20-weeks both

batches were assayed regularly using the radiolabelled tetrasaccharide to ensure they maintained activity (see Materials and Methods, Section 2.2.5).

6.2.3 EXPERIMENTAL ANIMALS, TREATMENT GROUPS AND ENZYME ADMINISTRATION FOR ERT

Fourteen pairs of 12-week old MPS IIIA male and female mice were paired on the same day, as were nine homozygous normal females with nine MPS IIIA males. This breeding programme produced affected and clinically normal mice, with at least 10 males in each group for MWM analyses. All pups used in this study were born in the same week and therefore considered age-matched. Five distinct groups of mice were analysed as summarised in Table 6.1. Each mouse was injected with an equivalent of 100 μ l of 1 mg/kg rhNS, diluted in PBS, pH 7.2, on the same day of every week for the duration of 20-weeks. For the mice injected from birth the initial injection was made into the superficial temporal vein (see Materials and Methods, Section 2.2.19), a prominent vessel located on either side of the head (Sands and Barker, 1999). After four-days of age, it becomes difficult to inject into this vein due to the development of pigment in darker strains of mice and the change in position of the vein. There were no other candidate veins for an intravenous injection at two-weeks of age, consequently the second infusion of enzyme was intraperitoneal. The remaining injections were made into the tail vein (see Materials and Methods, Section 2.2.19). The mice that began treatment from six-weeks of age all had injections made into the tail vein. All mice were monitored for up to 30-min after injection.

Table 6.1: Treatment group, dose regime and age of onset of mice in the study

Treatment group #	Genotype	Sex		Injection	Age @ onset of therapy †
		Males	Females		
Untreated normal	Heterozygote	10	7	PBS, pH 7.2	
Treated normal	Heterozygote	10	8	CHO rhNS, 1mg/Kg	Birth
Birth ERT MPS IIIA	MPS IIIA	16	9	CHO rhNS, 1mg/Kg	Birth
Delayed ERT MPS IIIA	MPS IIIA	16	7	CHO rhNS, 1mg/Kg	6 Weeks
Untreated MPS IIIA	MPS IIIA	18	8	PBS, pH 7.2	

† All mice used in this study were age matched ie: born in the same week

6.3 RESULTS

6.3.1 ENZYME DISTRIBUTION STUDIES IN MPS IIIA MICE

Six MPS IIIA mice were infused with rhNS 1 mg/kg into the superficial temporal vein on day one of life. One mouse was sacrificed at time points, one-hr, four-hr, six-hr, 24-hr, 48-hr and seven-days post-injection. Two mice not given enzyme were sacrificed for a time blank. The brain and liver were removed and assayed for the presence of enzyme but other organs were not removed since, at this age, they are very small and discrimination between organs is difficult. Similarly six MPS IIIA mice were each infused with rhNS 1 mg/kg into the tail vein at six-weeks of age. Mice were sacrificed at the same time points and the brain, liver, heart, kidney and spleen were removed and assayed for the presence of enzyme.

One hr after administration of 1 mg/kg rhNS in newborn MPS IIIA mice, 346 ng NS per mg of total protein was detected in the liver, and 75 ng NS per mg total protein was detected in the brain (Figure 6.1A). The amount of enzyme detected four-hr post-injection in the brain was nearly double that observed at one-hr. A small amount of rhNS was detected in the brain of newborn mice six-hr and even 24-hr post-infusion, 36 ng NS per mg total protein and 34 ng NS per mg total protein respectively. NS was detected in the liver 48-hr after infusion, insignificant levels were detected in the brain at this time. No NS was present in both tissues one-week after injection.

One-hr after administration of 1 mg/kg rhNS in six-week old MPS IIIA mice the majority of infused enzyme was detected in the liver (645 ng NS/mg total protein) relatively equal amounts were detected in the spleen and kidney, 270 ng NS per mg total protein and 325 ng NS per mg total protein, respectively. Low levels of enzyme were detected in the heart, and undetectable amounts in the brain (Figure 6.1B). RhNS content had almost doubled in the liver and spleen from one-hr to four-hr post- injection; significant amounts

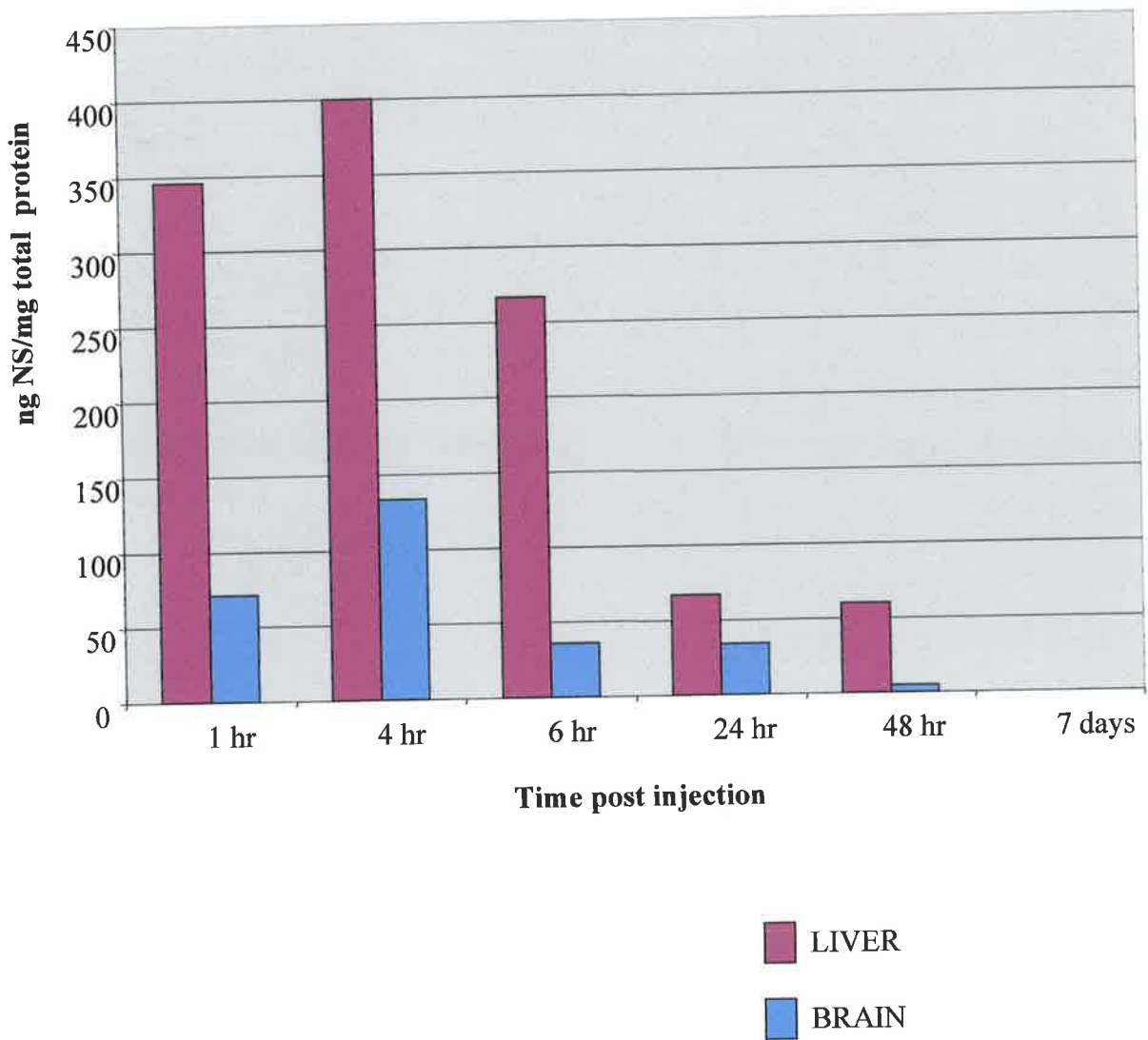
Figure 6.1: Distribution of 1 mg/kg rhNS intravenously injected into MPS IIIA mice.

A) Distribution in newborn MPS IIIA mice: rhNS was injected into the superficial temporal veins of MPS IIIA mice on day one of life. Mice were sacrificed by decapitation 0, one-hr, four-hr, six-hr, 24-hr, 48-hr and seven-days post-injection. Brain and liver were removed, weighed and homogenised. Homogenates were assayed directly for NS content using the immunoquantification method (see Materials and Methods, Section 2.2.6) and total protein. Results are expressed as ng NS/mg total protein.

B) Distribution in young adult MPS IIIA mice: rhNS was injected into the tail vein of six-week old MPS IIIA mice. Mice were sacrificed by cervical dislocation 0, one- hr, four-hr, six-hr, 24-hr, 48-hr and seven-days post-injection. Brain, liver, kidney, spleen and heart were removed, weighed and homogenised. Homogenates were assayed directly for NS content using the immunoquantification method (see Materials and Methods, Section 2.2.6) and total protein. Results are expressed as ng NS / mg total protein.

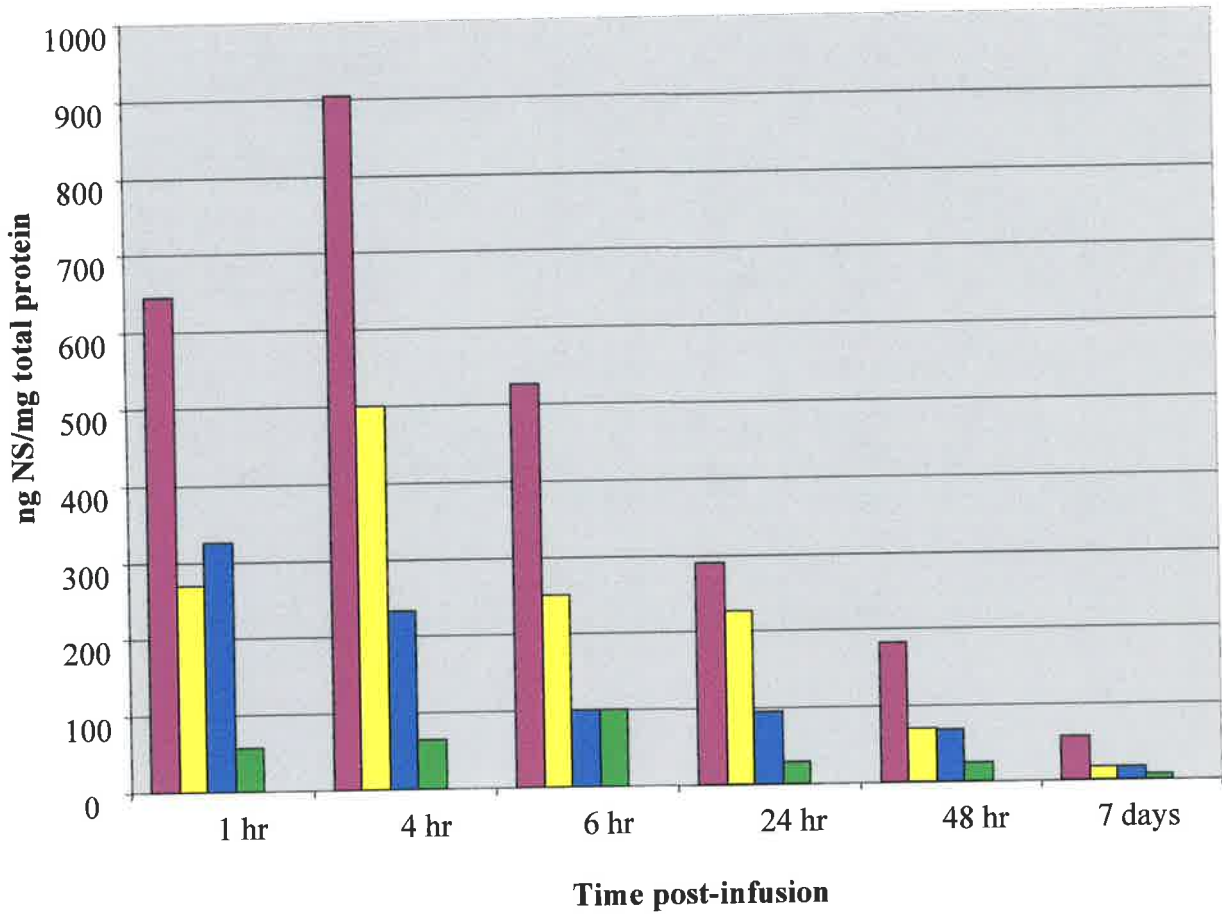
A)

DISTRIBUTION OF rhNS IN NEWBORN MPS IIIA MICE



B)

DISTRIBUTION OF rhNS IN SIX-WEEK OLD MPS IIIA MICE



- Liver
- Spleen
- Kidney
- Heart
- Brain

were also observed in the kidney and to a lesser extent in the heart. Up to 182 ng per mg total protein was detected in the liver 48 hr post- injection. Except for small amounts in liver, insignificant levels of rhNS content were observed in all tissues seven-days after infusion. Undetectable levels of rhNS were observed in the brain at all time points.

6.3.2 DISEASE PROGRESSION IN MPS IIIA MICE UNDERGOING ERT

Mice were sexed and weaned at three-weeks of age and were caged according to sex and treatment group. An average of five same-sex mice were caged together in a 50 cm x 20 cm cage. As expected from already documented observations, untreated male MPS IIIA mice began to display lesions on the rump by 10-weeks of age indicating fighting within the group. Similar observations were also made amongst the males belonging to the delayed ERT group. The victims were removed and singularly caged and by about 15-weeks of age all male mice from these two groups were individually caged (Table 6.2). Interestingly, the first sign of fighting amongst male mice in the birth ERT group was not until 18-weeks of age but it continued through until 20- weeks. Throughout the 20-week study normal male mice did not have to be separated.

In general MPS IIIA mice treated with enzyme displayed body weights close to that of normal mice. Generally there was no difference in the appearance of treated and untreated MPS IIIA mice throughout the 20-week study; this is not surprising as phenotypically MPS IIIA mice are normal up to 24-30 weeks of age (Bhaumik et al., 2000).

6.3.3 ANTIBODY RESPONSE

MPS IIIA mice subjected to ERT from birth showed no obvious adverse reactions throughout the study. The same was observed for delayed ERT MPS IIIA mice, although one mouse in this group (out of a total of 23) displayed a mild reaction immediately after injection at 10-weeks of age. The reaction was characterised by laboured breathing and lethargy, within approximately one-hr the mouse appeared normal.

Table 6.2 Caging of MPS IIIA male mice in ERT study

	MPS IIIA + ERT BIRTH			MPS IIIA + ERT FROM 6 WEEKS				MPS IIIA - ERT			
	AGE (WEEKS)			AGE (WEEKS)				AGE (WEEKS)			
CAGE #	3	17	20	3	10	12	15	3	10	12	15
1	6	4	2	6	3	0	0	6	4	2	0
2	6	3	0	6	3	2	0	6	4	3	0
3	4	4	2	4	2	2	0	6	3	0	0

At weaning male mice in the therapy study groups were caged according to therapy regime. Three cages of male mice were needed for each treatment group at weaning (three-weeks of age). The three-weeks of age number represents the starting number of mice in each cage (50 cm x 20 cm). The following numbers represent the numbers of male mice left in the original cage at various ages. Mice that were removed from the cage were housed singularly.

Serum was analysed by ELISA for the presence of antibodies against rhNS. Serum from delayed ERT MPS IIIA mice displayed the highest titre of 1,280; which was considered to be relatively insignificant. Birth ERT MPS IIIA mice and normal treated mice displayed low titres of 640. As expected mice that did not receive enzyme displayed no titre.

6.3.4 MORRIS WATER MAZE

Three days after the final injection at 20-weeks of age at least 10 male mice from each of the five treatment groups underwent the MWM test (refer to Chapter 5.0 for details). A search time of 90-sec to find the platform was allocated. On the first day of the acquisition phase no difference was observed between any of the groups in the time it took to locate the platform, and most mice required guidance to the platform (Figures 6.2A, E). On day two of the acquisition phase the search time for normal mice (treated and untreated) and birth ERT MPS IIIA mice had dramatically reduced by almost half for the normal mice and one-third for the birth ERT MPS IIIA mice. On the third, fourth and fifth days there was a significant decrease in search time of the treated/untreated normal mice and birth ERT MPS IIIA mice when compared with delayed ERT and untreated MPS IIIA mice (Figures 6.2A, E). Similarly, there was no significant difference between treated and untreated normal mice. Untreated MPS IIIA mice and delayed ERT MPS IIIA mice spent most of the allocated 90-sec swimming around the pool in circles. A slight difference was observed between birth ERT MPS IIIA mice and normal mice on day two with the birth ERT MPS IIIA mice taking longer to find the platform, but no difference between these groups was observed on the last three days (Figure 6.2E).

During the probe phase, treated and untreated normal mice and birth ERT MPS IIIA mice spent most of their time searching in the target quadrant, almost double that spent in the non-target quadrants (Figure 6.2B). There was no significant difference in the search times between these three groups. In comparison untreated MPS IIIA mice and delayed

Figure 6.2: MWM.

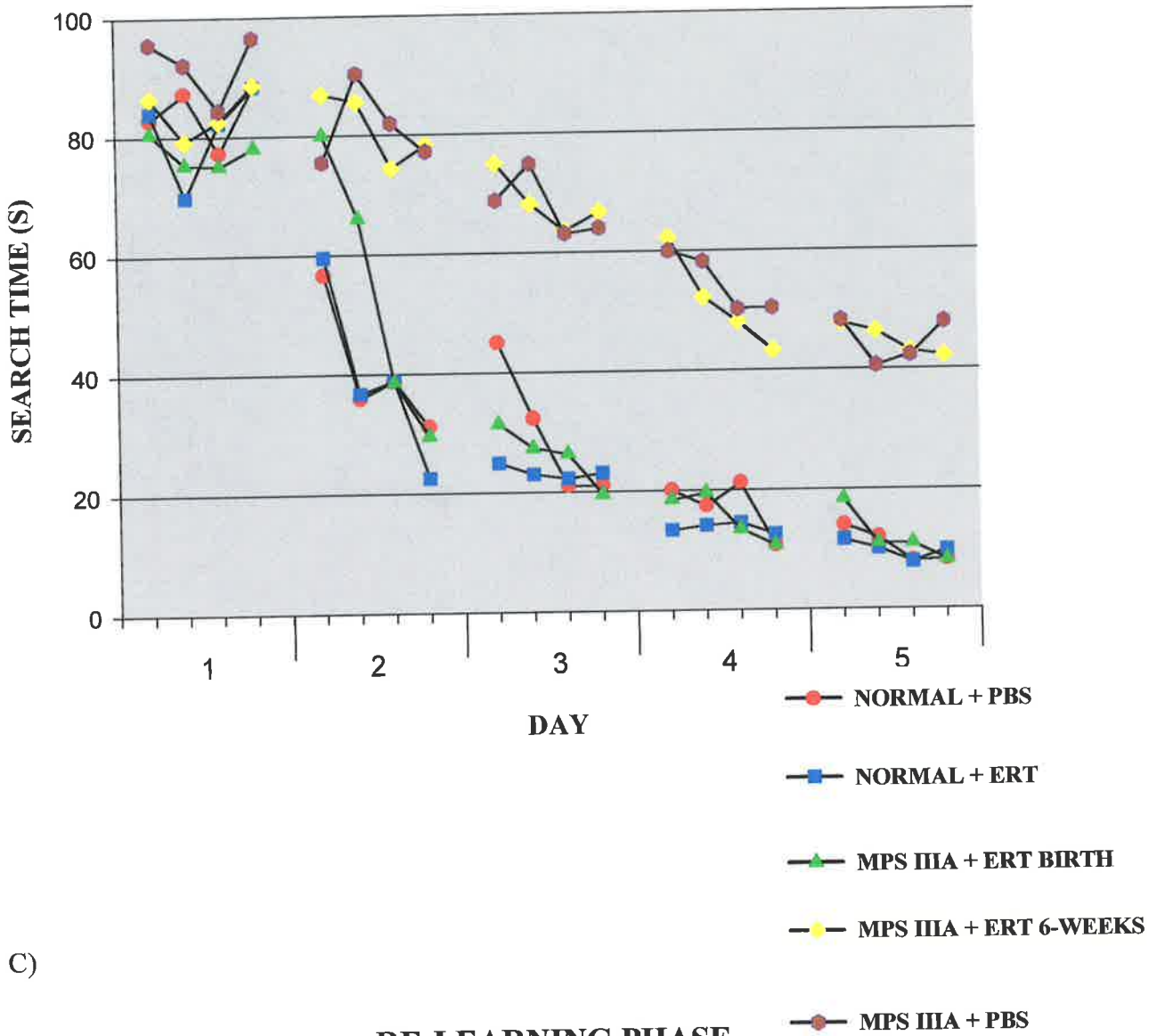
- A) Mean latency per trial for both groups during the acquisition phase of the MWM test (testing days one-five). From days one-five, the platform was placed in the NW quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice untreated (n=10), blue lines represent normal mice treated (n=10), green lines represent MPS IIIA mice treated from birth (n=11), yellow lines represent MPS IIIA mice treated from six-weeks of age (n=12), and brown lines represent MPS IIIA mice untreated (n=10).
- B) The probe test was performed on day six immediately after the acquisition phase. The time the mice spent in the target quadrant is shown. Blue bars indicate the time spent in the target quadrant and yellow bars represent the average time spent in each of the remaining three quadrants.
- C) Mean latency per trial for both groups during the re-learning phase of the MWM test (testing days seven-10). From days seven-10, the platform was replaced in the original quadrant (NW). Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice untreated (n=10), blue lines represent normal mice treated (n=10), green lines represent MPS IIIA mice treated from birth (n=11), yellow lines represent MPS IIIA mice treated from six-weeks of age (n=12), and brown lines represent MPS IIIA mice untreated (n=10).
- D) Mean latency per trial for both groups during the reversal phase of the MWM test (testing days 11-12). From days 11-12, the platform was relocated to the SE quadrant. Each animal was released from each of the four cardinal points once per day. The time needed to find the platform was recorded for each trial. Red lines represent normal mice untreated (n=10), blue lines represent normal mice treated (n=10), green

lines represent MPS IIIA mice treated from birth (n=11), yellow lines represent MPS IIIA mice treated from six-weeks of age (n=12), and brown lines represent MPS IIIA mice untreated (n=10).

E) Mean latency per day for both groups during each phase of the MWM test. Red lines represent normal mice untreated (n=10), blue lines represent normal mice treated (n=10), green lines represent MPS IIIA mice treated from birth (n=11), yellow lines represent MPS IIIA mice treated from six-weeks of age (n=12), and brown lines represent MPS IIIA mice untreated (n=10). Standard deviations are shown. *, Significant ($P < 0.01$) difference in latency between treated MPS IIIA mice and untreated or treated from six-weeks of age MPS IIIA mice.

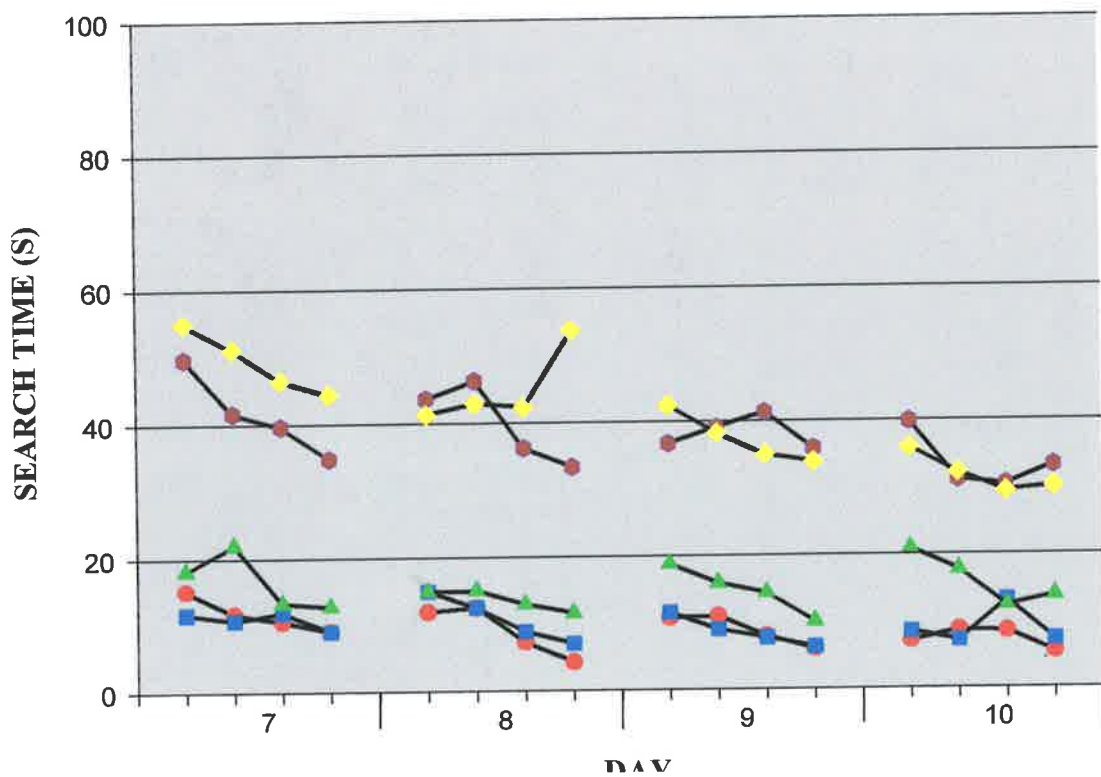
A)

ACQUISITION PHASE



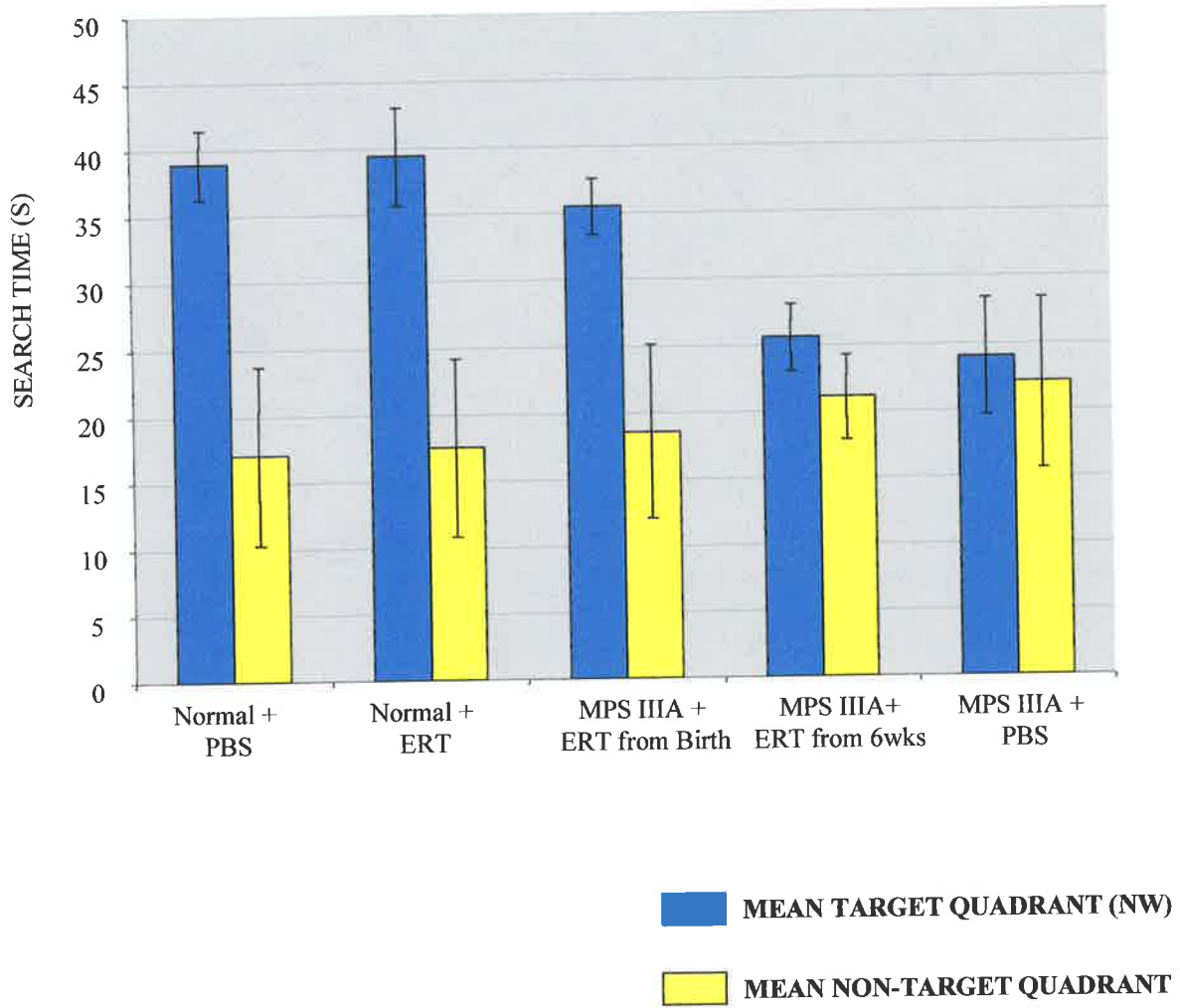
C)

RE-LEARNING PHASE



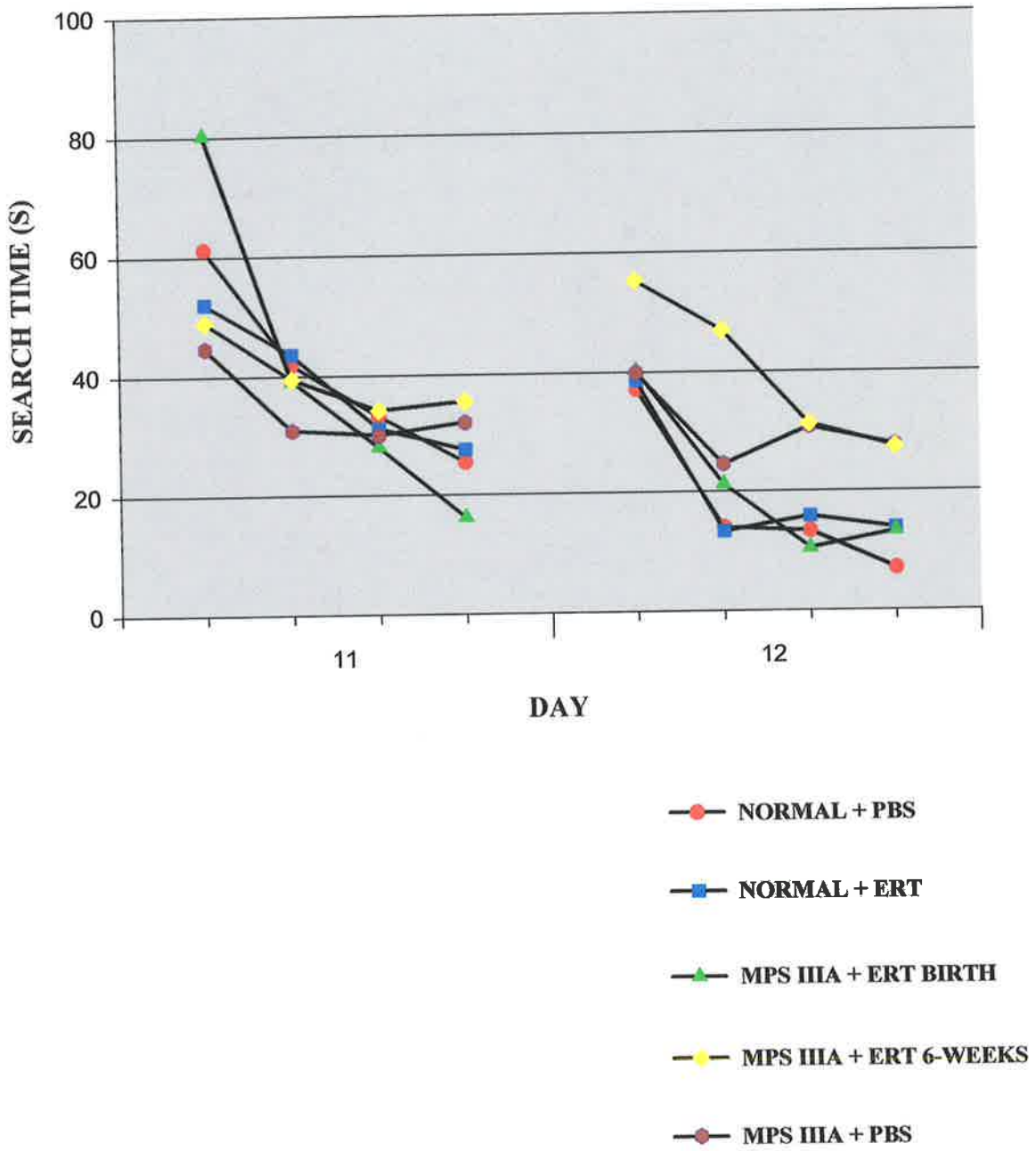
B)

PROBE PHASE



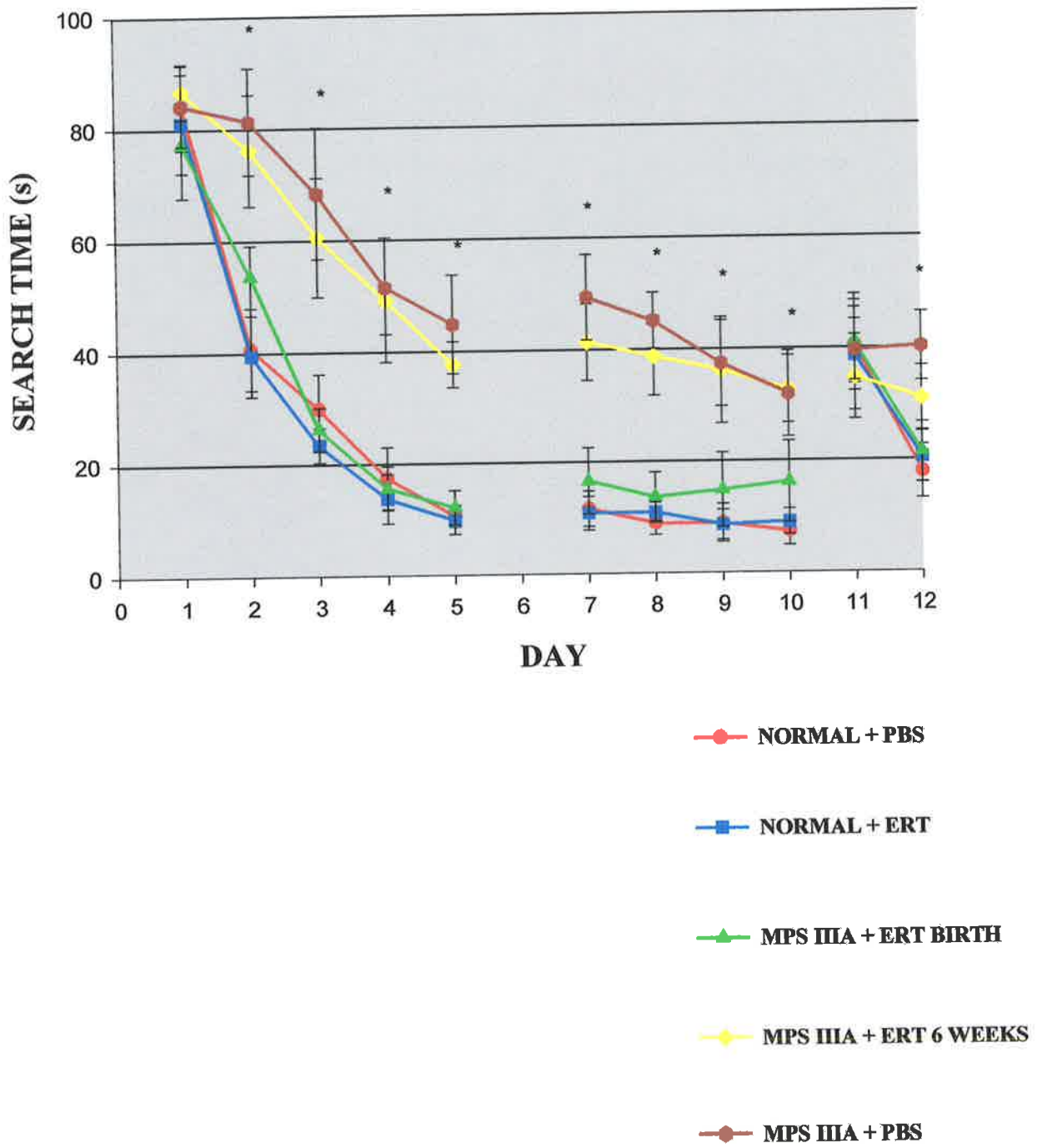
D)

REVERSAL PHASE



E)

MORRIS WATER MAZE



ERT MPS IIIA mice spent an equal amount of time searching in all four quadrants (Figure 6.2B).

During the re-learning phase there was no significant difference in search time between delayed ERT MPS IIIA mice and untreated MPS IIIA mice, taking these groups about 30% longer to find the platform than the other three groups (Figure 6.2C and 6.2E). Treated and untreated normal mice headed straight to the platform in each trial and were able to locate it within about 10-sec on each day of the re-learning phase. Birth ERT MPS IIIA mice performed notably worse during the re-learning phase in comparison with the last two days of the acquisition phase. However, whilst the birth ERT mice were distinct from the treated and untreated normal mice, this group still performed significantly better than the untreated MPS IIIA mice and delayed ERT MPS IIIA mice.

There was no significant difference on the first day of platform relocation between any of the five groups (Figures 6.2D, E). By the second day the search times of the treated and untreated normal mice and the birth ERT MPS IIIA mice had significantly decreased. In comparison the search time in the other two groups had not altered from that of the first day. In fact, the search times of the untreated MPS IIIA mice and delayed ERT MPS IIIA mice remained fairly constant, at an average of 50-sec on the last two days of the acquisition phase, the four days of the relearning phase and the two days of the reversal phase.

6.3.5 HISTOLOGY

One mouse from each of the five treatment groups was sacrificed at three-weeks, eight-weeks, 14-weeks, 18-weeks and 23-weeks. The brain was removed and immediately added to fixative (paraformaldehyde). A section of the cerebellum and cerebral cortex approximately 5 mm³ were removed and analysed. Samples were post-fixed in 1% osmium tetroxide (v/v) and processed in epoxy resin (see Materials and Methods, Section 2.2.24). Thick sections were stained with Toluidine blue for light microscopy. From these sections a region uniform in all samples was cut for electron microscopy (EM); thin

sections were stained by the uranyl acetate/lead citrate method. Toluidine blue sections required prompt processing as a result of technical problems encountered with the fading of the dye used, thus making cell types hard to observe. Consequently, EM sections were only evaluated for histological analysis. Caution should therefore be taken when interpreting these results because the number of cells available for scoring from EM sections was markedly reduced, in comparison to the amount of cells that could be scored under light microscopy. A minimum of 20 cells in total (i.e. not of each cell type) from each of the sections were graded. One EM section per brain region per mouse was evaluated.

Neurons, glial cells, perivascular cells and non-nucleated cells were analysed and scored from the cerebral cortex sections (Table 6.3). Non-nucleated cells were included in the evaluation to provide a larger sample size to analyse; these cells, however, made up the minority of cells scored from cortex sections. Purkinje cells were analysed from cerebellum sections. Cerebellum sections were not graded, as too few cells were observed under EM. A three-tier grading system was employed, with sections graded according to the number of storage vacuoles observed, the number of cells containing storage vacuoles and finally the type of inclusion observed. Results are summarised in Table 6.3.

A moderate number of storage vacuoles were present in neurons, glial and perivascular cells in untreated MPS IIIA mice at three-weeks of age; the lysosomal inclusions were electron lucent, membrane-bound vacuoles (Figure 6.3A). Inclusions in Purkinje cells of these mice were much less common; four out of the nine Purkinje cells observed had small cytoplasmic inclusions (Figure 6.4A). In the birth ERT MPS IIIA mice, a dramatic reduction of storage to almost negligible levels was observed in the cells of the cerebral cortex at three-weeks of age (Figures 6.3B, C). Only 17% of neurons observed contained inclusions, compared to 73% in untreated MPS IIIA mice. Lysosomal inclusions were not observed in perivascular cells scored from the treated MPS IIIA mice (Figure 6.5B).

Table 6.3: Lysosomal storage in different cell types of the cerebral cortex in untreated and treated MPS IIIA mice, and untreated and treated normal mice at various ages.

EM sections of a region in the cerebral cortex uniform in all mice were analysed and scored according to the amount of storage vacuoles, number of cells observed with storage vacuoles and the nature of the inclusion. Cell types graded included neurons, glial, perivascular and non-nucleated cells.

Amount of storage vacuoles

0 = no vacuoles.

$1/2+$ = very few vacuoles

1+ = few vacuoles.

2+ = moderate vacuoles

3+ = abundant vacuoles.

Number of cells with storage vacuoles

F = few cells

M = moderate cells

D = diffuse involvement

Type of inclusion

OP = open (empty) vacuoles

WH = flocculent whorls &
stacks

Treatment group

1 = Normal + PBS

2 = Normal + ERT

3 = MPS IIIA + ERT from birth

4 = ERT from six-weeks

5 = MPS IIIA + PBS

* NA – not available

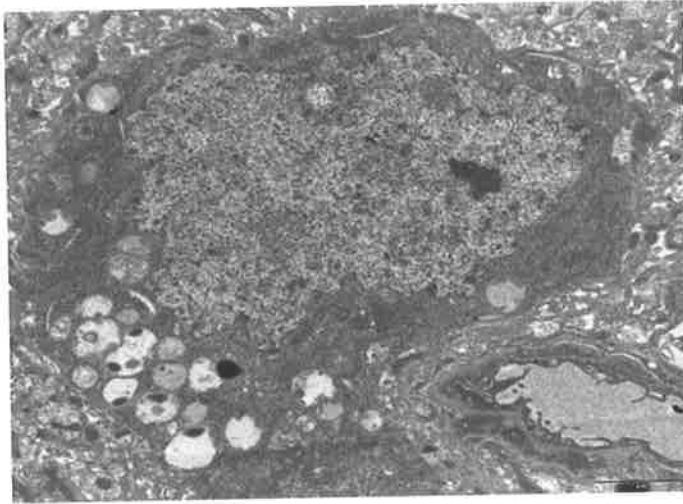
Table 6.3: Lysosomal storage in different cell types of the cerebral cortex in untreated and treated MPS IIIA mice at various ages

Treatment Group	Cell type	Age				
		3 weeks	8 weeks	14 weeks	18 weeks	23 weeks
1	Neurons	0	0	0	0	0
	Glial	0	0	0	0	0
	Perivascular	0	0	0	0	0
	Non-nucleated	0	0	0	0	0
2	Neurons	0	0	0	0	0
	Glial	0	0	0	0	0
	Perivascular	0	0	0	0	0
	Non-nucleated	0	0	0	0	0
3	Neurons	1/2 + F (OP)	1+ M (OP)	2+ M (OP)	2+ D (OP/WH)	2+ D (WH)
	Glial	1/2+ F (OP)	1+ M (OP)	2+ M (OP)	2+ M (OP)	2+ D (OP/WH)
	Perivascular	0	2+ M (OP)	3+ M (OP)	3+ D (OP)	3+ D (OP)
	Non-nucleated	0	1+ F(OP)	2+ F (OP)	3+ M (OP&WH)	3+ M (WH)
4	Neurons	N/A *	2+ D (OP)	3+ D (OP/WH)	3+ D (WH)	3+ D (WH)
	Glial	N/A	2+ D (OP)	2+ D (OP/WH)	3+ D (WH)	3+ D (WH)
	Perivascular	N/A	3+ M (OP)	3+ D (OP)	3+ D (WH)	3+ D (WH)
	Non-nucleated	N/A	2+ F (OP)	3+ M (OP/WH)	3+ M (WH)	3+ D (WH)
5	Neurons	2+ M (OP)	2+ D (OP)	3+ D (OP/WH)	3+ D (WH)	3+ D (WH)
	Glial	2+ M (OP)	2+ D (OP)	2+ D (OP/WH)	3+ D (WH)	3+ D (WH)
	Perivascular	2+ M (OP)	3+ M (OP)	3+ D (OP)	3+ D (WH)	3+ D (WH)
	Non-nucleated	1+ F (OP)	2+ F (OP)	3+ M (OP/WH)	3+ M (WH)	3+ D (WH)

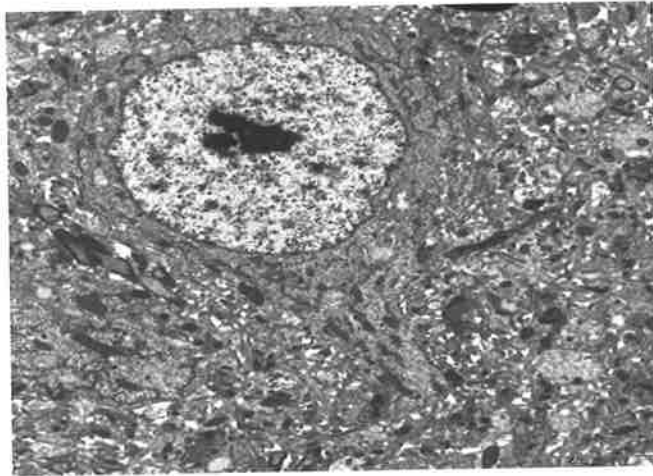
Figure 6.3: EM of cortical neurons representative of three-week old MPS IIIA mice in selected treatment groups.

- A) EM of a cortical neuron in an untreated MPS IIIA mouse, showing a moderate number of empty lysosomal vacuoles. (Note, not all neurons at this age contained storage vacuoles, but those that were storing were to the extent represented in this figure.) Uranyl acetate/lead citrate; 4,600X
- B) EM of a cortical neuron in an MPS IIIA mouse treated from birth. Of note is the absence of storage vacuoles and the intact shape of the neuron. Uranyl acetate/lead citrate; 4600X.
- C) EM of a cortical neuron representative of the few neurons observed with storage vacuoles in MPS IIIA mice treated from birth. Uranyl acetate/lead citrate; 4,600X.

A)



B)



C)

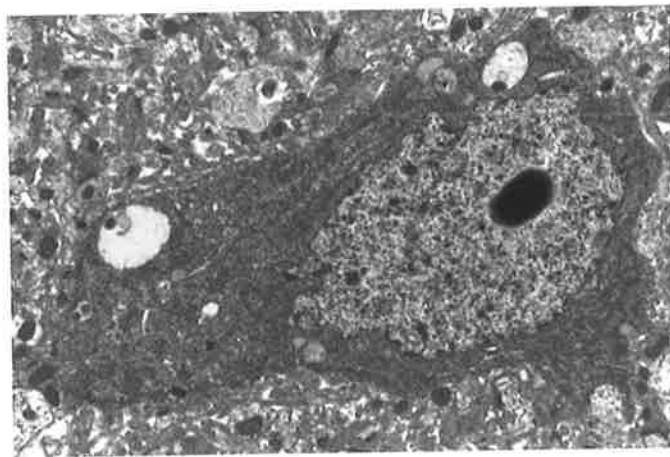
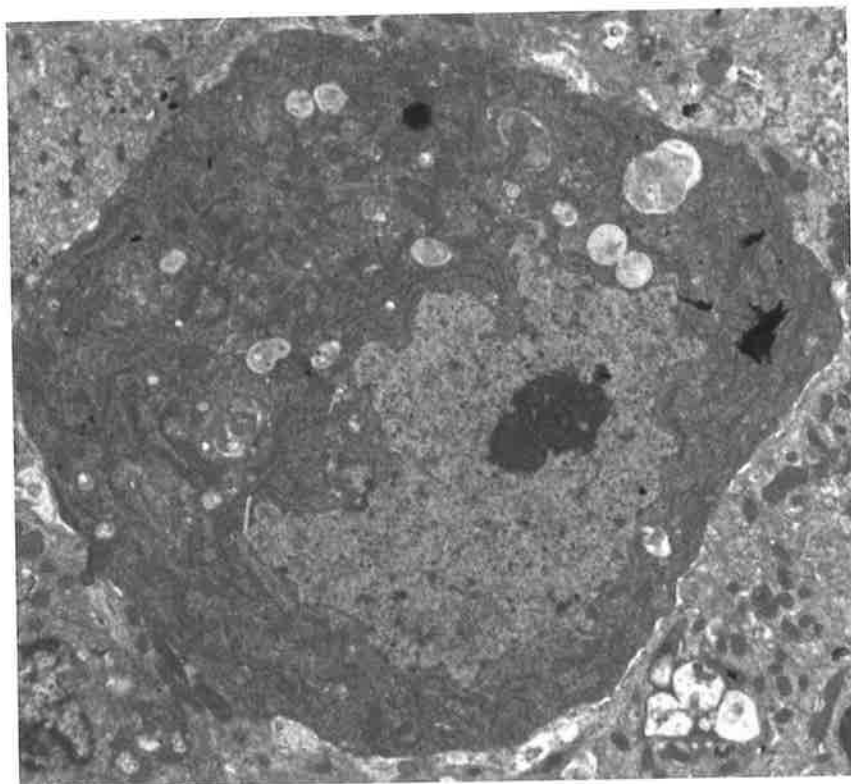


Figure 6.4: EM of cerebellum Purkinje cells representative of three-week old MPS IIIA mice in selected treatment groups.

- A) EM of a Purkinje cell storing small cytoplasmic inclusions in the cerebellum of untreated MPS IIIA mice. Uranyl acetate/lead citrate; 2,600X
- B) EM of a typical Purkinje cell in MPS IIIA mice treated with enzyme from birth. There was no evidence of storage vacuoles in any of the Purkinje cells observed from this section. Uranyl acetate/lead citrate; 1,450X

A)



B)

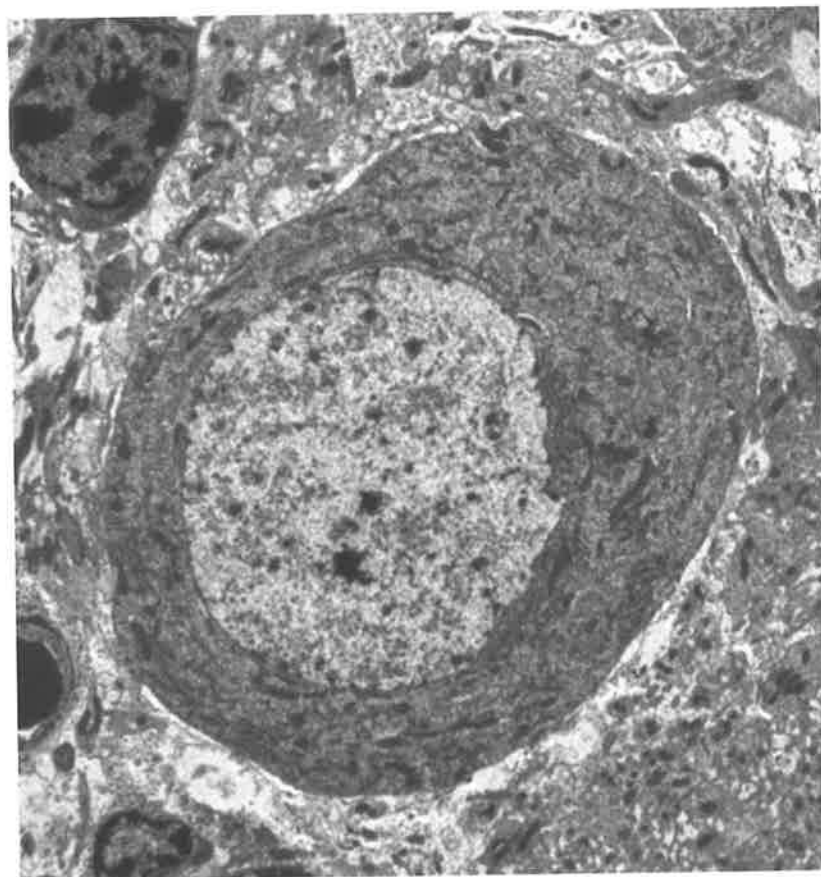
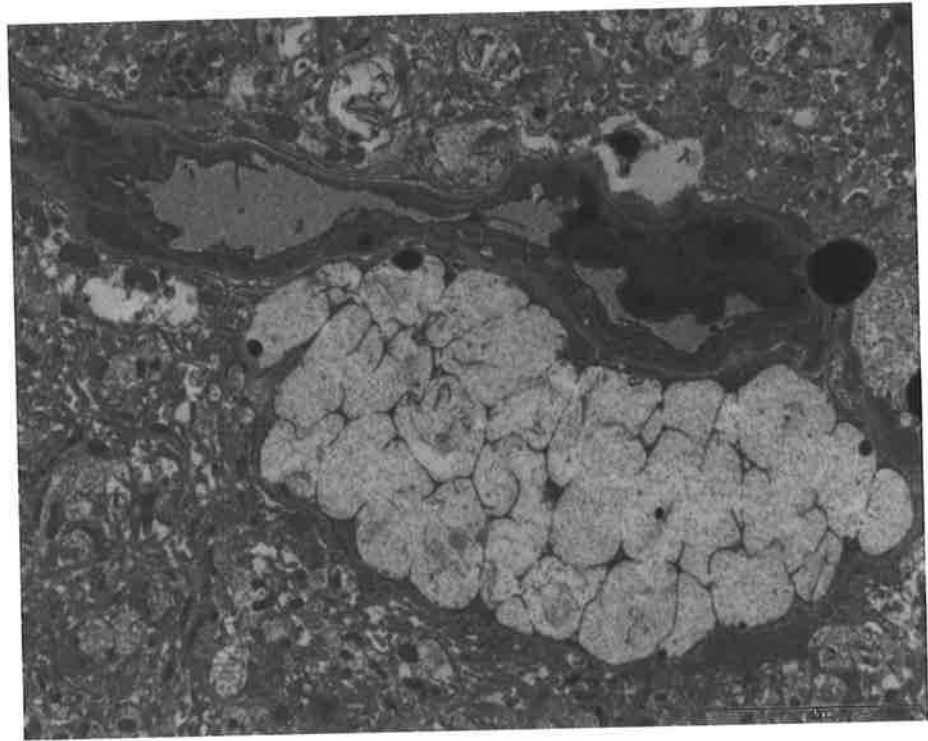


Figure 6.5: EM of perivascular cells representative of three-week old MPS IIIA mice in selected treatment groups.

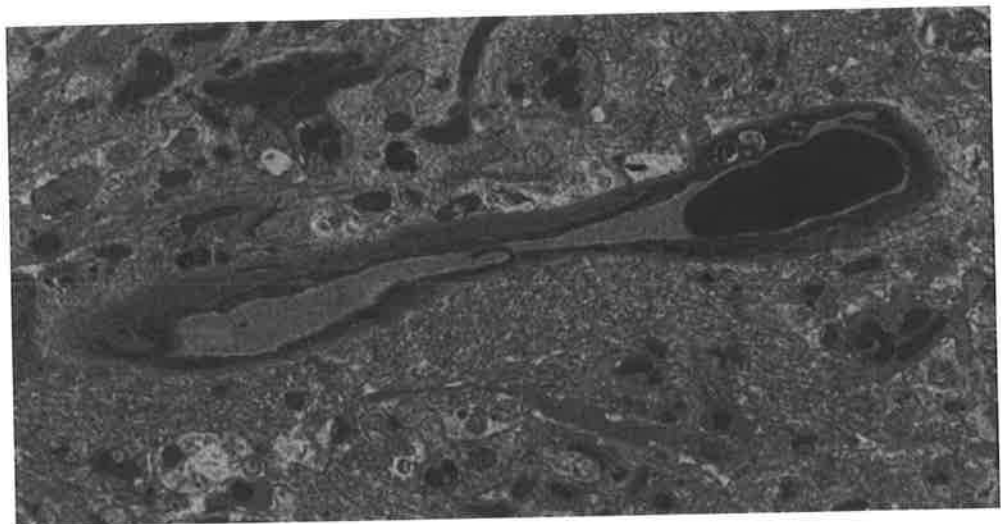
- A) EM of a typical perivascular cell in an untreated MPS IIIA mouse, showing numerous large empty lysosomal vacuoles. Uranyl acetate/lead citrate; 3,400X.

- B) EM of a typical perivascular cell in an MPS IIIA mouse treated with enzyme from birth, cleared of storage vacuoles. Uranyl acetate/lead citrate; 4,600X.

A)



B)



Purkinje cells of the cerebellum in treated from birth MPS IIIA mice did not contain storage vacuoles at three-weeks of age (Figure 6.4B).

By eight-weeks of age storage vacuoles had started to return to the cells of the cerebral cortex in birth ERT MPS IIIA mice, with the extent of vacuolation being moderately less than that observed in untreated MPS IIIA mice at three-weeks of age (Figures 6.6A, C). The number of cells with storage vacuoles in untreated MPS IIIA mice had increased dramatically by eight-weeks of age when compared with three-week old mice, with nearly all neuron and glial cells graded containing medium to large clear, empty inclusions. This pattern of vacuolation was also observed in delayed ERT MPS IIIA mice (Figure 6.6B). In comparison to neurons observed in normal mice, the architectural shape of neurons containing storage vacuoles in the untreated and delayed ERT group by this age had started to change (Figure 6.6D). Neurons appeared swollen and were distended.

The pattern of storage observed at 14-weeks of age in birth ERT MPS IIIA mice was similar but slightly less than that seen in untreated MPS IIIA mice at eight-weeks of age (Figure 6.7C). Most cells graded contained a moderate number of storage vacuoles that were empty inclusions. In contrast, untreated and delayed ERT MPS IIIA mice demonstrated severe storage in most cells graded and, apart from the perivascular cells, the inclusions observed were membranous whorls and stacks which were very flocculent in nature (Figures 6.7A, B). A higher magnification of the stack nature of these inclusions can be seen in Figure 6.7C. The perivascular cells still contained clear open vacuoles. By 14-weeks of age the Purkinje cells in untreated MPS IIIA mice and delayed ERT MPS IIIA mice contained cytoplasmic inclusions that were much larger than those observed in cortical neurons from these mice (Figures 6.8A, B). The content of these inclusions also appeared different to what was observed in the inclusions of cortical neurons. In contrast to either the empty or whorl and stack nature of lysosomal inclusions observed in cortical neurons, the cytoplasmic inclusions of the Purkinje cells in these mice displayed a very granular, foamy-like appearance. The Purkinje cells in birth ERT MPS IIIA mice

Figure 6.6: EM of cortical neurons representative of eight-week old MPS IIIA mice in selected treatment groups.

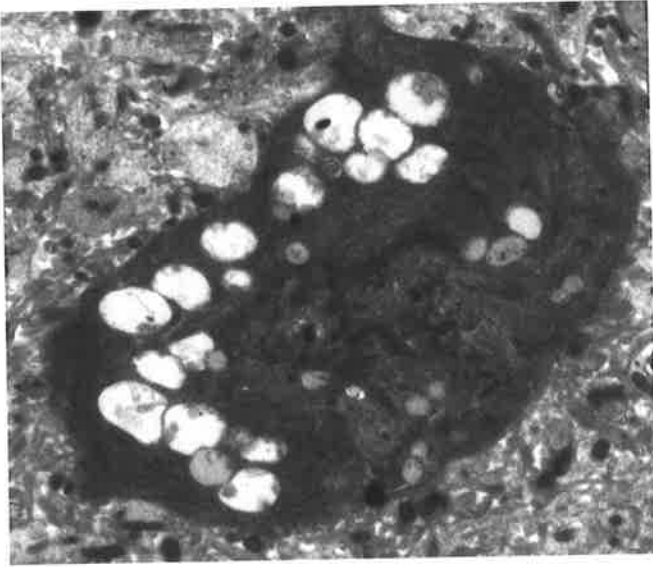
- A) EM of a neuron in an untreated MPS IIIA mouse, showing numerous large empty lysosomal vacuoles. Uranyl acetate/lead citrate; 4,600X.

- B) EM of a neuron in an MPS IIIA mouse treated from six-weeks of age; numerous large empty lysosomal vacuoles are observed, just as in (A) above. Uranyl acetate/lead citrate; 4, 600X.

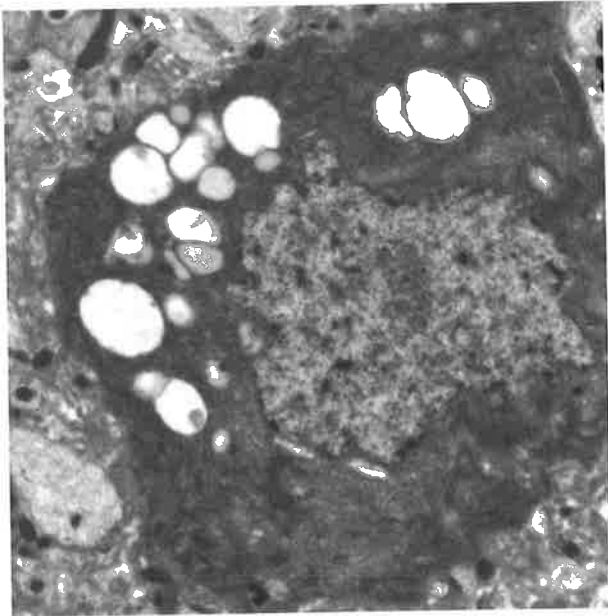
- C) EM of a neuron in an MPS IIIA mouse treated from birth; few empty storage vacuoles are observed in comparison to either (A) or (B) above. Uranyl acetate/lead citrate; 4,600X.

- D) EM of a typical neuron in an eight-week old normal mouse, for comparison. Note the nice architectural shape of the cell. Uranyl acetate/ lead citrate; 4,600X.

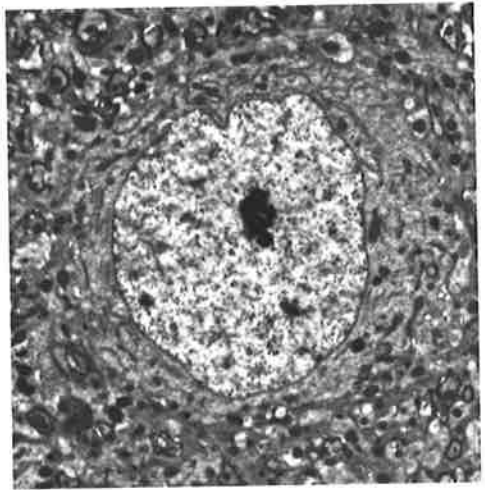
A)



B)



D)



C)

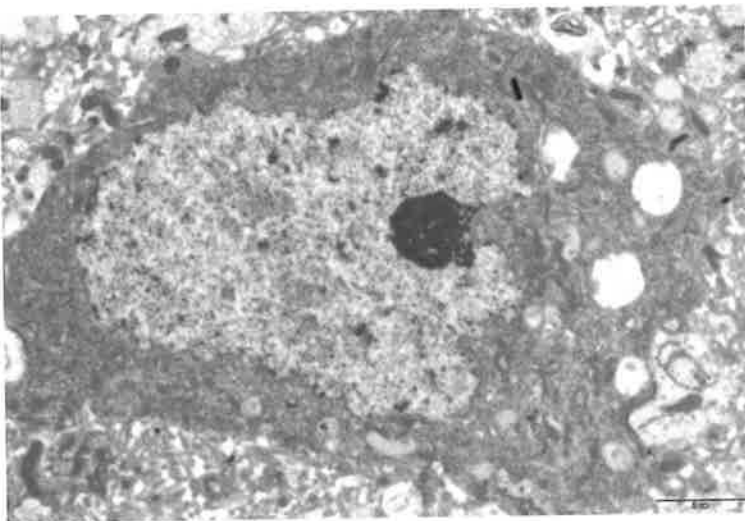
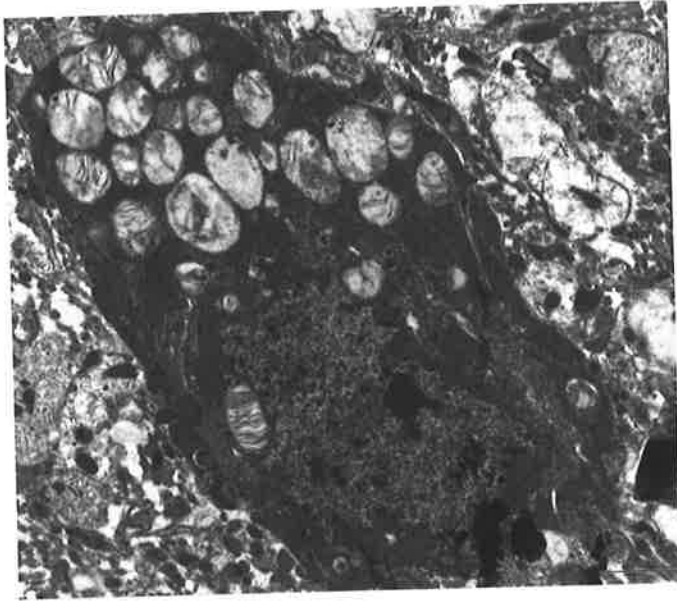


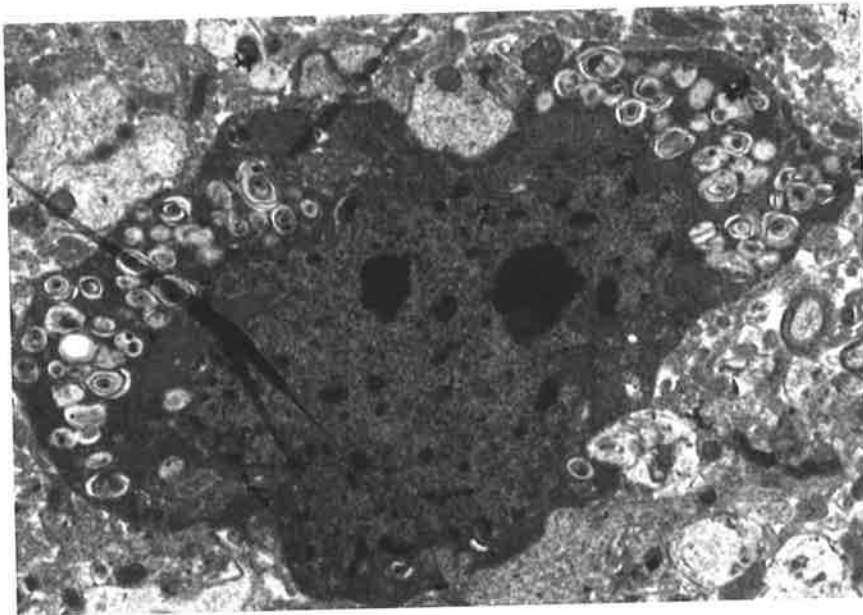
Figure 6.7: EM of cortical neurons representative of 14-week old mice in selected treatment groups.

- A) EM of a neuron in an untreated MPS IIIA mouse. Numerous large lysosomal inclusions with a flocculent appearance are observed. Uranyl acetate/lead citrate 3,400X.
- B) EM of a neuron in an MPS IIIA mouse treated from six-weeks of age, also showing numerous storage stacks and whorl vacuoles, a pattern characteristic of storage of lipid-like material. Uranyl acetate/lead citrate; 3,400X.
- C) EM of a neuron in an MPS IIIA mouse treated from birth, showing a moderate number of clear, empty lysosomal inclusions. Uranyl acetate/lead citrate; 4,600X.
- D) (Next page) High magnification of inclusion in untreated MPS IIIA mouse (from A above). Uranyl acetate/lead citrate; 25,000X.

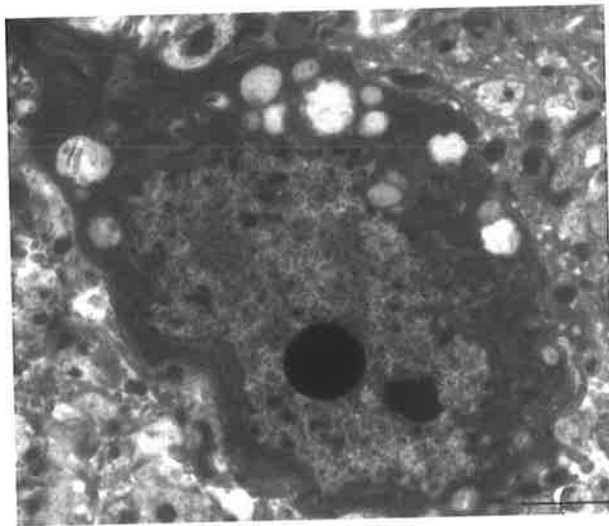
A)



B)



C)



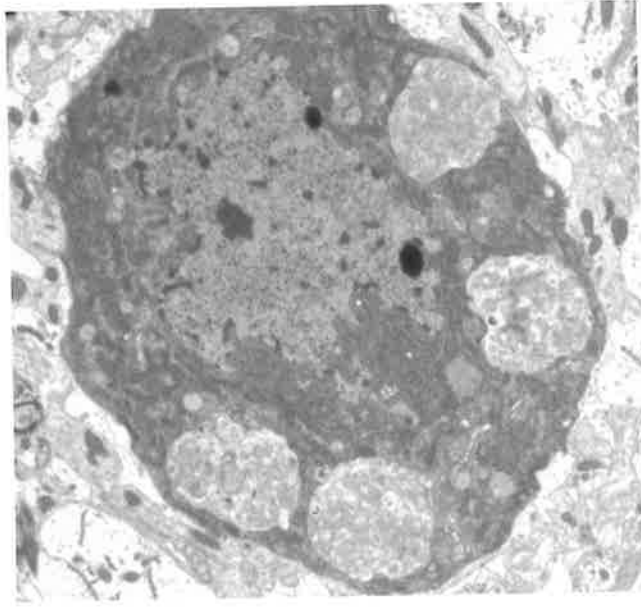
D)



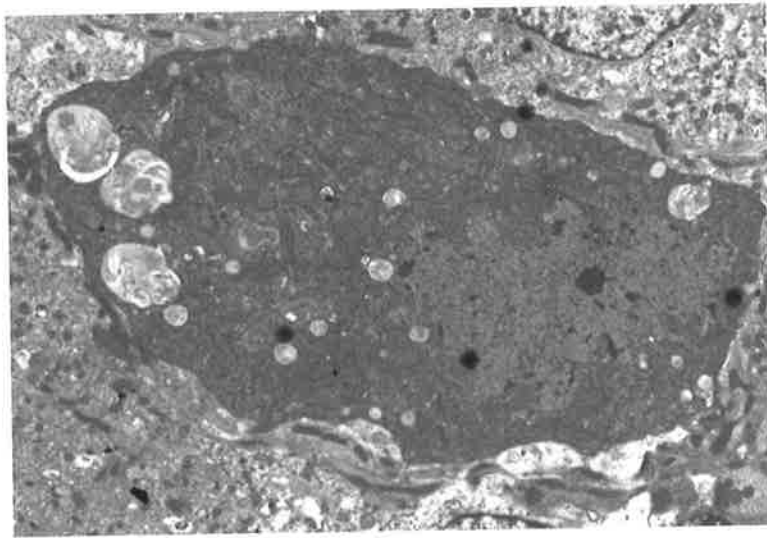
Figure 6.8: EM of cerebellar Purkinje cells representative of 14-week old MPS IIIA mice in selected treatment groups.

- A) EM of a Purkinje cell in an untreated MPS IIIA mouse; huge cytoplasmic inclusions with a granular appearance are observed. Uranyl acetate/lead citrate; 3,400X.
- B) EM of a Purkinje cell in an MPS IIIA mouse treated from six-weeks. As in (A) above; large and also small cytoplasmic inclusions with a granular appearance are noted. Uranyl acetate/lead citrate; 2,600X.
- C) EM of a Purkinje cell in an MPS IIIA mouse treated from birth; big, open lysosomal inclusions are observed. Granular cytoplasmic inclusions observed in (A) and (B) above, appear to be absent from these cells. Uranyl acetate/lead citrate; 3,400X.

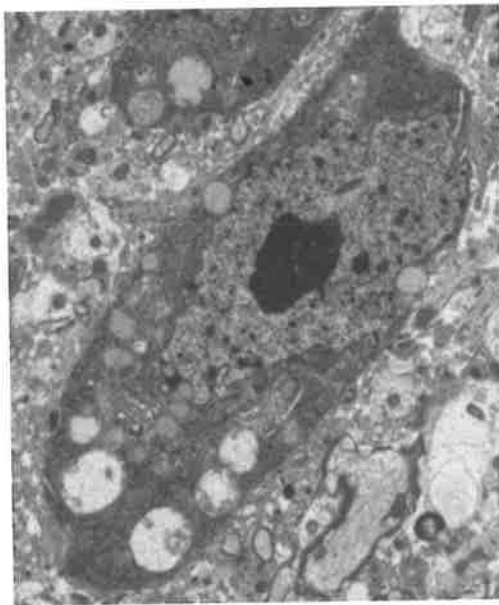
A)



B)



C)



contained large, relatively empty membrane-bound vacuoles (Figure 6.8C), although a slight granular appearance was noted in some vacuoles.

One cortical neuron, observed in the 14-week of age untreated MPS IIIA mice, contained a large cytoplasmic body with small- to medium bodies surrounding it (Figure 6.9A). This body had a foamy appearance, similar to that observed in the Purkinje cells, however, upon higher magnification (Figure 6.9B), small whorl-like structures within the membrane-bound body were observed.

Membranous inclusions of whorls and stacks present in the cells of 14-week old untreated MPS IIIA mice were becoming apparent in the same cell types as seen in the birth ERT mice by 18-weeks of age (Figure 6.10C). However, in the cortical neurons at least, the majority of cells contained a mixture of open and flocculent vacuoles. Eighteen-week old untreated and delayed ERT MPS IIIA mice contained numerous storage vacuoles that were abundant in all cells graded and all inclusions were membranous whorls (Figures 6.10A, B). A higher magnification inclusion can be seen in Figure 6.10C. A more whorl-like pattern is observed in these inclusions, in comparison to the stack-like nature of 14-week old untreated or delayed ERT MPS IIIA mice. The pattern of storage in Purkinje cells was similar to that observed at 14-weeks of age.

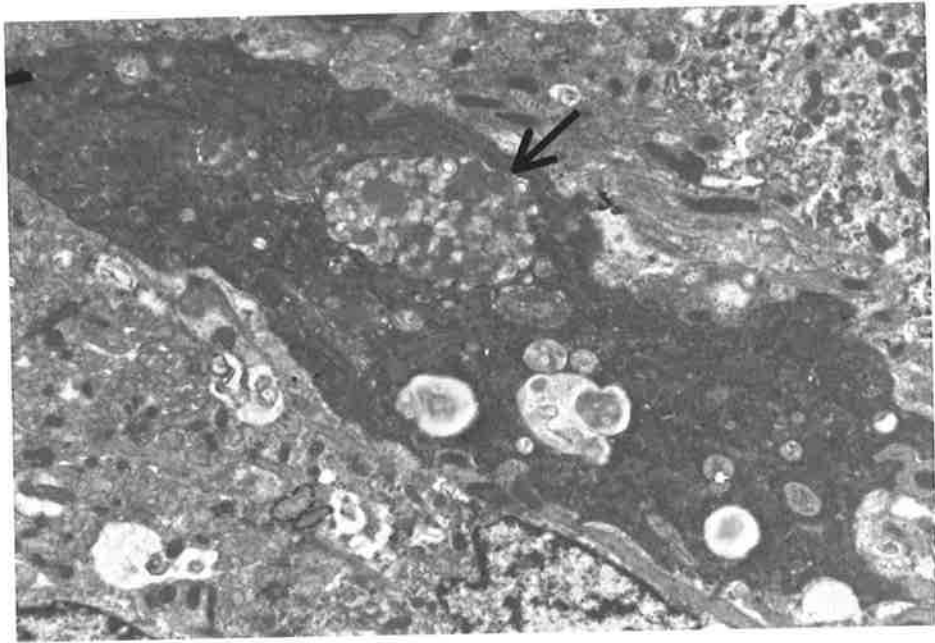
It was generally difficult to observe differences in the amount and type of storage vacuoles between birth ERT, delayed ERT or untreated MPS IIIA mice, by 23-weeks of age. MPS IIIA mice treated with enzyme from birth were storing membranous lipid containing vacuoles in their neurons by this age, but not to the same extent seen in untreated or delayed ERT mice (Figures 6.11A, B, C). The whorl pattern observed in the vacuoles of untreated or delayed ERT MPS IIIA mice at this age were more concentrated than what was observed at 18-weeks of age (Figure 6.11D compared with Figure 6.10C). However, the grading system employed was not able to distinguish these finer differences which were visible by eye.

Figure 6.9: EM of a cortical neuron from a 14-week old untreated MPS IIIA mouse.

- A) Neuron containing large cytoplasmic inclusions. A huge cytoplasmic inclusion with a foamy-like appearance is highlighted by the arrow head and is magnified in (B). Uranyl acetate/lead citrate; 2,600X.

- B) Higher magnification of foamy-like cytoplasmic inclusion. Uranyl acetate/lead citrate; 7,900X.

A)



B)

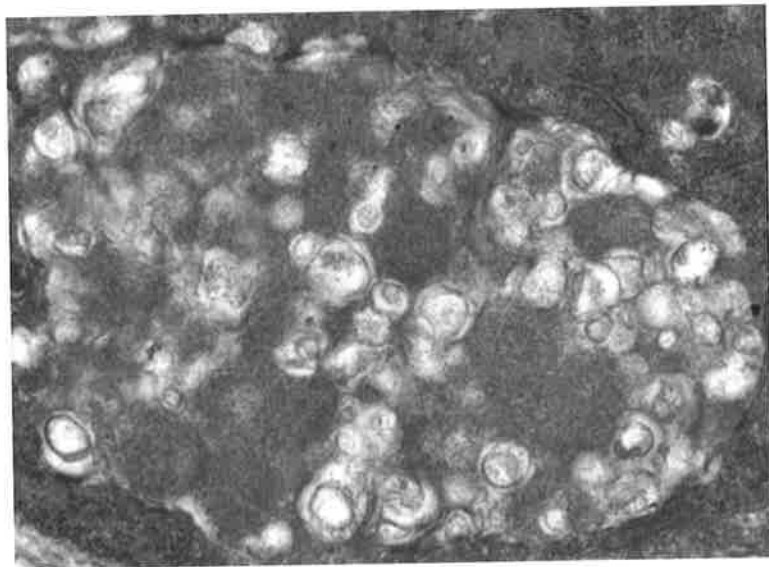


Figure 6.10: EM of cortical neurons representative of 18-week old MPS IIIA mice in selected treatment groups.

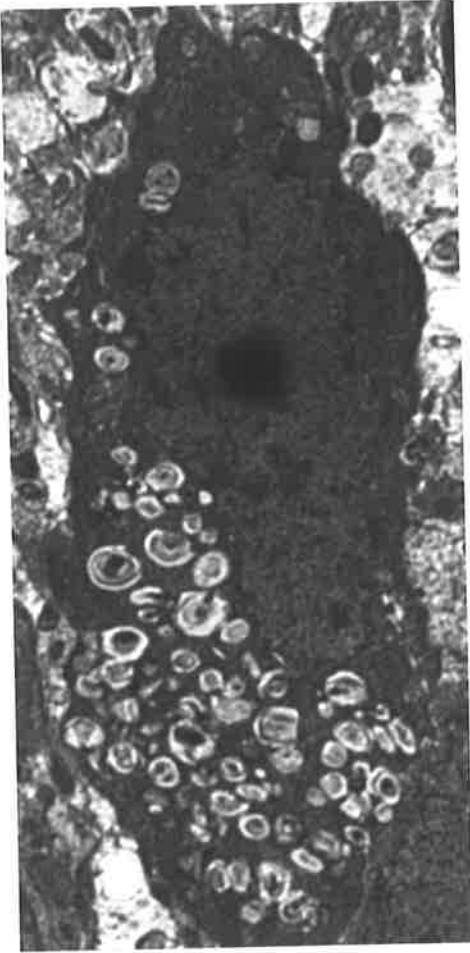
- A) EM of a neuron in an untreated MPS IIIA mouse, demonstrating abundant storage vacuoles with a characteristic whorls and stack appearance. Uranyl acetate/lead citrate; 3,400X.

- B) EM of a neuron in an MPS IIIA mouse treated from six-weeks, showing numerous storage vacuoles with a whorl appearance, a characteristic pattern of ganglioside storage. Uranyl acetate/lead citrate; 3,400X.

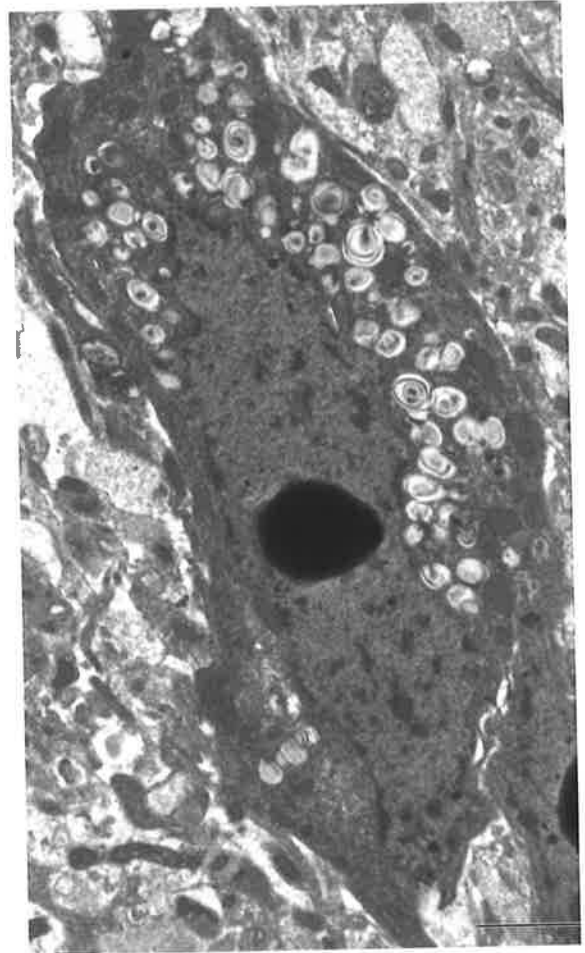
- C) EM of a neuron in an MPS IIIA mouse treated from birth. A moderate number of storage vacuoles are observed, some demonstrating whorls characteristic of ganglioside storage. Uranyl acetate/lead citrate; 3,400X.

- D) (Next page) EM of enlarged lysosomal vacuole from (A) above. The pattern is typical of ganglioside storage. Uranyl acetate/lead citrate; 19,000X.

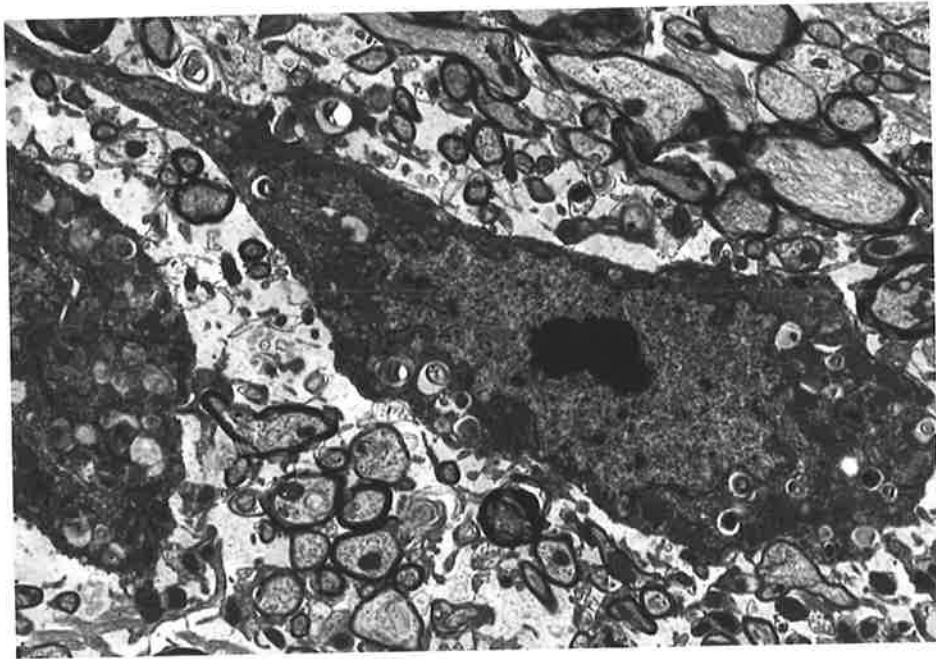
A)



B)



C)



D)

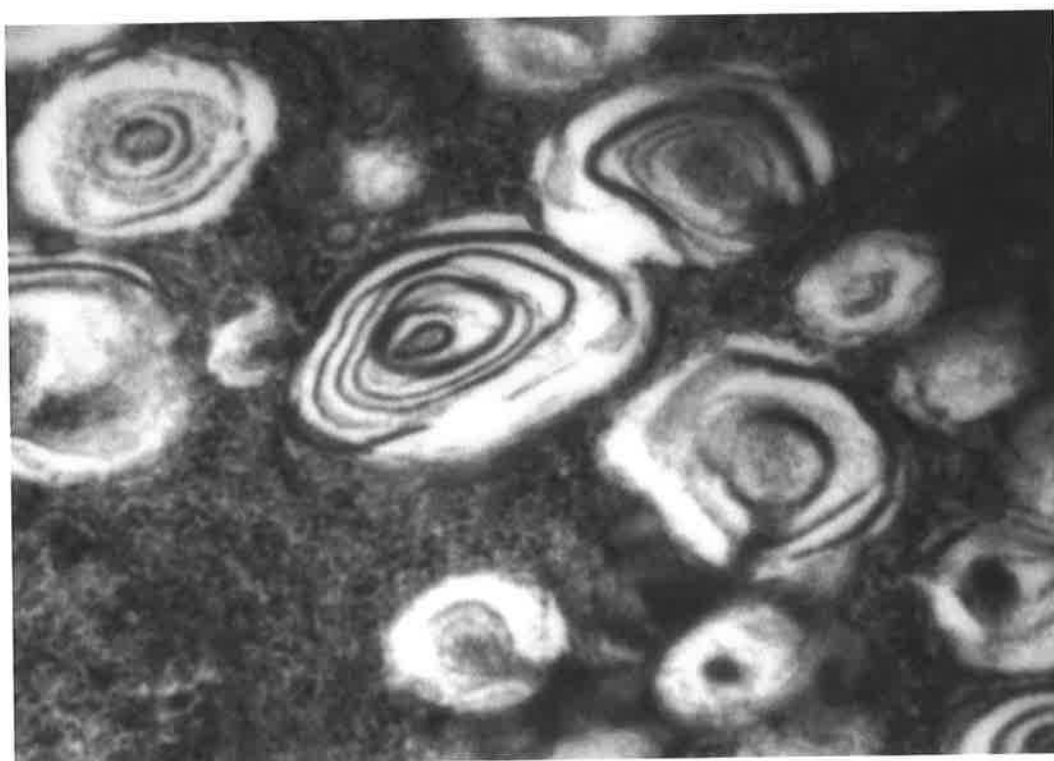


Figure 6.11: EM of cortical neurons representative of 23-week old MPS IIIA mice in selected treatment groups.

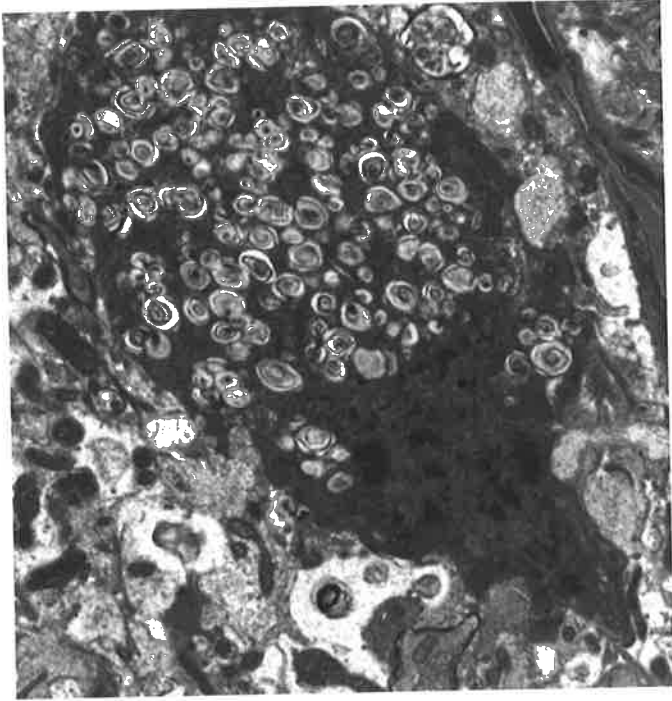
- A) EM of a neuron in an untreated MPS IIIA mouse, demonstrating abundant storage vacuoles, with whorl inclusions characteristic of lipid/ganglioside storage. Uranyl acetate/lead citrate; 3,400X.

- B) EM of a neuron in an MPS IIIA mouse treated from six-weeks, also showing numerous vacuoles with whorl inclusions. Uranyl acetate/lead citrate; 3,400X.

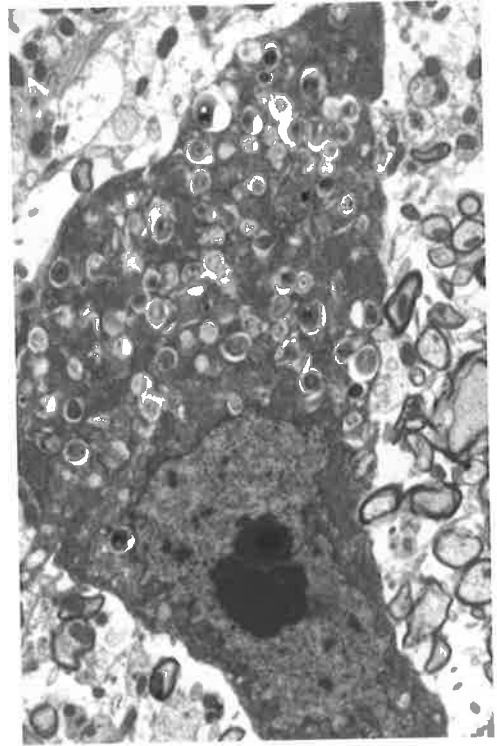
- C) EM of a neuron in an MPS IIIA mouse treated from birth. Most of the lysosomal inclusions observed have a whorl and stack appearance, but to a lesser extent to that observed in (A) and (B) above. Uranyl acetate/lead citrate; 4,600X.

- D) (Next page) High magnification of inclusion from (B) above. Uranyl acetate/lead citrate; 64,000X.

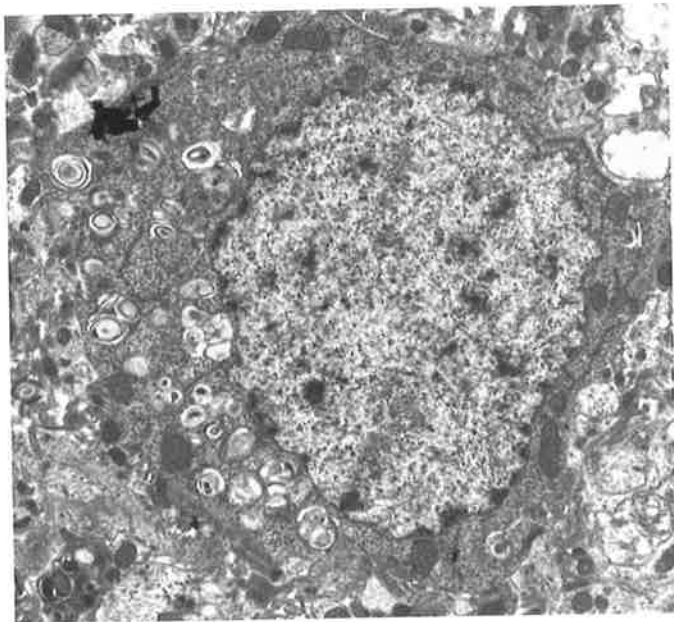
A)



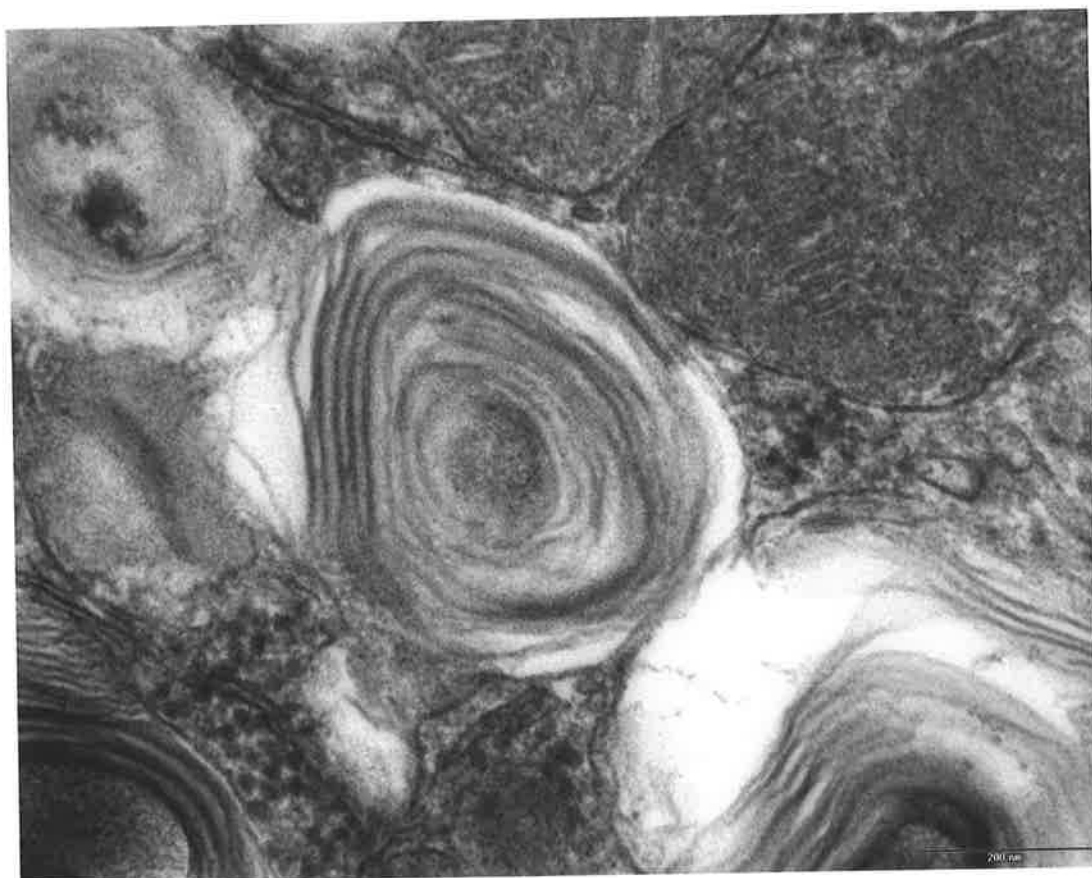
B)



C)



D)



As expected, all of the sections graded from the two groups of normal mice did not contain storage vacuoles in any CNS cells at all of the ages studied.

Liver sections from 14-week old mice in all five treatment groups were analysed for the presence or absence of storage vacuoles by EM. Untreated MPS IIIA mice displayed abundant storage in hepatocytes and Kupffer cells; inclusions were electron lucent and membrane-bound. In comparison, MPS IIIA mice treated from birth or from six-weeks, demonstrated a marked reduction in storage vacuoles in the same cell types. However, the delayed ERT group demonstrated relatively more vacuoles than was observed in the birth ERT group

6.4 DISCUSSION

The major aim of experiments described in this chapter was to evaluate and compare the efficacy of weekly ERT from birth or from six-weeks of age in MPS IIIA mice for a duration of 20-weeks at a preliminary dose rate of 1 mg/kg rhNS.

As mentioned previously evidence suggests that enzyme administered to newborn mice should gain access to the CNS due to the presence of an immature BBB in the first two-weeks of life (see Section 6.1). Enzyme distribution studies in newborn and six-week old MPS IIIA mice demonstrate the presence of rhNS activity in the brain of newborn mice but not in the brain of six-week old mice. From a study comparing NS protein activity from fibroblasts of 35 MPS IIIA patients, presenting from a few years to 40-years of age, it was proposed that ERT achieving a correction of approximately 10% of normal NS activity is required to avoid the onset of an MPS IIIA clinical phenotype (Perkins, et al., 2001). One-hour after infusion of 1 mg/kg rhNS in newborn MPS IIIA mice, enzyme levels were detected in the brain, with higher levels in the liver; enzyme levels in the brain had doubled by four-hr post-infusion, and then levels started to decrease, with insignificant levels observed 48-hr post-infusion. In comparison, one-hr after infusion of 1 mg/kg rhNS in six-week-old MPS IIIA mice, widespread tissue uptake of enzyme had occurred, with the highest levels observed in the liver followed by spleen, kidney, heart; no enzyme was detected in brain.

A reduction in lysosomal storage material in neurons of the cerebral cortex in the birth ERT MPS IIIA mice correlates with the observed improvements in behaviour. A striking difference in the second behavioural developmental stage, which is characterised by an aggressive phenotype in male MPS IIIA mice, was observed between the birth ERT and untreated or delayed ERT mice. The aggressive nature of MPS IIIA mice first observed in untreated or delayed ERT mice at 10-weeks of age, was delayed in the birth ERT MPS IIIA mice until 17-weeks of age. Unfortunately, aggression in the MPS IIIA mice was not quantitated in this study and was solely determined by the development of bites on the lower part of the abdomen. A better understanding of this behavioural phenotype would

be beneficial. A test to measure aggressiveness in mice would provide another behavioural test to complement the MWM to assess the efficacy of ERT. Such tests have been established in rodents. There are several stages to an aggressive attack in mice, all of which can be measured (Miczek *et al.*, 2001). The *attack bite* is directed preferentially at the back or flanks of the opponent, and bites toward the back are accompanied by kicking movements of the rear legs. This is followed by a *sideways threat*, which is a lateral rotation of the body; it entails short steps, directed toward an opponent. The *tail rattle* is a rapid vibration of the tail and typically occurs just before and during the attack flurry. The tail rattle reflects a state of high arousal and is preferentially seen in dominant mice. Measurement of aggressive behaviour begins with simply tallying: (1) the proportion of animals fighting, (2) the latency for the first attack bite; and (3) the duration of attack bouts or flurries. These three measurements are sufficiently basic and only require the use of timers and counters.

It is unclear whether ERT decreased the hyperactive stage observed in three-week old MPS IIIA mice. The enormous numbers of mice used for experimentation were all weaned on the same day and made assessment of this developmental trait difficult. Generally there was no observable difference when handling the mice from birth to weaning between the experimental groups.

Both treatment groups of MPS IIIA mice were very tolerant to enzyme therapy via administration of rhNS. No significant antibody titres were observed and generally no side effects to the treatment by enzyme were noted. Overall a decrease in body weight approaching normal was observed in both the birth ERT and delayed ERT MPS IIIA mice. By the age of approximately 25-weeks both of the treated groups of MPS IIIA mice still developed coarse fur, similar to that observed in the untreated MPS IIIA mice. One possible explanation for this could be that the enzyme dose rate of 1 mg/kg is insufficient in overcoming this phenotype. Alternatively, enzyme injections ceased by 20-weeks of age and possibly this was too soon to see an improvement in phenotypic appearance. As most of the mice were sacrificed by approximately 30- to 40-weeks of age, we are unable

to speculate whether or not ERT is successful in increasing the lifespan of MPS IIIA mice. MPS VII mice treated with only six weekly intravenous injections of GUSB from birth remained alive after one-year of age. MPS VII mice usually die before one-year of age, so this is a remarkable result (Vogler et al., 1996).

Birth ERT MPS IIIA mice performed significantly better than untreated or delayed ERT MPS IIIA mice in every phase of the MWM test. The performances of birth ERT MPS IIIA mice were similar to normal mice during the acquisition phase, however performance diminished during the re-learning phase. Interestingly, the pattern observed during the re-learning phase between birth ERT MPS IIIA mice and normal mice was similar to what was observed between MPS IIIA mice (untreated) and normal mice at 15-weeks of age during the preliminary MWM studies (discussed in Chapter 5.0).

With the hindsight of histology performed in this chapter we can now make some interesting comparisons between the amount and type of lysosomal storage accumulating with age, and performance in the MWM. CNS lysosomal storage is established by three-weeks of age in MPS IIIA mice and gradually increases in severity with age. The majority of storage in neurons is located within membrane-bound lysosomes in the cell soma. Before 14-weeks of age the inclusions appeared as clear, empty vacuoles. Presumably these vacuoles would be storing HS, which is extracted during the EM processing method. After 14-weeks of age inclusions appeared as membranous whorls and stacks, a pattern characteristic of ganglioside storage. MPS IIIA mice are reported to secondarily store G_{M2} and G_{M3} gangliosides (Bhaumik et al., 1999) (see Section 1.14). This secondary storage product may result from GAG interference with ganglioside catabolism (Baumkötter and Cantz, 1983; Avila and Convit, 1975). Sulphated GAG such as HS have been reported to inhibit G_{M3} sialidase and other studies have shown an inhibitory effect by HS on 27 different lysosomal hydrolases (Avila et al., 1975). Another explanation is that certain GAG structures may form complexes with gangliosides, therefore preventing effective enzymatic degradation (Lullmann-Rauch et al., 1995). Other perturbations in the ganglioside catabolic pathway could involve activator proteins and alternate pathways

related to variable specificities of the involved lysosomal hydrolases. Membranous whorls and stacks have been reported in the wire-haired Dachshund, and New Zealand Huntaway dog MPS IIIA models (Fischer et al., 1998; Jolly et al., 2000) and also in the MPS IIID goat (Jones et al., 1998). Severity of the neurological disorder in murine MPS IIIA appears to parallel the accumulation of the CNS gangliosides. It has previously been proposed that the accumulation of gangliosides is probably the cause of the neurological deficits observed in MPS that secondarily store these compounds (Walkley, 1998). It is possible that the accumulation of G_{M2} and G_{M3} may have devastating effects on one or more processes in which gangliosides are important, such as dendritogenesis, modulation of numerous growth factor receptors and second messenger systems, and also cell adhesion properties (Walkley et al., 1995; Goodman and Walkley, 1996). From our observations, untreated MPS IIIA mice start accumulating glycolipids, most likely to be gangliosides at about 14-weeks of age; it was at 15-weeks of age that a difference in performance in the MWM was observed between MPS IIIA and normal mice, taking the MPS IIIA mice longer to find the platform in every phase of the test. From this information we can hypothesise that the primary storage of HS may not be solely responsible for the observed cognitive deficits in MPS IIIA mice, and that the secondary accumulation of gangliosides may contribute to these deficits.

We can correlate this finding in the MPS IIIA mice to what is observed in human MPS III patients. The morphological abnormalities seen in the CNS of MPS IIIA mice are similar to those reported for human Sanfilippo and MPS syndromes with HS accumulation (Hadfield et al., 1980; Ghatak et al., 1977; Kriel et al., 1978; Jones et al., 1997). An epidemiological survey performed on parents of MPS III children, which focused on the developmental acquisition and subsequent loss of cognitive and social/adaptive behaviour milestones and behaviour problems arising during the course of the disorder (Nidiffer and Kelly 1983), demonstrated that MPS III children begin to deteriorate cognitively between the ages of 3¹/₂ and 6¹/₂ years, and that by eight-years of age, marked cognitive and developmental milestone losses were inevitable. This included specifically the loss of language and memory functions as the first significant sign of cognitive decline. There is

little information available on CNS histology with age in MPS III patients, however tissue samples obtained at post-mortem examination of an eight-year old MPS III patient (Constantopoulos et al., 1980) reveal loss of Purkinje cells and also focal dendritic swelling. Cells in the cerebellar cortex contained lysosomes exhibiting granular content, densely packed membranes and characteristic whorls and stacks (Constantopoulos et al., 1980). Neurons from an eight-year old patient with MPS IIIA (Ferrer et al., 1988) demonstrated markedly increased levels of the gangliosides G_{M2} , G_{M3} and G_{D3} . The correlation between a dramatic decline in cognitive function and the accumulation of gangliosides in the CNS cells of MPS III patients parallels what we see in the MPS IIIA mice.

Quantitation of gangliosides and HS in the CNS in mice would give added information about the relative levels of storage products. It is possible to analyse gangliosides using electrospray ionisation-tandem mass spectrometry (Whitfield et al., 2000). Preliminary results demonstrate a 30-fold increase in G_{M2} ganglioside and a 50-fold increase in G_{M3} ganglioside in the brain of MPS IIIA mice over normal mice (Singh, H., unpublished observations). The Department of Chemical Pathology has recently developed specific methods to quantitate sulphated oligosaccharides that represent partially degraded HS. These oligosaccharides have been shown to be elevated 1,000-fold in tissues and urine from MPS IIIA mice (King, B., Fuller, M. and Ramsay, S., unpublished observations).

We can presume that probably only the first two infusions of enzyme were successful in reaching the CNS of mice treated from birth, resulting in a decrease in lysosomal storage. Negligible storage was demonstrated in these mice at three-weeks of age, the earliest age analysed histologically. It remains unknown if storage vacuoles are present at birth in MPS IIIA mice and are therefore being cleared by NS infusions, or if the enzyme infusions are preventing the accumulation of storage material at this age. A persistent reduction in storage vacuoles in the cerebral cortex was observed up to 18-weeks of age in MPS IIIA mice treated from birth, in comparison to untreated MPS IIIA mice. However, the accumulation of glycolipids presumed to be gangliosides did begin to occur

in the cells of these mice by 18-weeks of age, although the extent was minimal compared with that observed in 14-week old MPS IIIA untreated mice. This initiation of glycolipid (presumed to be ganglioside) accumulation may be responsible for the slight decline in performance of the birth ERT MPS IIIA mice during the re-learning phase, when compared to normal mice in the MWM. Even though a slightly increased latency (10-sec) to find the platform was observed in these mice over normal mice, it was clear that birth ERT MPS IIIA mice were far superior in their ability to locate the hidden platform than the untreated or delayed ERT MPS IIIA mice.

The unusual cytoplasmic body observed in the untreated MPS IIIA mouse at 14-weeks of age could possibly be one of a number of oddities reported in the storage of CNS cells of certain neuronal storage diseases, such as a meganeurite or an axonal spheroid body. Meganeurites have been identified in a feline model of G_{M1} gangliosidosis (Purpura et al., 1978) and the murine MPS VII model (Levy et al., 1996). These anomalies have also been reported in the brain of patients with G_{M1} and G_{M2} gangliosidosis (Walkley, 1998). Meganeurites contain storage material consistent with the specific defective lysosomal hydrolase. Axonal spheroids, however, contain a vast array of different materials with ultrastructural features consisting of tubulovesicular profiles, dense bodies, mitochondria and related materials (Walkley, 1998). Spheroid bodies have been reported to be abundant in gray matter areas like the cerebral cortex. Also, one type of neuron most susceptible to spheroid formation is the cerebellar Purkinje cell, a cell type that is vulnerable to cell death in storage disease (Walkley, 1998).

A loss of Purkinje cells has been reported in the MPS IIIA wire haired Dachshund (Jolly et al., 2001) and New Zealand Huntaway dog (Jolly et al., 2000). Due to the limited number of Purkinje cells present in the EM sections of the cerebellum it was not possible to state whether the same occurs in the MPS IIIA mice and, if so, whether or not it is corrected by ERT. The nature of the unusual and huge cytoplasmic inclusions present in the Purkinje cells of 14-week or older MPS IIIA mice has not been reported in either of the MPS IIIA dog models. The nature of storage material in these inclusions remains

unclear and it is possible that these inclusions are spheroid bodies as mentioned above. However, MPS IIIA mice have been reported to store autofluorescent material and free cholesterol secondary to G_{M2} and G_{M3} gangliosides, therefore tertiary to HS (Walkley et al., 2002). Furthermore, these storage products accumulate in neurons, where the degree of intraneuronal storage of these non-GAG compounds appears to exceed that of HS and, in many instances, occurred in separate vacuoles (Walkley et al., 2002). It would be interesting to carry out *in situ* studies on the Purkinje cell sections, such as immunohistochemistry, to detect gangliosides and filipin histochemistry to detect cholesterol to determine the nature of the storage products.

No difference in performance was observed between normal mice treated and untreated during the MWM, suggesting that an excess of NS does not have a deleterious effect on cognitive function in mice. In contrast, an excess of GUSB was found to induce spatial learning deficits in normal mice when assessed via the MWM (O'Connor et al., 1998).

In conclusion, ERT was effective in restoring (or maintaining) cognitive deficits characteristic in MPS IIIA mice, but only if initiated at birth. ERT was unsuccessful in altering the neurological impairment in mice where treatment was delayed until six-weeks of age. An interesting relationship between the onset of learning and memory loss and the accumulation of secondarily stored gangliosides was highlighted in this chapter, and, for a more prolonged therapeutic response, needs to be evaluated further. The finding that enzyme entering the brain in the first two-weeks of life, resulted in remarkable improvements in the MWM not performed until 20-weeks of age. This suggests that human MPS patients exhibiting CNS pathology may only require enzyme in the CNS early in life followed by monthly (or longer) infusions of enzyme into the CNS to give significant improvements in quality of life. MPS VII mice who received weekly intravenous injections during the first five-weeks of life and then monthly enzyme replacement beyond this time displayed a persistent therapeutic response as indicated by a decrease in lysosomal storage in the liver, spleen, bone, meninges and cortical neurons (Sands et al., 1997). The evidence that storage vacuoles will accumulate in the cells

where enzyme is not being continuously supplied such as in the CNS, require the development of alternate therapeutic protocols that could be used in conjunction with ERT, with the aim to give a more prolonged response. A combination therapy of ERT performed weekly from birth to five-weeks of age, followed by syngeneic BMT in MPS VII mice, provided a more complete therapeutic response than did either ERT or BMT alone (Sands et al., 1997). We would anticipate that the prevention of ganglioside accumulation would have a positive response on CNS pathology in MPS IIIA mice. Substrate deprivation therapy has enhanced survival in both Tay-Sachs and Sandhoff disease mouse models. Sandhoff disease mice treated with a combination therapy of substrate deprivation (NB-DNJ) and BMT survived significantly longer than those treated with BMT or NB-DNJ alone (Jeyakumar et al., 2001). Potentially, a combination therapy of ERT from birth followed by substrate deprivation in MPS IIIA mice may result in prolonged maintenance of neurological function

CHAPTER 7.0

CONCLUSIONS AND FUTURE WORK

7.1 INTRODUCTION

At the outset of this study no effective therapy existed for patients with MPS III and only preliminary short-term ERT trials had been investigated in MPS III animal models (see Section 1.12.1). ERT has proven to be an effective means of treating some LSD patients who do not exhibit CNS pathology and has been successful in treating MPS VII mice if initiated in neonates, before the closure of the BBB. The mouse model of MPS IIIA was chosen as a good model for evaluation of ERT as disease in the mice closely parallels the same disease in humans.

The main objective of this thesis was to evaluate the efficacy of ERT initiated in newborn mice compared with ERT initiated in young adult mice. Before this aim could be achieved it was necessary to characterise MPS IIIA in the mice, and also characterise the D31N mutation giving rise to disease in these animals. The establishment of the MWM to test cognitive function in mice was also needed for critical assessment of ERT.

7.2 SUMMARY

When evaluating therapy protocols in MPS animal models it is advantageous to understand the nature of disease-causing mutations. The identification of mutations that cause disease in animal models allow for the development of screening methods to identify homozygotes and heterozygote carriers, leading to efficient management of the colony. The identification of the mutation that results in MPS IIIA in mice (D31N) permitted the development of a rapid PCR-based screening assay to genotype the mice at birth.

The ability to mate MPS IIIA mice was advantageous in being able to obtain large numbers of affected mice for assessment.

The characterisation of disease-causing mutations allows for a better understanding of the enzymatic and molecular nature of mutant proteins. The D31N mutation was found to have only 1.5% of wild type mNS activity when expressed in CHO-K1 cells. This tells us that the mutant protein expressed in the mice has a very small amount of residual NS enzyme activity. The mutant D31NmNS was shown to have a similar maturation pathway to wild type mNS as analysed by pulse-chase experiments. Wild type mNS display a similar maturation pathway to hNS (Perkins et al., 1999). rmNS also display similar kinetic properties to rhNS (Bielicki et al., 1998). This suggested that the MPS IIIA mice may be tolerant to enzyme therapy by the administration of rhNS. The efficacy of therapy is subject to many factors, including antibody-mediated responses. The potential of an antibody-mediated response is more likely in models where a null mutation is present; in this case the animals have absolutely no active enzyme, mutant or otherwise. A specific IgG response was observed in MPS I dogs when injected with autologous gene-corrected myoblasts (Shull et al., 1996). The MPS I dog contains a null mutation (Menon et al., 1992) in the α -L-iduronidase gene, leading to a rapid decline in circulating α -L-iduronidase, rendering therapy unsuccessful. To some extent this was overcome by the use of anti-rejection drugs. MPS IIIA mice undergoing ERT with weekly injections of rhNS did not develop specific antibodies to this enzyme.

A decline in cognitive function in MPS IIIA mice as assessed by the MWM test, was first observed at 15-weeks of age. A dramatic loss of learning and memory skills in the MPS IIIA mice was demonstrated by 20-weeks of age; interestingly, it is also at this age that MPS IIIA mums will kill and/or eat their babies. This progressive decrease in cognitive function with age correlates with the increasing accumulation of storage in CNS in MPS IIIA mice, particularly with the accumulation of secondary storage product of glycolipids most likely to be gangliosides.

Enzyme distribution studies following the infusion of rhNS in newborn MPS IIIA mice demonstrated a significant amount of NS in the brain and liver. Following infusion of rhNS into six-week old MPS IIIA mice, no NS was detected in the brain, with the majority of enzyme being targeted to the liver, spleen, kidney and, to a lesser extent the heart. These results confirmed the presence of a 'leaky' BBB in neonatal mice, which became fully intact in young adult mice.

ERT initiated in MPS IIIA mice from six-weeks of age was unable to reverse or alter progression of CNS pathology. However, significant improvements in CNS pathology were observed in MPS IIIA mice treated with NS from birth. These studies demonstrated that long-term ERT had minimal or no side effects on the mice, with only one mouse developing an anaphylactic reaction, which only lasted 30-minutes.

7.3 CONCLUSIONS AND IMPLICATIONS

The major conclusion from this work is that ERT with rhNS from birth is able to significantly alter the progression of CNS disease in MPS IIIA mice. Enzyme replacement is successful in removing inclusions present in three-week old mice and is able to delay the accumulation of HS in adult mice, therefore having the downstream effect of delaying the secondary accumulation of glycolipids most likely to be gangliosides. These studies are not directly applicable to human clinical application because the BBB in humans is formed early in fetal development. However, as therapy protocols had not been investigated in MPS IIIA animal models it was uncertain at the outset of this study whether or not active enzyme in the brain would have an effect on the CNS pathology at all. Despite the knowledge gained from ERT in newborn MPS VII mice, it was still necessary to perform these baseline studies in the MPS IIIA mice. The studies reported in this thesis provide useful information about the efficacy of ERT. Importantly the studies demonstrate that the presence of NS in the CNS is capable of reducing storage vacuoles, which in turn is effective in restoring or maintaining the behavioural and cognitive deficits characteristic of MPS IIIA mice. In addition, ERT in MPS IIIA mice appears to have no side-effects and is safe to use during developmental growth.

The coincidence of glycolipid (most likely ganglioside) accumulation in the lysosomes of CNS cells and the diminution in cognitive function in the mice lead us to contemplate therapy protocols aimed at preventing or reducing the build-up of gangliosides rather than focusing on the primary storage of HS. Perhaps a combined therapy of ERT and substrate deprivation (see Section 1.11.3) will have a more lasting effect on maintaining cognitive abilities in MPS IIIA mice.

7.4 FUTURE WORK

The studies outlined in this thesis provide information about the clinical progression of disease in MPS IIIA mice and the biochemical characteristics of the D31N mutation. The ERT trial has formed a foundation for the evaluation of other potential therapies in MPS IIIA mice. The positive response to ERT in MPS IIIA mice provides much incentive to investigate a wide range of other therapeutic protocols in this very useful animal model.

For it to be a successful therapy enzyme replacement is reliant upon a number of factors, particularly the efficient delivery of enzyme to the various sites of pathology in sufficient amounts at the appropriate time in development. For this reason, it would be logical to follow-up with experiments to optimise enzyme dose rates and the number of enzyme injections in the MPS IIIA mice to achieve maximal response to ERT. Also, various dose regimes could also be investigated; a constant dose rate of 1 mg/kg was investigated in this thesis, but a dose rate that is proportional to growth rate may be beneficial. Therapy could be initiated with a very high dose rate that is reduced with increasing age. For example, MPS VII mice were injected with 28,000 unit dose of GUSB enzyme; this equals approximately 7 mg/kg in the newborn mice and only 0.7 mg/kg in the adult mice (Sands et al., 1997). A marked reduction in lysosomal storage in the CNS of these mice was observed. Correct dosage also has other implications such as making ERT more cost effective; the small birth weight of babies means less enzyme is needed, hence the reduced cost. Reduced amounts of enzyme and/or fewer injections would reduce the burden or expense of the therapy.

An obvious and essential problem to address is overcoming the BBB. A potential way of doing this is by coupling the non-transportable polypeptide pharmaceutical to a BBB transport vector, making a pro-drug (see Section 1.13.1.2). P97 has been identified as a BBB transport vector. P97 is claimed to be able to target to the lysosomes of neurons (<http://www.biomarinpharm.com/>). One proposed method of investigating P97 as a pro-drug treatment for MPS IIIA would be to conjugate P97 to rhNS or prepare a P97/NS fusion protein and intravenously administer the construct to MPS IIIA mice and evaluate its ability to cross the BBB.

It still remains unknown if already established CNS pathology, including cognitive deficits, can be reversed. This is an important area to study because, to date, there are no effective screening methods to detect patients affected with MPS at birth. The majority of MPS patients are not diagnosed until clinical pathology has already been established. This is certainly the case in MPS III, due to the period of normal development and mild somatic phenotype. A recent study aimed at addressing this issue demonstrated that when recombinant feline immunodeficiency virus based vectors were unilaterally injected into the striatum of adult MPS VII mice with already established impairments in spatial learning and memory, there was a dramatic recovery of behavioural function (Brooks et al., 2002). These encouraging results suggest that enzyme replacement may not only be effective in restoring GUSB activity in the lysosome but may also improve plasticity and spatial learning in adult MPS VII mice. To gain an understanding of whether or not it is possible to reverse CNS pathology in MPS IIIA mice, rhNS should be intrathecally injected into the brains of adult MPS IIIA mice at various ages throughout development. These mice should then be assessed using the MWM test. Despite the promising results obtained by Brooks et al (2002), a poor understanding still remains on the issue of whether or not there is a critical threshold that is reached in storage accumulation. The Brooks et al (2002) study looked only at MPS VII mice at one age (16-weeks). An abundant amount of accumulated storage ^{product} has a serious effect on the normal functioning of cells in the CNS, and whilst it seems very plausible that this storage can be removed by ^{product}

enzyme replacement, there may be a critical time at which the functioning of the cell becomes lost forever. It has also been reported that by end-stage disease, neuronal cell loss is observed in many types of LSD (Walkley 1998), and that Purkinje cells are vulnerable to cell death earlier in some storage diseases. A poor understanding remains on why neurons die in storage disorders. Storage is widespread and occurs in many other tissues as well as the brain, however disease-related cell death predominates only in the cerebral cortex. It is therefore necessary to assess whether pathology is reversible after the onset of neuronal cell death.

It is also important to study CNS pathology reversibility in MPS IIIA mice before and after the accumulation of the secondary storage product of G_{M2} and G_{M3} gangliosides. Ganglioside accumulation may be responsible for the loss of cognitive function in MPS IIIA mice. Ganglioside accumulation possibly results from the inhibition of neuraminidase and sialidases responsible for G_{M2} and G_{M3} degradation as a result of HS accumulation (Baumkötter and Cantz, 1983). Injections of rhNS into the brain of adult MPS IIIA mice should be investigated to see if the removal of HS storage is successful in this inhibitory effect on ganglioside-degrading enzymes.

Following the evaluation of therapies aimed at the introduction of enzyme into the CNS of MPS IIIA mice, it would be interesting to upscale these experiments to a larger animal model such as one of the MPS IIIA dog models. Whilst mouse models are an invaluable resource for developing therapeutic strategies for the MPS and other disorders due to their low maintenance cost, short breeding time and efficient management, they do have limitations. Mice have a short life-span, therefore less time for symptoms to develop, which may not accurately reflect human disease progression. Mice also have a much smaller brain in comparison to humans and a leaky BBB at birth, also not present in humans. These differences mean that studies of diffusion of enzyme throughout the brain of mice could not be correlated to humans. Therefore, to allow for full evaluation of therapy protocols before clinical application in humans, evaluation in a larger animal model with a brain size comparable to that of humans would be ideal.

In conclusion, the studies detailed in this thesis have illustrated the importance of having a well characterised animal model (for the same disorder in humans) for the evaluation of therapies. These studies have increased our understanding of the effect of reducing lysosomal storage following ERT in the CNS of MPS IIIA mice, and highlight the significance of initiating therapy as early in life as possible. Further study of this model will enhance our knowledge about the reversibility of impaired cognitive function which still remains unresolved.

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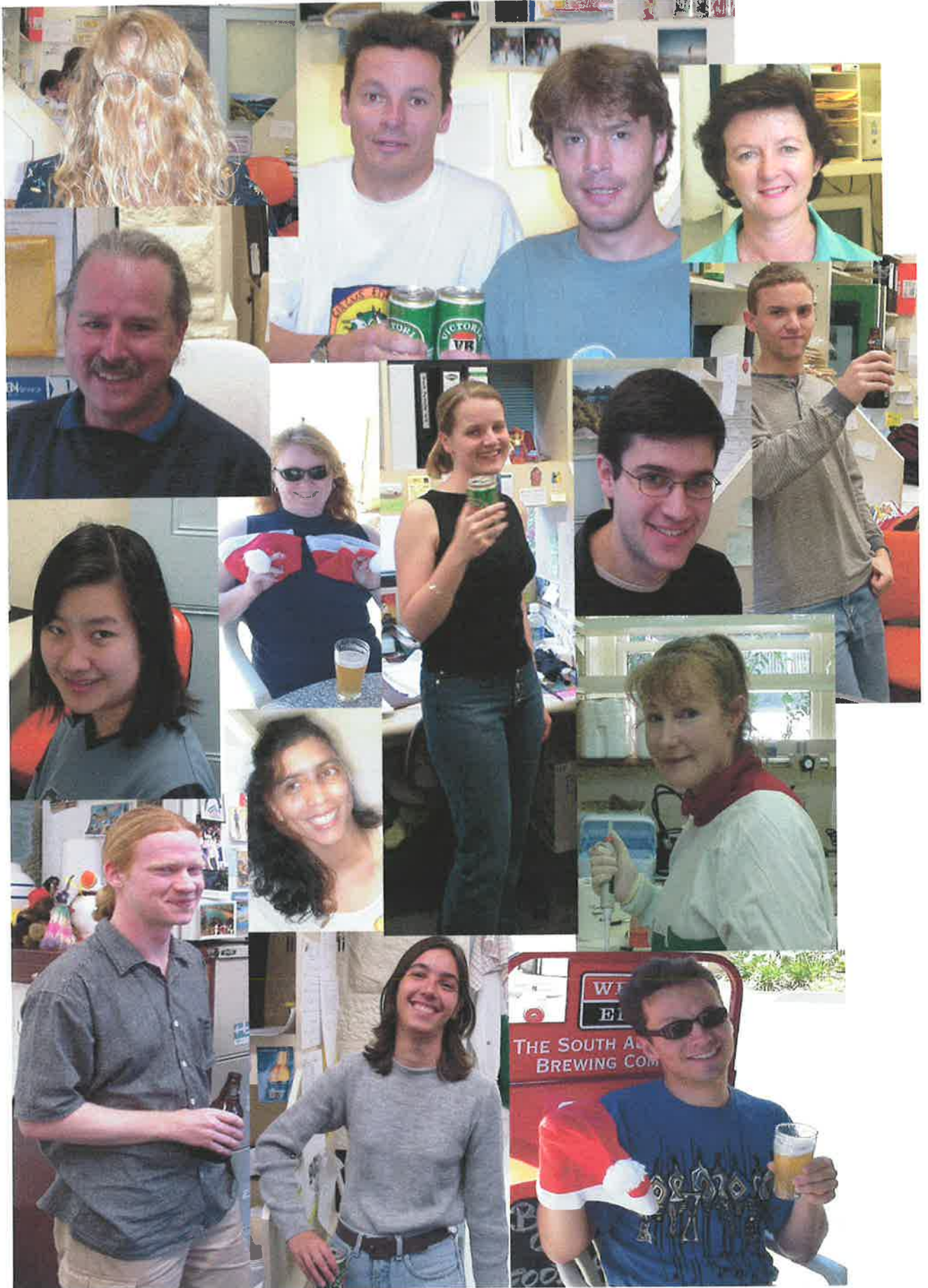
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1999-2002**



**ADDENDA**

P VI: add abbreviations.

ASN Asparagine
ER Endoplasmic Reticulum
GlcNS Glucosamine-N-sulphate

P55: section 2.2.3: Radiolabel used was $^{35}\text{S}(\text{Na}_2^{35}\text{SO}_4)$, from Perkin Elmer, Life Sciences

P56: section 2.2.5: The rate of NS activity was determined by multiplying the substrate to product conversion by the amount of substrate used per reaction, and dividing this by the time of the incubation and the total mg amounts of protein in each sample assayed. To give results expressed as pmol/min/mg protein.

P58: section 2.2.7: nature of EXPRESS ^{35}S was methionine, from Perkin Elmer, Life sciences

P58: section 2.2.7: sodium cholate; instead of NaCholate;

P59: section 2.2.10: COONS/DMEM from JRH, Australia.

P61, section 2.2.13&P88, section 4.2.6: reference to Lineweaver-Burk plots: Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**: 658.

P62: section 2.2.18.1: change the word eppendorf to tube.

P71: section 3.2.1 Reference PCR method; see section 2.2.18.3

P82: section 4.2.3: Two-hour instead of Two-hr.

P84: section 4.2.5: 1-2% DMSO instead of 1-2% DMEM

Table 6.2: mice were housed 'singly', instead of 'singularly'

P117: section 6.3.1: NS activities in the liver of normal mice is 0.135 nmol/min/mg compared to 0.004 nmol/min/mg in MPS IIIA mice. NS activities in the brain of normal mice is 0.054 nmol/min/mg compared to 0.002 nmol/min/mg in MPS IIIA mice (Bhaumik, et al 1999).

Figure 6.5B: storage vacuoles are present in cortical neurons of MPS IIIA mice from day one of life.

P143: section 6.4: Description of meganeurites and axonal spheroids: Meganeurites are structural enlargements proximal to the initial segment of the axon and contain storage material consistent with the specific defective lysosomal hydrolase. Axonal spheroids are enlargements distal to the initial segment of the axon and contain a distinct array of materials with ultrastructural features that are the same across many types of storage disorders (Walkley, 1998).

P144: section 6.4: GUSB was in excess ~ 30% over normal levels

P153: change Aronovich, et al 2001 to Aronovich, et al 2000.