BIOCHEMICAL AND CELL CULTURE APPROACH TO STUDY THE MOLECULAR ASPECTS OF METACHROMATIC LEUKODYSTROPHY

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Gutachters

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Publications

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Saravanan K, Gieselmann V, Bussow H, Franken S. (2005). Establishment and characterization of spontaneously immortalized Schwann cell culture to study the molecular aspects of metachromatic leukodystrophy. (To be submitted shortly)

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Contents

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IV. Abbreviations

µg – Microgram

2D – Two dimension

ADMEM - Advanced Dulbecco's Modified Essential Medium

APS – Ammonium persulphate

ASA – Arylsulfatase A

BA - ß-actin

BDNF - Brain derived neurotrophic factor

bp – base pair

BPE - Bovine pituitary extract

BSA – Bovine serum albumin

CGT - Ceramide galactosyltransferase

CNS – Central nervous system

CO_2 - Carbon dioxide

DAPI - 4,6-Diamidino-2-phenylindole

DD- Double distilled

DIGs – Detergent insoluble glycosphingolipid enriched microdomains

DMSO – Dimethyl sulphoxide

DNA – Deoxy ribonucleic acid

E.g. - Example

dNTP –Deoxynucleoside-triphosphate

EDTA – Ethylene diamine tetra acetic acid

FCS – Foetal calf serum

GDNF - Glial cell line derived neurotrophic factor

hr(s) – Hour(s)

HBSS – Hank's balanced salt solution

KO – Knockout

LAMP - Lysosomal associated membrane protein

MA - milliampere

MAL- Myelin and lymphocyte protein

MBP – Myelin basic protein

min – Minutes

MLD - Metachromatic leukodystrophy

mm – Millimeter

MW – Molecular weight

NS – Non-storing

NGF – Nerve growth factor

NGS - Normal goat serum

nm – Nanometer

NT - Neurotrophin

NTR – Neurotrophin receptor

OH – Hydroxyl

PAGE- Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PLP - Proteolipid protein

pmol – Picomole

PMP – Peripheral myelin protein

PMSF - Phenyl methyl sulfonyl fluoride

P0 – Protein zero

PS – Phospho serine

PT – Phospho threonine

PY – Phospho tyrosine

RBC's- Red blood cells

RT – Room temperature

SC(s) – Schwann cell(s)

SDS- Sodium dodecyl sulfate

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec – Seconds

TBS – Tris buffered saline

TLC – Thin layer chromatography

Trk – Tropomycin-related kinase

WT – Wild type

1. Abstract

Metachromatic leukodystrophy (MLD) is a lysosomal lipid storage disease caused by arylsulfatase A deficiency. In MLD patients, the sphingolipid sulfatide increasingly accumulates leading to progressive demyelination. In this study, arylsulfatase A (ASA) knockout (KO) mice were analysed and it was shown that accumulation of sulfatide is not restricted to the lysosomal compartment but occurs in myelin itself. Although this sulfatide storage did not affect the overall composition of most myelin proteins, it specifically caused a severe reduction in myelin and lymphocyte protein (MAL). This demonstrates a regulatory link between sulfatide accumulation and MAL expression and indicates the existence of regulatory mechanisms between lipid and myelin protein synthesis in oligodendrocytes. In addition, in cultured renal epithelial cells, sulfatide accumulation diverts MAL to the late endosomal/lysosomal compartment and thus also affects the intracellular distribution of MAL. The specific reduction and mistargeting of MAL protein as a reaction to sulfatide overload may contribute to the pathogenic mechanisms in metachromatic leukodystrophy. To investigate further the cellular pathomechanism of MLD, spontaneously immortalised Schwann cell (SC) lines from the arylsulfatase A knockout (ASA KO) mice were established. Cultures of SC derived from peripheral sciatic nerves of 2-week-old ASA KO mice were maintained for 6 months and those colonies that spontaneously developed were expanded further and characterised. One of the cell lines, designated SC KO, showed distinct SC phenotype and it was passaged once a week and maintained for over 10 months without phenotypic alterations. The SC KO cells were genotyped and characterised. They showed marked sulfatide storage in the late endosomal/lysosomal compartments. This was demonstrated immunologically, ultra structurally and by biochemical analysis. The stored sulfatide responded to ASA treatment and hence could be a suitable model to study this disease at a molecular level. Preliminary molecular analysis of these cells has paved way to investigate in detail for neurotrophins and their receptors in the sciatic nerve. RT-PCR analysis of sciatic nerve has shown that there is an upregulation of the neurotrophic receptors tyrosine kinase A (TrkA) and tyrosine kinase B (TrkB) and a downregulation of neurotrophins namely, nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), which are necessary for the normal myelination process. The down regulation of neurotrophins might be the reason for the demyelination observed in the peripheral nervous system (PNS) of the two year old mouse. Finally, the method developed to load sulfatide in the sulfatide non-storing SCs (SC KO NS) can be used as a comparative cell culture model to study the disease further for possible therapeutic approaches.

2. Introduction

The leukodystrophies are genetically determined progressive disorders that affect the brain, spinal cord and peripheral nerves. The term leukodystrophy is derived from the Greek words "*leuko*" meaning white and referring to the white matter of the nervous system and "*dystrophy*" meaning imperfect growth or development. When the term *dystrophy* is used in medicine, it is meant to imply a condition which is progressive; that is, the condition tends to get worse as the patient gets older.

MLD is a lysosomal storage disorder caused by the deficiency of the enzyme ASA. ASA catalyses the first step in the degradation of the sphingolipid, cerebroside 3-sulfate (sulfatide) (Gieselmann, 2003; Kolodny, 1989) (Fig 1).

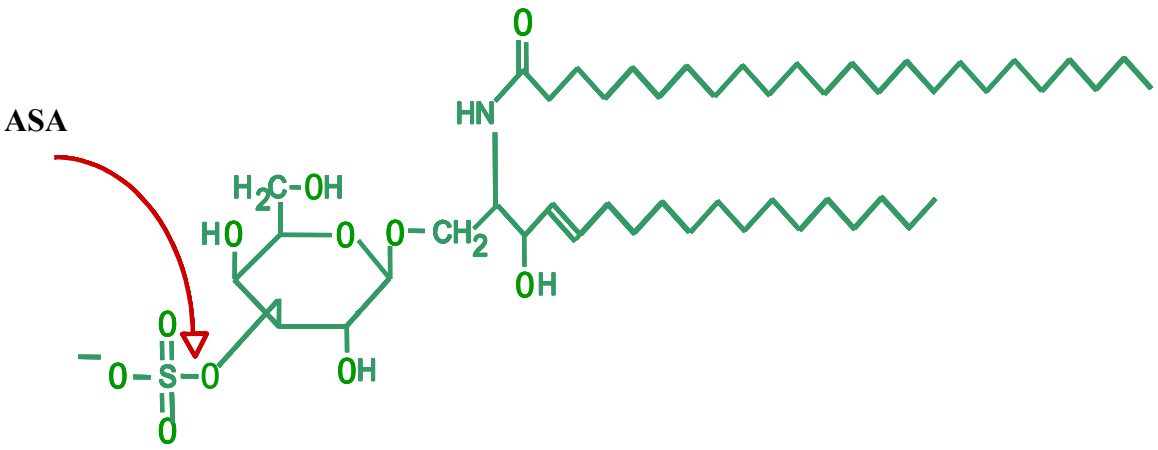


Fig 1: Cerebroside 3-sulfate (Sulfatide). Sulfatide is a sulfate ester of cerebroside. Sulfatide has a sphingosine back bone with 3-O–beta–sulfo-D-galactose head group. Sulfate is joined by an ester linkage to the C3 hydroxy group of galactose. It contains equimolar concentration of cerebronic acid, sphingosine, galactose and sulfate. Desulfation by ASA is the first step in the degradation of sulfatide.

Sulfatide is a major component of myelinating cells in the nervous system. The genetic deficiency of this enzyme results in defective desulfation of sulfatide and other 3-O-sulfogalactosyl containing glycolipids. This pathology leads to progressive demyelination of the nervous system, which in turn causes severe neurological symptoms. Patients usually die within a few years after the onset of the disease. The early pathological changes and course of development of pathology is unknown. The frequency of the disease is estimated to be about 1 : 40,000 newborns (Gustavson and Hagberg, 1971).

The first description of a patient with MLD was published in the year 1910 by the German neurologist Alzheimer. In 1921, Witte described a patient with metachromatic granules in liver, kidney and testis apart from brain. MLD was known for many years as a diffuse brain sclerosis, but it was renamed as MLD by Einarson and Neel in 1938 (von Figura et al., 2001). In 1963, Austin reported that the enzyme ASA is deficient in MLD patients and two years later, Mehl and Jatzkewitz showed a block in the metabolism of sulfatide (Mehl and Jatzkewitz, 1965). During 1975, there were reports of healthy individuals with a family history of MLD having very low levels of ASA. Subsequently it was established that there is a relatively common allele of the ASA gene that leads to low expression of the enzyme (von Figura et al., 2001).

2.1. Classification of MLD based on clinical forms and symptoms

Depending on the age of onset, MLD can be classified into 3 types. Late infantile (6 months - 4 years), juvenile (4 -16 years) and adult (beyond 16years) (Gustavson and Hagberg, 1971). In the classical form of MLD, patients develop progressive gait disturbances, ataxia and spastic paresis around the age of two years. Pathological hallmark of this disease is a progressive demyelination, which finally results in death. Spastic quadriplegia and optic atrophy are also some of the other symptoms of the disease (Gieselmann et al., 1994). In patients, macroscopically, reduced volume of white matter and in severe cases spongiform or cystic degeneration are noted. ASA deficiency leads to progressive sulfatide storage in glial cells and some neurons as well as in kidney and bile duct epithelia. Functionally, sulfatide storage has negligible effects on kidney and bile ducts, but it severely affects the nervous system. Microscopically, loss of myelin sheaths, a reduction in number of oligodendrocytes and accumulation of metachromatic granules are observed. The early pathological changes and course of development of pathology is unknown. Patients finally die in a decerebrated state (Gieselmann et al., 1994).

2.2. Pathology

The metachromatic granules are spherical masses of about 15-20 μm in diameter. Electron microscopy shows that the inclusions are surrounded by a membrane which is expected for their lysosomal nature. The morphology of the inclusions varies, but prismatic and tuff stone like profiles are characteristic (Wittke et al., 2004). In CNS, accumulation of metachromatic

granules is also observed in macrophages, oligodendrocytes and neurons. Demyelination in brain stem, spinal cord and cerebellum is prominent. There is a marked reduction in Purkinje and granular cells, retinal cells also show polymorphic lysosomal inclusions. Demyelination of the peripheral nervous system with the presence of metachromatic granules in Schwann cells is observed. Sural nerves show reduced myelin sheath thickness for all fibres. The sensitivity of demyelination of peripheral nerves correlates with the onset and duration of the disease. Tissues with excretory functions are particularly affected; these include kidney, gall bladder, liver, islets of Langerhans, anterior pituitary, adrenal cortex, testes and sweat glands. Biochemically, significant increase in the content of sulfatides in the white matter together with marked decrease in the content of myelin lipids like cholesterol and sphingomyelin is observed (von Figura et al., 2001). The galactocerebroside : sulfatide ratio which is approximately 3 in normal white matter, is reduced to 1 in late infantile forms of MLD (Harzer and Kustermann-Kuhn, 1987). The concentration of lysosulfatide (deacylated form of sulfatide) was found to be increased fifty to hundred times compared to control brains (Krendel et al., 1994). Sulfatide concentration is increased in liver, kidney, gall bladder and urine of MLD patients. The level of sulfatide is increased ten to seventy five fold as that of normal kidney (Malone and Stoffyn, 1966).

2.3. Sulfatide biochemistry

In order to understand the concept of this work, it is necessary to have an overview about sulfatide. Thudichum first showed the sulfur containing glycolipid and named it sulfatide. Sulfatide accounts for three and half to four percent of the total lipids of myelin (Norton and Poduslo, 1982). In 1933, Blix showed that sulfatide contains equimolar amounts of cerebronic acid, sphingosine, galactose and sulfate (von Figura et al., 2001). Sulfatide is a sulfate ester of galactocerebroside with a sulfate joined by an ester linkage to the C-3 hydroxyl of galactose (Stoffyn and Stoffyn, 1963). The sphingosine base of sulfatide consists predominantly of C-18 sphingosine (Stoffyn, 1966). Both sulfatide and galactocerebroside contain high proportion of long chain fatty acids and of fatty acids that contain 2-hydroxy groups. Nearly all of the 2-hydroxy fatty acids found in brain are of these 2 glycolipids. The kidney is second in relative abundance of sulfatides, but its concentration is only about one-tenth as that of the brain.

Sulfatides are synthesised through sulfation of galactosylceramide (also designated as galactocerebroside) by a reaction with 3'-phosphoadenosine -5'-phosphosulfate (PAPS). The

reaction is catalysed by microsomal sulfotransferase (Farrell, 1974). UDP galactose : ceramide galactosyltransferase (CGT) catalyses the biosynthesis of galactosylceramide which is the precursor of sulfatide. 3'-phosphoadenosine-5'-phosphosulfate-cerebroside sulfotransferase (CST), sulfates galactosylceramide to sulfatide. Its synthesis is maximum during myelination and proceeds more slowly in adults. Established cells lines from renal tubule epithelium have also been shown to synthesise sulfatide (Klein et al., 2005). Microscopically, in affected cells from MLD patients and mouse model, sulfatide is visualised as bizarre shaped bulky inclusions. Lamellated, prismatic or tuff stone profiles are also often observed (Fig 2).

Like other membrane lipids, sulfated glycolipids have the capability of hydrophilic and hydrophobic interactions. Their anionic charge allows combination with inorganic cations or organic amines to maintain electrical neutrality of membranes. Sulfatide is located at the surface of the myelin membrane and is bound to myelin basic protein (MBP) and proteolipid protein (PLP) by strong ionic interactions. Together with galactosylceramides, sulfatides maintain the insulator function of the membrane bilayer (Arvanitis et al., 1992; Norton and Poduslo, 1973; Vacher et al., 1989). Sulfatides are believed to be involved in active sodium transport serving as a cofactor for Na/K ATPase (Rintoul and Welti, 1989). Various cellular adhesion molecules bind specifically with high affinity to sulfatides. E.g. laminin, thrombospondin, tenascin R etc. Sulfatides are also believed to have anticoagulant activity in serum.

2.4. Arylsulfatase A (ASA)

ASA is a house keeping enzymes expressed in all tissues and has been purified from various sources like liver, placenta and urine. The enzyme has a low isoelectric point. All forms of MLD are inherited as autosomal recessive traits and are caused by allelic mutations of the ASA locus except for few cases of saposin B deficiency. In the period of myelination, ASA activity increases concomitantly with that of CST. After completion of myelination, the latter decreases, whereas ASA activity does not change. ASA gene is located on chromosome number 22. It is a small gene of about 3 kb with 8 exons (Kreysing et al., 1990). ASA mRNA of about 2.1 kb is responsible for the synthesis of ASA polypeptides. The translational efficiency of larger transcripts is lower. The ASA mRNA is translated into a 507-amino-acid precursor at the rough endoplasmic reticulum. ASA receives several post-translational modifications in the endoplasmic reticulum. A linear sequence of 16 amino acid residues

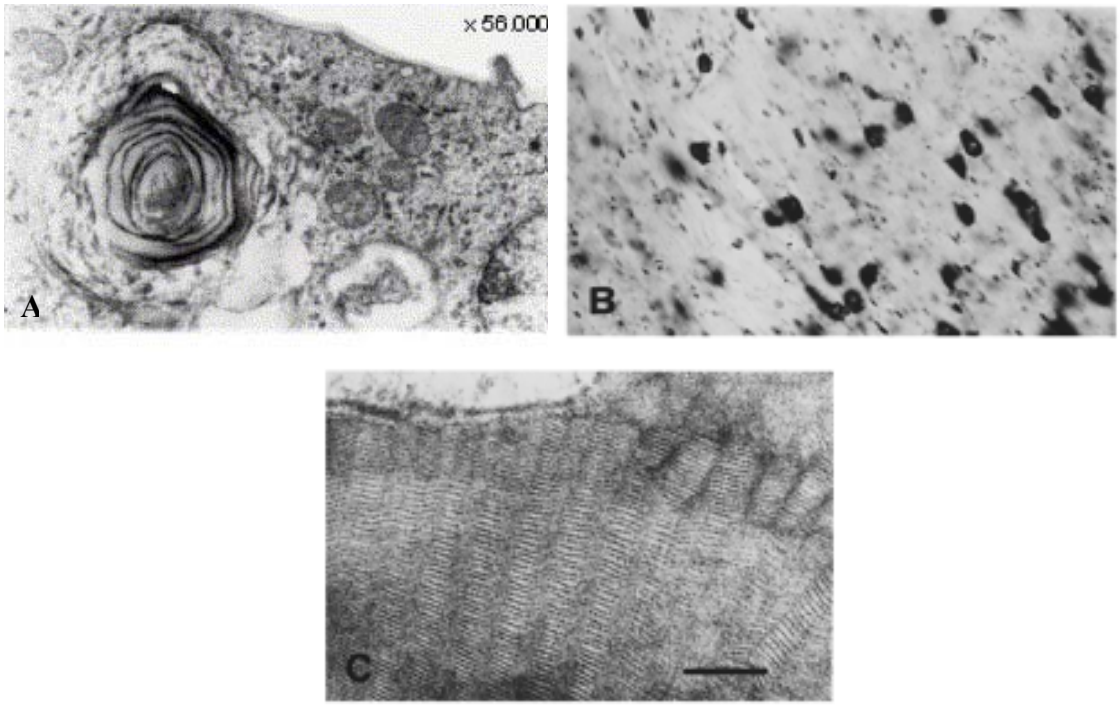


Fig 2: Microscopical view of sulfatide storage. (A) Ultrastructure of lamellated storage material in cultured ASA KO kidney cells (Klein et al., 2005). (B) White matter (hippocampal fimbra) sections of ASA KO mouse stained for alcian blue shows cells filled with large storage granules and linearly arranged small cellular processes with small granules. (C) Ultra structure of storage material. Prismatic herringbone-like inclusions in an astrocyte of ASA deficient mouse. (Bar = 100 nm.)(Hess et al., 1996).

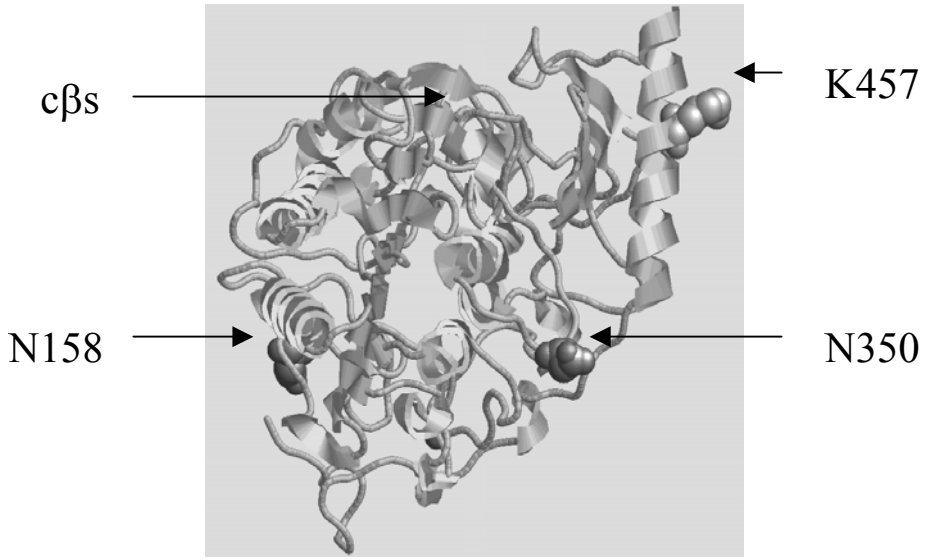


Fig 3: Three dimensional structure of ASA. ASA is synthesized as a 62-kDa polypeptide and bears three *N*-linked oligosaccharide side chains. Two of these oligosaccharide side chains (at N-158 and N-350) are accessible by the phosphotransferase. These two sites are needed for the activity of ASA. Cβs depicts the central β-pleated sheet and K457 depicts the lysine in the N-terminal helix of the enzyme (Yaghootfam et al., 2003).

surrounding Cys69 is sufficient to direct post translational oxidation of cysteine residue 69. This modification is required for sulfatase activity. Eighty different mutations in ASA are known till date to cause MLD (von Figura et.al., 2001).

ASA cleaves various sulfate containing substrates. The major physiological substrates are sulfatides. Other 3-O galactosyl sulfates hydrolysed by ASA are lactosyl ceramide 3-sulfate, seminolipid and psychosine sulfate.

Within the lumen of the endoplasmic reticulum, the signal peptide is cleaved and the enzyme receives N-oligosaccharide side chains at each of the three potential N-glycosylation sites. After completion of folding, ASA is transported to the Golgi apparatus, where it is recognised as a lysosomal enzyme by the lysosomal enzyme phosphotransferase (von Figura et.al., 2001). Detailed examination of the three N-linked oligosaccharide side chains of ASA revealed that mannose-6-phosphate residues are found predominantly on the first and the third oligosaccharide side chains, whereas the second N-glycosylation site is only ineffectively phosphorylated. Transport to the lysosome is accomplished via a prelysosomal acidic compartment by mannose 6 phosphate receptor pathway (Braulke et al., 1990; Kelly et al., 1989). Recombinant human ASA polypeptide is available in our lab and is produced by the method described in (Matzner et al., 2000). The human ASA produced by this method has been widely used in this study.

2.5. Animal models of MLD

A naturally occurring animal model has not been described for MLD. For that reason, an ASA KO mouse model was generated through specific disruption of the murine ASA gene (Hess et al., 1996). This animal model displays several characteristics of the human disease, but has comparatively mild phenotype. These animals store sulfatide in the same tissues as humans, including oligodendrocytes and SCs (Coenen et al., 2001; Gieselmann et al., 1998; Lullmann-Rauch et al., 2001). With increasing age, the animals accumulate increasing amounts of sulfatide. The degenerative phenotype of the KO mice is mild. Mice older than 18 months show degeneration of up to 20% of fibers in peripheral nerves. However, except for some fibres of the peripheral nervous system and the acoustic ganglion, the animals do not show the widespread demyelination characteristic for humans. The reason for this discrepancy is unknown.

Independent of lack of widespread demyelination, these animals develop progressive neurological symptoms, particularly in the second half of their life. The pathological features for the progressive neurological symptoms are not entirely clear. They might be due to neuronal sulfatide storage and to the generally decreased axonal diameters found in ASA deficient mice. Although the mice develop progressive neurological symptoms, their life span is normal. The periventricular alterations found in 12 months old mice in MRI was similar to the one in human patients. Pathological and behavioural studies suggest that the animals resemble the early stages of the human disease (D'Hooge et al., 1999; D'Hooge et al., 2001).

2.6. Myelin and Schwann cells

2.6.1. Myelin

Myelin is a highly specialised plasma membrane synthesised by oligodendrocytes and Schwann cells. It enwraps axons thereby acting as an insulator and allowing the fast axonal propagation of the action potential. The specific molecular organisation of myelin is essential for the proper function of the nervous system. Myelin is a multi lamellar structure with a particular lipid composition being rich in cholesterol and the glycosphingolipids, galactosylceramide and sulfatide. Examinations of mice deficient in the synthesis of these lipids proved their importance in the formation and maintenance of myelin (Bosio et al., 1996; Coetzee et al., 1996; Honke et al., 2002) .

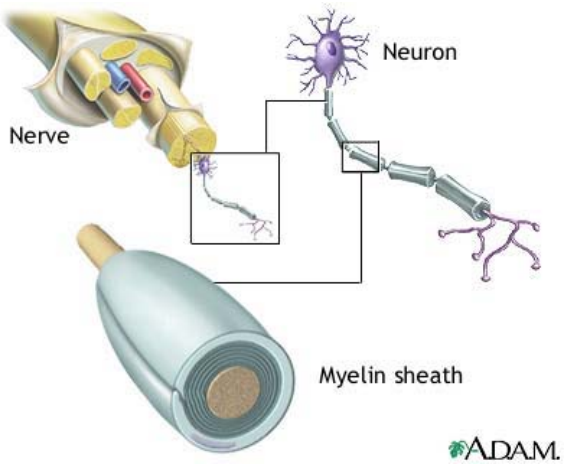


Fig 4: Neuron and Myelin. Diagrammatic illustration of the nerve cell neuron and its axonal insulation by myelin sheath. Source: http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/9682.jpg

A number of hereditary as well as acquired diseases affect myelin architecture, which usually causes severe neurological symptoms. Genetic diseases may be caused by defects in genes of myelin proteins (Anderson et al., 1998; Nave, 1994; Werner et al., 1998) or enzymes affecting myelin lipid metabolism. The latter involves, in particular, two lysosomal storage diseases - Krabbe disease and MLD (Gieselmann, 2003; Suzuki, 2003; von Figura et al., 2001; Wenger et al., 2001). Patients suffering from Krabbe disease (globoid cell leukodystrophy) lack ß-galactocerebrosidase and are unable to degrade galactosylceramide. Prevention of demyelination in MLD can help in the treatment of the disease to a great extent.

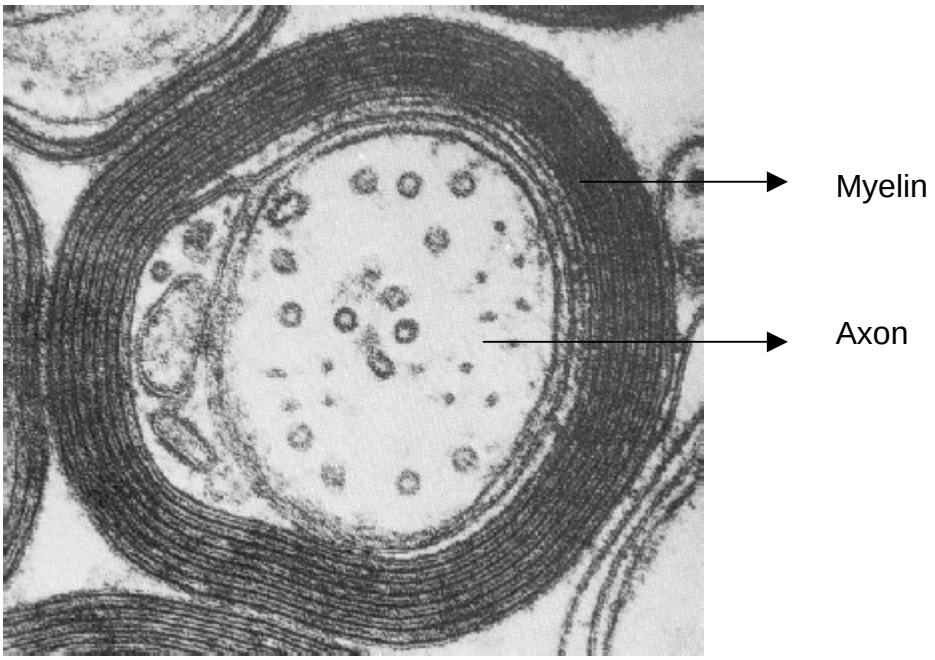


Fig 5: Ultrastructure of myelin. Electron micrograph of myelin surrounding an axon.
Source: www.cytochemistry.net

2.6.2. Schwann cells (SCs)

To date, it is not clear how sulfatide storage affects metabolism of a cell at the molecular level and an appropriate model of sulfatide storing cell culture system is not available (Gieselmann et al., 2003). However little is known about the abnormal metabolic pathway of the causative molecule and the mechanism of demyelination. Previous cell culture experiments indicate that metabolic defect of cells deficient for an individual soluble lysosomal enzyme can be cross corrected by an exogenously applied enzyme (Neufeld, 1991). The enzyme is internalised

through mannose 6-phosphate receptors into the lysosomal compartment and causes hydrolysis of substrate present in the lysosomes. The mannose 6-phosphate dependent endocytosis of lysosomal enzymes has been demonstrated for glial and neuronal cells (Schluff et al., 1998; Stewart et al., 1997).

SCs, the principal accessory cells of the peripheral nervous system, sustain neurons and play an important role in the growth of axons, its regeneration and myelination. Myelin is formed by oligodendrocytes in the central nervous system and by SCs in the peripheral nervous system. It facilitates salutatory nerve conduction (Guenard et al., 1994). A 38 year old man presented with weakness of lower limbs along with low ASA activity in leukocytes and fibroblasts culture showed de- and remyelinating lesions and characteristic lamellar inclusions in SCs and macrophages (Fressinaud et al., 1992). Similar reports with peripheral neuropathy in MLD patients were published by (Coulter-Mackie et al., 2002; Hansen et al., 1994; Martinez et al., 1975).

There have been reports that spontaneously immortalised SCs of new-born rats can be maintained in culture for several months in serum containing medium. Similar treatment was adopted for SCs derived from mice. These cells were not contact inhibited and they form ball shaped masses (Eccleston et al., 1990; Eccleston et al., 1991). Using high doses of serum, new-born mouse SC line was established by (Boutry et al., 1992). Previous studies obtained from new-born rat SCs have shown that these cells are quiescent and grow very slowly even in FCS. Forskolin shows a synergetic activity with growth factors such as PDGF, FGF, TGF-β and IGF-I (Davis and Stroobant, 1990; Eccleston et al., 1989; Ridley et al., 1989; Schumacher et al., 1993; Weinmaster and Lemke, 1990). However, it has been reported that new-born rat SCs proliferate in a dose dependent manner in medium containing rat serum instead of FCS (Maertens, 1994).

Researchers have also established a spontaneously immortalised cell line designated TwS1 from long term cultured SC derived from DRG and peripheral nerves of the Twitcher mouse (Shen et al., 2002). These established cells demonstrated distinct SC morphology and phenotypes positive for SC associated molecules demonstrated by immunofluorescence and RT-PCR analysis. These cells exhibited a marked reduction in galactosylceramide activity and accumulation of psychosine. Electron microscopically, varieties of cytoplasmic inclusions

were demonstrated in this cell line and the results indicated that these cells sufficiently represent the *in vivo* pathological feature of the twitcher mouse (Suzuki, 1995 #158).

Large number of purified SCs expressing the twitcher phenotypes can readily be obtained for biochemical analysis of myelin related lipids and proteins (Shen et al., 2002). Relatively high doses of forskolin inhibits the proliferation of immortalised new born rat SCs (Yoshimura et al., 1994). Although the detailed mechanism of spontaneous immortalisation of SC lines in still unknown, it is likely that the spontaneous immortalisation of long term cultured SCs is a general phenomenon in both wild type and mutated mice (Watabe et al., 2003). It has been reported that rat SCs can divide indefinitely under appropriate culture conditions (Mathon et al., 2001).

Several methods for the preparation of pure population of neonatal SCs have been described and are in use in many laboratories. Similar cell culture models are also available for Niemann-Pick disease type C (Watabe et al., 2001). To date, more than fifteen SC lines have been established by spontaneous immortalisation or transfection with oncogenes (Bolin et al., 1992; Boutry et al., 1992; Chen et al., 1987; Goda et al., 1991; Jirsova et al., 1997; Li et al., 1996; Porter et al., 1987; Ridley et al., 1989; Tennekoon et al., 1987; Thi et al., 1998; Toda et al., 1994; Watabe et al., 1995; Watabe et al., 1990). The degree of phenotype expression and differentiation differ from each other (Hai et al., 2002). All these reports aided to establish a technique to isolate SCs from ASA KO and wild type (WT) mice for an *in vitro* cell culture system for MLD.

The overall aim of this study was initially to investigate the ASA KO mice and compare it with the WT mice for any malformations or dysfunction in protein and lipid compositions in the myelin. The results obtained from the first part of the work encouraged to establish a sulfatide storing *in vitro* cell culture system to study the molecular aspects of the disease.

2. Materials and Methods

Any modifications in the materials and methods have been mentioned in the results.

3.1. Materials and solutions

Unless otherwise stated, chemicals were purchased from Serva (Heidelberg, Germany), Sigma (Deisenhofen, Germany), Roche (Basel, Switzerland), Fermentas (St.Leon-Rot, Germany), Merck (Darmstadt, Germany) or Invitrogen Life Technologies, (Karlsruhe, Germany).

3.2. Frequently used equipment

ABI prism310 Genetic analyser	-Perkin Elmer
Beckmann DU 640 Spectrophotometer	-Beckmann Coulter
Bio Max MR film	-Kodak
CAMAG automatic TLC sampler and scanner3	-CAMAG
Ice machine AF-10	-Scotsman
Electrophoresis set up for agarose gels	-Hoefer Scientific Instruments
Polyacrylamide electrophoresis set-up	-Bio-Rad
ELISA reader	-Bio-Rad
Gel dryer	-Savant
Heating block model III Thermostat 5320	-Eppendorf
Hyper cassette	-Amersham
Laminar flow system – Type CA/REV6	-Clean Air
Magnetic stirrer	-Janke & Kunkel
Microscope	
Axiovert M (Epifluorescence)	
Inverted light microscope Axiovert 135	
Axioplan 2 Imaging	-Zeiss
Gel blot system	-Bio-Rad
Digital pH meter	- Knick
Shaking water bath	-Köttermann
Thermo-Cycler Gen Amp Model 9600	-Perkin-Elmer
Ultrasonicator	-Branson Ultrasonic SA
UV hand lamp	-Konrad Bender

Vortexer	-Bender and Hobein
Digital balance	-Typ1264MP;1265-Sartorius
Water purifier Milli-Q plus	-Millipore
Centrifuge	
Eppendorf 5810 R, 5417 R and 5415 D	-Eppendorf
Optima TLX Ultracentrifuge	-Beckman Coulter
Ultracentrifuge L7-65	-Beckman Coulter
Sorvall GS3, JA-10 suppressed	-Heraeus Sorvall
Cell counter	-Neubauer
Freeze drier (LYO VAC)	-Amsco/finn-Aqua
Two dimension electrophoresis system	-Bio-Rad
IR laser scanner	-LI-COR

3.3. Bacterial strains

1. DH5αF^{é} : (F`/endA1 hsdR17($r_k^-m_k^+$) supE44 thi-1 recA1 gyrA (NaI^r) relA1Δ(lacZYA-argF)u_{169}(m80lacZΔM15)

2. BL21 Star·(DE3) : (F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3).

3.4. Animals

129 SV / ola mice - ASA knockout mice (ASA KO) and 129 SV / ola wild type mice (WT) of different age were used for the experiments. All experiments related to animals were carried out according to the laws prescribed by the Federal Government and the ethical committee.

3.5. Cells

Astrocytes: (17-/-) and (11+/+), Chinese hamster ovary cells (CHO K1), Schwann Cells: Schwann cells isolated from ASA knockout mice (SC KO) and Schwann cells isolated from wild type mice (SC WT) were used for the study.

3.6. Standard buffers

Table 1

Name of the buffer	Ingredients
Phosphate buffered saline (PBS)	50 mM Na_2HPO_4/NaH_2PO_4(pH 7.4) 150 mM NaCl
10X HEPES buffer	100mM HEPES 20mM EGTA (pH 7.4)
Tris-(hydroxymethyl)-aminoethane buffered saline (TBS)	20mM Tris-HCl (pH 7.4) 150 mM NaCl
Glycine buffer (GBS)	100 mM glycine/HCl (pH 2.5) 150 mM NaCl
10X TE buffer	100mM Tris/HCl (pH 7.6) 10mM EDTA
TNE buffer	10 mM Tris–HCl (pH 7.4); 150 mM NaCl; 5 mM EDTA and 1 mM PMSF
Hank's balanced salt solution (HBSS)	Sigma (Taufkirchen, Germany)
50X TAE	2M Tris-base 100mM EDTA (pH 8.0)

3.7. Protease and phosphatase inhibitors

These inhibitors were added to the buffers for many of the protein preparations

3.7.1. Protease inhibitors

2mM PMSF
200mM pefabloc
1µg/ml leupeptin
1µg/ml pepstatin
1 µg/ml aprotonin
5 mM EDTA

3.7.2. Phosphatase inhibitors

10 mM sodium orthovanadate (Na_3VO_4)
100 mM sodiumfluoride (NaF)
5 mM sodium pyrophosphate ($Na_4P_2O_7$)

3.8. Antibodies and their source and dilutions used

Table 2

Lyn (1:400), Fyn (1:400), rat anti - neural cell adhesion molecule (NCAM)(1:200), anti-2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) (1:500) Rabbit polyclonal anti-myelin basic protein (MBP) (1:5000)	Chemicon (Hofheim, Germany)
Mouse anti-human MBP (67-74) and mouse monoclonal anti-MBP (1:5000)	Serotec (Düsseldorf, Germany)
Lysosomal associated membrane protein - 1 (LAMP-1) (1D4B) and LAMP-2 (GL2A7) (1:1000)	Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA)
NCAM (BSP-2) (1:500)	(Hirn et al., 1981)
F3 (1:1000), mouse anti-Proteolipid protein (PLP) (1:10,000)	J. Trotter, Heidelberg, (Koch et al., 1997)
Myelin associated glycoprotein (MAG): L-MAG(1:5000) and S-MAG (1:1000)	(Butt et al., 1998)
Sulfatide (1:100) and p75 (1:100)	(Fredman et al., 1988)
PLP (1:5000)	K.-A. Nave., (Jung et al., 1996)
Myelin and oligodendrocyte protein (MOG) (1:1000)	R. Reynolds
Anti-MAL rabbit antiserum (1 :500)	(Frank et al., 1998)
Rabbit anti-L-MAG (1 :5000)	(Erb, 2003 #440)
Mouse anti-glial fibrillary acidic protein (GFAP) (1:100), monoclonal anti-phosphoserine (1:4000), monoclonal anti--phosphothreonine(1:4000)	Sigma, (Taufkirchen, Germany)
Monoclonal anti-phosphotyrosine(1:3000)	Transduction laboratories

All peroxidase and fluorescently labelled secondary antibodies (1:5000-1:10,000) : Jackson IR Laboratories (West Grove, PA, USA), Amersham Bioscience (Freiburg, Germany), Dianova (Hamburg, Germany)

3.9. Protein Chemistry

3.9.1. Protein Estimation (Bio-Rad Dc reagent kit)

The Bio-Rad *DC* (detergent compatible) protein assay is a colorimetric assay for protein determination following detergent solubilisation. The principle of this assay is based on the well-documented Lowry assay (Lowry et al., 1951). This assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. There are two steps in this assay which lead to color development. The reaction between protein and copper in an alkaline medium and the subsequent reduction of Folin reagent by the copper-treated protein. Color development is primarily due to the amino acids tyrosine and tryptophan and to a lesser extent, cystine, cysteine and histidine. Proteins effect a reduction of the Folin reagent by loss of 1, 2 or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

Five different concentrations of bovine serum albumin (BSA) standards in linear range, namely, 0.125; 0.25; 0.5; 1.0 and 2.0 mg/ml were solubilised with the same solvent used to solubilise the sample. They were added in quadruplicate in a 96 well plate (5µl / well) along with the same volume of negative control. To that, 20µl of Dc assay reagent A (20µl of DcS / ml DcA) and 200µl of Dc assay reagent B were added and incubated in dark at room temperature for 5 min. The plates were read using a Bio-Rad microplate reader at 655 nm. The protein concentrations were calculated in mg/ml or µg/µl.

3.9.2. Myelin Preparation (Caroni and Schwab, 1988) (Erne et al., 2002)

To avoid proteolytic degradation of proteins, the whole preparation was done at 4°C. All solutions were prepared in advance and stored at 4°C. All centrifuge tubes and rotors were pre-cooled.

The principle of this method relies on the low density of myelin compared to other cellular membranes due to its high content of lipids. Hence, myelin can be isolated as a band at the interface between 10.5% and 30% sucrose. Contaminating axolemma from the myelin

preparation was removed by subjecting the isolated myelin through several osmotic steps and a second sucrose gradient centrifugation.

WT and KO brain was separately homogenised in 10.5% sucrose in water and centrifuged at 17,000 x g for 45 min. The supernatant was collected and labelled as Sup of 17,000 x g and stored for future analysis. 1-2 ml of 30% sucrose was added to the pellet and the white layer of the pellet was dislodged leaving the red blood corpuscles (RBC's) and yellow layer underneath. This was transferred to a homogeniser and homogenised with 30% sucrose. The homogeniser was rinsed with the same buffer to make a total volume of 8 ml. The combined solutions were transferred to another centrifuge tube, overlaid with 6 ml of 30% sucrose followed by 10.5% sucrose up to 1-2 mm below the rim. This was centrifuged at 68,000 x g for 50 min. Myelin was carefully removed from the interface using a micropipette and transferred to new centrifuge tubes.

DD water was added to the myelin and mixed. This osmotic shock step loosens the membranes and the contaminating axolemma is washed out. Spun at 68,000 x g for 30 min, this water wash step was once again repeated. The pellet of the last wash step was taken in 30% sucrose, overlaid with 10.5% sucrose, centrifuged at 68,000 x g for 50 min. Myelin in the interface was removed, washed twice with water using an ultracentrifuge at 68,000 x g for 30 min. The final pellet was resuspended in cold DD water and frozen in aliquots.

3.9.3. Preparation of rafts from myelin (Erne et al., 2002)

Lipid rafts (otherwise designated as DIGs) are specialised membrane domains enriched in sphingolipids, cholesterol and proteins. These DIGs can be isolated easily by sucrose gradient centrifugation. Myelin equivalent to 500 µg protein was allowed to thaw on ice and sonicated under cold conditions. Myelin was extracted in 1 ml extraction buffer [2% Triton X-100, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 1 mM PMSF] by vortexing shortly once in 5 min for about 30 min at 4°C. One millilitre portions of the total detergent extracts were adjusted to 40% sucrose by adding equal volumes of 80% sucrose in 10 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 5 mM EDTA and 1 mM PMSF (TNE buffer). This was mixed gently and transferred to a Beckman ultracentrifuge tube (12 ml). For the step gradient, 5 ml of 30% sucrose in TNE was layered over the 40% sucrose lysate mix. Additional 5 ml of 5% sucrose in TNE was placed onto the 30% sucrose layer. Gradients were centrifuged for 20

hrs at 150,000 x g at 4°C. After centrifugation, 1 ml fractions were collected from the top of the tube. If not immediately used, the fractions were frozen in small aliquots at −20°C until further use.

3.9.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Franken et al., 2003)

Proteins are charged due to the acidic and basic amino acids and in PAGE, the migration depends on the protein charge. In SDS-PAGE, proteins are separated primarily by their molecular weights. Negatively charged SDS molecules bind along the polypeptide chain and mask the charges in the protein. During electrophoresis, migration distance of the reduced SDS-protein complex is proportional to its molecular weight and not dependent on protein charge. Separating gels of different percentages and stacking gels were prepared as given in table 3, 4 and 5.

3.9.4.1. Reagents for SDS PAGE separating gel

Total volume: 10ml
All measurements are in ml

Table 3

Ingredients	Gel Percentage				
	6%	7.5%	10%	12.5%	15%
H_2O	5.79 ml	5.42 ml	4.79 ml	4.17 ml	3.54 ml
40% Acrylamide	1.50 ml	1.88 ml	2.50 ml	3.13 ml	3.75 ml
1.5 M Tris-HCl pH 8.8	2.50 ml	2.50 ml	2.50 ml	2.50 ml	2.50 ml
10% SDS	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
10% APS	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
TEMED	0.01 ml	0.01 ml	0.01 ml	0.01 ml	0.01 ml

3.9.4.2. Reagents for SDS PAGE gradient gel (18 x 20cm)

Total volume: 26 ml

All measurements are in ml

Table 4

Ingredients	Gel percentage			
	5%	**10%**	**15%**	**20%**
40% Acrylamide	1.6	3.2	4.8	6.4
DD water	8.1	-	-	-
70% Glycerol	-	6.5	4.9	3.3
4 x Tris buffer pH 8.6	3.3	3.3	3.3	3.3
10% SDS	0.13	0.13	0.13	0.13
10% APS	0.05	0.05	0.05	0.05
TEMED	0.005	0.005	0.005	0.005
Total	13.2	13.2	13.2	13.2

3.9.4.3. Reagents for 5% SDS PAGE stacking gel

Table 5

Ingredients	Volumes for 10 ml
H_2O	6.04
40% Acrylamide	1.25
0.5 M Tris-HCl pH6.8	2.5
10% SDS	0.1
10% APS	0.1
TEMED	0.01

An appropriate amount (30-50 µg total proteins) of the protein samples to be analysed from brain or myelin or cell homogenates were denatured in one fourth the volume of 4x Laemmli sample buffer (4% SDS, 0.5% bromophenol blue, 1% β-mercaptoethanol, 0.5 % glycerol, 0.5 M Tris-HCl pH 6.8) at 95°C for 5 min. After a brief centrifugation, the samples were loaded onto SDS-PAGE 12.5 % gel in an apparatus containing running buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, pH 8.3) in both chambers. A constant voltage of 90 V was applied to the gel until the tracking dye entered the separating gel. Then the voltage was increased to 250 V until the dye reached the bottom of the gel and proteins were blotted onto nitrocellulose membranes.

3.9. 5. Two Dimensional (2D) - Iso Electric Focusing (IEF)

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in which proteins are separated according to charge by IEF in the first dimension and according to size by SDS-PAGE in the second dimension. 2D has a unique capacity for the resolution of complex mixtures of proteins, permitting the simultaneous analysis of hundreds or even thousands of gene products.

Bio-Rad protocol was followed to prepare the IPG strips and perform 2D. The first dimension, IEF, was performed in individual IPG gel strips, 3 mm wide and cast on GelBond (Bio-Rad). The minimal protein concentration required for 2D sample should be about 10 µg/µl. 50 µg equivalent protein was used for the rehydration. For 1ml of rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS), 2.8 mg of DTT and 2.5 µl of biolyte or ampholyte (IPG buffer; for 1 ml rehydration buffer 20 µl of biolyte® or ampholyte® was used) was added. In case of 7 cm strips, 130 µl of rehydration buffer was used as an end volume along with sample, whereas in case of 18 cm strips, 330 µl of rehydration solution was used. The IPG strip tray was washed and cleaned to remove any contaminating protein. The rehydration solution was layered on the groove of the tray and the strip was placed gently with the gel side in contact with the rehydration solution. It was made sure that there are no trapped air bubbles between the strip and the tray. The rehydration solution was distributed throughout the groove for equal rehydration of the strip. The maximum sample volume of 10 µl can be used with the rehydration buffer. Larger volumes dilute the buffer solution, which in turn hinder focussing. For better sample entry, a low voltage gradient (50V) was applied across the gel for the first 12 hrs during rehydration. Later, the voltage was increased up to 8000 V for definite volt hours depending upon the size of the strips as specified in the manufacturer's protocol.

After focussing, the strip was equilibrated with few ml of equilibration buffer (50 mM Tris HCl pH 8.8; 6 M urea; 30%v/v glycerol; 2%v/v SDS, trace of bromophenol blue) to cover the strip completely. The strip was rocked gently with the equilibration buffer for 10 min, then washed in a thin stream of DD water and the strip was placed on the second dimension SDS-PAGE gel and fixed with 0.75% agarose and separated according size.

3.9.6. Silver Staining (Bassam et al., 1991)

Silver staining is the most sensitive method for permanent visible staining of proteins in polyacrylamide gels. This sensitivity, however, comes at the expense of high susceptibility to interference from a number of factors. Precise timing, high-quality reagents and cleanliness are essential for reproducible and high-quality results. In silver staining, the gel is impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. This reduction is promoted by protein.

3.9.6.1. Fixing solution I:

30% ethanol

10% acetic acid

60% DD water

3.9.6.2. Fixing solution II:

30% ethanol

0.5M sodium acetate

0.2% sodium thiosulfate

100% with DD water

3.9.6.3. Staining solution

0.1% silver nitrate (from 30% stock)

3.9.6.4. Developing solution

2.5% sodium carbonate in DD water and 2.5% sodium carbonate in 0.15% of 37% formaldehyde in DD water

3.9.6.5. Neutralising solution

5% and 1% acetic acid in DD water

3.9.6.6. Procedure

The gel was transferred to the primary fixing solution and was left overnight or for a minimum of 20 min and replaced with secondary fixing solution for 20 min. Washed 3x with DD water and stained for 20 min with silver nitrate solution and washed once with DD water for 2 min and rinsed with 2.5 % sodium carbonate. The gel was developed with sodium carbonate formaldehyde mix and the reaction was stopped with 5% acetic acid. The gel was left in 1% acetic acid with 0.05 % glycerol. Finally, the gel was placed in water containing 0.05% glycerol mix before drying.

3.9.7. Coomassie staining

Table 6

Staining solution	Wash solution 1	Wash solution 2
1%(w/v) Coomassie Blue 35051	-----	-----
40%(v/v) Methanol	50%(v/v) Methanol	10%(v/v) Methanol
10%(v/v) Acetic acid	10%(v/v) Acetic acid	10%(v/v) Acetic acid
50%(v/v) Water	40%(v/v) Water	80%(v/v) Water

The gel was soaked in Coomassie blue staining solution (Table 6) for 20 min, destained with wash solution 1 until bands became visible, then washed in wash solution 2 until background disappeared, later scanned and dried.

3.9.8. Western Blot (Franken et al., 2003)

Western blot is a method in biochemistry to detect protein in a sample by using antibody specific to that protein. It also gives information about the size of that protein. In general, a complex protein mixture (such as a cell lysate or extract or a purified protein preparation) is fractionated on a gel by electrophoresis. After separation, proteins are transferred to a membrane, which can be nitrocellulose, polyvinylidene fluoride (PVDF) or nylon. Nitrocellulose and PVDF membranes are most commonly used for protein analysis. Usually, specific antibodies, known as primary antibodies, are used to detect specific protein antigens on the membrane. These are further probed using chemically or fluorescently labelled secondary antibodies.

Following SDS-PAGE, proteins were transferred onto a nitrocellulose membrane with a pore size of 0.45 μm (Schleicher & Schuell) in a semi-dry blotting apparatus at 0.8 mA/cm^2 for 90 min using Tris/glycine/methanol (10 mM Tris, 100 mM glycine, pH 8.5 and 20 % methanol) blot buffer. After transfer, the membrane was washed twice with TBS (20 mM Tris, 137 mM NaCl, pH 7.6) and stained with Ponceau S to ensure proper loading and blotting as recommended by the supplier (Roth, Karlsruhe, Germany). Free protein binding sites on the

membrane were blocked by incubation in 5% skimmed milk in TBS containing 0.05% Tween 20 (TBS-T) at RT for 1 hr or over night at 4°C. This was followed by incubation with affinity purified primary antibody for 1 hr at RT or overnight at 4°C. Unbound antibodies were removed by triplicate wash for 10 min each with TBS-T and then the membrane was incubated for 1 hr at RT with appropriate secondary antibody diluted in TBS-T containing 0.5% skimmed milk. After three washings for 10 min each in TBS-T, bound antibodies were visualised by the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's protocol or using indigenous ECL.

3.9.9. Preparation of ECL substrate for Western blotting

3.9.9.1. Solution A: Luminol (Fluka 09253) 250 mM in dimethylsulphoxide (DMSO) (MW: 177.16) i.e., 5 g in 112 ml DMSO solution. This was stored as 2 ml aliquots at –70°C.

3.9.9.2. Solution B: Coumaric acid (Fluka 28200) 90 mM in DMSO (MW: 164.16) i.e., 1 g in 67 ml DMSO solution. From this, 1 ml aliquot were prepared in eppendorf tubes and stored at –80°C.

3.9.9.3. Solution C: 1 M Tris HCl (pH 8.5)

For 20ml: 200 µl of solution A was added to 89 µl of solution B. To this mixture, 2 ml of solution C was added and the volume was made up to 20 ml with DD water. 6.1 µl of 30% hydrogen peroxide was added just before membrane incubation. Usually the incubation period is for 1 min.

3.9.10. Western blot development with infrared imaging system

Advanced infrared (IR) imaging gives a new way to analyse Western blots. High sensitivity and direct detection on membranes eliminate traditional problems with other fluorescent or chemiluminescent imaging systems. Odyssey™ (Li-COR) is uniquely equipped with two infrared channels for direct fluorescence detection on membranes. Two detection channels simultaneously helps to probe two separate target samples on the same membrane.

After the antigen is transferred to the membrane, an unlabelled primary antibody is bound to the antigen. For detection, an IR-labelled secondary antibody is subsequently bound to the primary antibody. For two-color scans, two labelled secondary antibodies (Alexa 680 and Alexa 800) can be hybridised together in the same reaction. The labelled antibodies are detected directly on the membrane.

The procedure is similar to the conventional Westerns. The modifications are, the secondary antibody incubation is done under dark conditions and the membrane is washed finally twice with TBS T and twice in TBS without Tween20 for 10 min each to prevent auto fluorescence.

3.9.11. Dot blot

It is a simple and convenient method for detection of proteins in crude lysates or solutions without the need for separation by SDS-PAGE. Protein samples (1µl) were applied to nitrocellulose membrane and allowed to dry at room temperature. The membrane was blocked in blocking buffer and processed as described for Western blot.

3.10. Molecular biology methods

3.10.1. Spectrophotometric quantitation of nucleic acids

Quantification of DNA by spectrophotometric methods is reliable if the sample is pure (sample containing insignificant amounts of proteins, agarose or phenol). DNA and RNA absorb light in the UV range (200-400 nm) with the absorption peak at 260 nm. Proteins have an absorption peak at 280 nm. Hence, spectrophotometric readings were taken at both wavelengths and calculated by Kalckar's formula (OD_{260}/OD_{280} ratio) to estimate the purity of the nucleic acid. Pure preparations of DNA or RNA have OD_{260}/OD_{280} values of 1.8 to 2.0 respectively. If there is contamination with proteins, the ratio will be significantly less than the values given above. The concentration of DNA or RNA can be estimated by Beer-Lamberts law.

$$A = \varepsilon \cdot c \cdot l$$

A = absorbance
ε = extinction coefficient
c = concentration of sample
l = length of light pathway in cm

An $OD_{260\ nm}$ measurement of 1.0 with l = 1 cm corresponds to approximately

50 µg/ml double stranded DNA or

40 µg/ml single stranded DNA or RNA or

20 µg/ml single stranded oligonucleotides

Procedure

The sample was diluted in DD H_2O or TE buffer and the absorbance was then measured at 260 and 280 nm in a spectrophotometer. Quartz cuvettes were used for the measurement since plastic absorbs light in the ultraviolet range. Diluents were used as a blank to calibrate the spectrophotometer.

3.10.2. Polymerase chain reaction (PCR)

The PCR is an *in vitro* technique, where a given deoxy ribonucleicacid (DNA) sequence is identically copied. The number of copies rises exponentially, since every newly synthesized DNA-sequence is also the template for the next copy. PCR amplifies segment(s) of DNA that are situated between two regions of a known sequence. Two short oligonucleotides are used as primers for a series of synthetic reactions that are catalysed by a DNA polymerase. Two primers are complementary to the two known sequences at the end of the segment(s) to be amplified.

The template DNA is first denatured by heating in the presence of a large molar excess of each of the two primers and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequence. After annealing, the primers are extended in a reaction catalysed by the DNA polymerase, which synthesises the complementary strand. The cycle of denaturation, annealing and DNA synthesis is repeated many times. The major product of the reaction is a segment of double stranded DNA, whose termini is defined by the 5'-termini of the oligonucleotide primers and whose length is defined by the distance between the two primers. Twenty cycles of PCR amplification increases the amount of the target sequence around one-million fold with high specificity.

If not otherwise stated, PCR reactions were performed using the following protocol. The forward and the reverse primers were diluted to 10 pmol final concentration with HPLC grade water. The reaction mixture was prepared as described in Table 7.

Table 7

PCR reaction mix

1µl	10 ng DNA template
5µl	10x Pfu buffer with $MgSO_4$
5µl	2 mM dNTPs (0.2 mM end concentration)
1µl	10 pmol forward primer (0.4 pmol end concentration)
1µl	10 pmol reverse primer (0.4 pmol end concentration
1µl	1 U Pfu DNA polymerase
36µl	HPLC grade water

A typical program used for amplification is as follows

95°C	3 min	
95°C	30 sec	
55°C	30 sec	36 cycles
72°C	30 sec	
72°C	5 min	

The extension time was calculated depending upon the number of base pairs. As a standard, 60 sec was used for every 500 bp. PCR products were analysed using agarose gel electrophoresis.

3.10.3. Digestion of DNA with restriction enzymes

Restriction enzymes recognise specific, often palindromic sequences in double stranded DNA and cleave these sequences by hydrolysis of the phosphodiester bonds in DNA. These enzymes are classified into three groups - type I, type II and type III. Neither type I nor type III are widely used in molecular cloning. Typical type II restriction enzymes recognise specific DNA sequences that are four, five or six nucleotides in length. The location of cleavage sites within a sequence differs from one enzyme to the other. Some enzymes cleave both strands exactly in the middle of the sequence creating fragments with blunt ends, while others cleave at similar locations which are some base-pairs apart on the opposite strands of the DNA creating DNA fragments with single stranded termini (sticky ends). One unit of restriction enzyme is defined as the enzyme amount needed to digest 1 µg λDNA at 37°C in one hour. It is recommended to use 2 U/µg DNA for small-scale preparations and plasmids. In large-scale preparations, less than 1 U/µg DNA was used if the incubation time was increased up to 2 hrs. More than one restriction enzyme can be used simultaneously, but the restriction enzyme(s) volume was maintained to a volume not to exceed 1/10th of the volume in the final reaction mixture.

Procedure

The following procedure was developed for a typical single standard reaction, containing 0.2 – 5 µg DNA. For digestion of larger amounts of DNA, the reaction mixture should be scaled up appropriately. It is important to optimise the temperature, incubation time, pH and salt concentration for optimal digestion of DNA. Buffer, incubation time and temperature are as those recommended by the manufacturer (Fermentas).

The DNA was mixed with DD water and restriction buffer and then restriction enzyme(s) were added. A typical reaction contains 1-5µl vector (2-5 µg DNA), 1-2µl restriction enzyme (1 U/µg DNA) 2µl of 10x restriction buffer (recommended by manufacturer) and the volume was made up to 20 µl. The reaction mixture was incubated for the time and at the temperature required for optimal restriction by the restriction enzyme, usually 1 hr at 37°C. The digestion reaction was analysed using agarose gel electrophoresis.

3.11. Northern blot

3.11.1. Isolation of RNA

All solutions were prepared in diethylpropyl pyrocarbonate (DEPC) treated sterile water. Brains from ASA KO and WT mouse were isolated, frozen in liquid nitrogen and stored at –80°C. Total RNA was purified by the caesium chloride (CsCl) method as described by (Chirgwin et al., 1979). Briefly, frozen mouse brain was homogenised in GIT buffer (4 M guanidiniumisothiocyanate (Sigma), 25 mM sodium acetate (pH 4.8) and 0.1 M β-mercaptoethanol) for 1 min at 4°C using a potter homogeniser. Homogenate was depleted from tissue debris by centrifugation in a SS-34 rotor at 30,000 x g at 4°C for 30 min. Supernatant was then laid onto 3 ml of 5.7 M CsCl and centrifuged overnight at 12°C in a SW41 rotor at 92,500 x g. The supernatant was carefully removed and the RNA pellet was re-suspended in DEPC-treated water. RNA solution was depleted from proteins by phenol/ chloroform precipitation and centrifuged at 15,700 x g for 5 min at RT. The RNA solution was once again precipitated with one volume of chloroform and centrifuged at 15,700 x g at RT for 5 min. RNA was finally precipitated in absolute ethanol, dried at RT. Finally RNA pellet was solublised in DEPC-treated water, quantified at 260 nm and stored at –80°C.

3.11.2. Northern blotting of MAL, PLP, MBP and β-actin mRNA

20 µg of total RNA isolated from mouse brain was separated in a 1% denaturing agarose gel containing 20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde. The transfer of RNA to a Hybond-N+ membrane (Amersham Pharmacia) was achieved using 20x sodium citrate buffer (SSC) and was immobilized on the membrane by heating at 80°C for 2 hrs.

3.11.3. Preparation of cDNA templates

cDNA probes encompassing the coding regions of MAL, PLP, MBP and beta-actin (BA) was generated by RT-PCR analysis. 5 µg of total RNA was mixed to 100 pmol RNAse free oligo-dT primer and denatured for 5 min at 70°C. Single strand DNA was generated at 42°C for 1

hr by incubating the denatured RNA mixture with 1 mM dNTPs in 25 mM Tris-HCl (pH 8.3) supplemented with 25 mM KCl, 4 mM MgCl2, 10 mM DTT, 20 U of ribonuclease inhibitors and 40 U reverse transcriptase (Fermentas). RNA was depleted from the mixture by hydrolysis in the presence of 5 U RNAse H for 20 min at 37°C. The PCR was performed for MAL, PLP, MBP and beta-actin using 1µg of cDNA as previously described. In short, an initial denaturation for 2 min at 94°C and 30 cycles of 30 sec at 94°C, 30 sec 55°C, 1 min at 70°C and a final extension for 10 min at 70°C. PCR product was gel purified, quantified using λ DNA Hind III marker (Fermentas) as reference following the instructions of the manufacturer. The mouse MBP (1.3 kb) cDNA was released from a pCMV-sport6 plasmid using Bstx I enzyme. Hind III/BamHI enzymes were used to digest the PLP (800 bp) cDNA from a pEGFP plasmid and the MAL (600 bp) insert was removed from a dsRed2 plasmid using EcoRI/Sal I.

3.11.4. Synthesis of [α^{32}P]-labelled probes

The synthesis of [α^{32}P]-labelled DNA probes was carried out by mixing 25ng DNA with 5µl of random primers and the volume was made up to 50µl with DD water and incubated at 95°C for 5 min and at RT for 10 min. To this, 10 µl of Buffer (5x), 5 µl of [α^{32}P]-dCTP (10µCi) and 2 µl of Klenow enzyme (1 U/µl) was added and incubated for 10 min at 37°C. Labelled-probes were purified using G-50 sephadex column by centrifugation at 3200 rpm for 5 min. Labelled DNA fragments were denatured at 95°C for 5 min.

Membranes were pre-hybridized for 1 hr at 42°C (0.2 ml hybridization solution per cm² of the membrane) in the presence of 100 µg heat denatured salmon sperm DNA/ml hybridization solution. The hybridization was done with 2 million cpm/ml hybridization solution of [α^{32}P]-labelled mouse MBP, MAL or PLP overnight at 42°C. After the hybridization, membranes were washed twice for 10 min at RT in 2x SSC/0.1% SDS and twice for another 10 min at 65°C in 0.2x SSC/0.1% SDS and exposed on pre-flashed Fuji Imager screen (Fuji Photo film Co., Kanagawa, Japan) followed by exposure to X-ray films. Before re-probing, the membranes were stripped for 30 min at 65°C in 0.1M Tris-HCl, pH 8.0, 1% SDS and 50% formamide.

3.12. RT PCR [RevertAid H Minus First Strand cDNA Synthesis Kit™ (Fermentas Life Sciences, Leon-Rot, Germany)]

To 1µg of template RNA, 1µl of oligo(dT)$_{18}$ primer was added and the volume was made up to 12 µl using nuclease free water (DEPC water). The mixture was incubated at 70°C for 5 min, chilled on ice and was briefly centrifuged. The tube was placed on ice and to this, 1 µl of ribonuclease inhibitor, 4 µl of 5x reaction buffer and 10mM dNTP mix was added and centrifuged briefly. The mixture was incubated at 37°C for 5 min and then 1 µl of RevertAid™ H Minus-MuLV RT (200u/µl) was added. The volume was made upto 20 µl and incubated at 42°C for 60 min. The reaction was stopped by incubating at 70°C for 10 min. DNA was quantified and used for PCR with various set of primers. PCR analysis was carried out using the oligonucleotide primers listed in Table 8 and 9. The PCR programme is similar to the one described elsewhere. The PCR products were subjected to 2% agarose gel electrophoresis stained with ethidium bromide.

3.13. Preparation of RNA from sciatic nerve

Sciatic nerves from mice were chopped into pieces and homogenised with 2 ml of Trizol® using a 2 ml potter homogeniser at 4°C. The completely homogenised sample was incubated at RT for 5 min. To that, 400 µl of 1-bromo-3-chloro-propane (BCP) was added and vortexed for 30 sec and incubated at RT for 3 min. The sample was centrifuged at 12,000 x g for 15 min at 4°C. The clear RNA phase on the top was gently removed into a new falcon tube. To that, 2 ml of isopropanol (0.5 ml/ml Trizol) was added, vortexed and incubated for 10 min at RT. This was spun as above for 10 min and supernatant was gently discarded. The pellet was washed with 75% ethanol, pelleted by centrifugation as above and the final pellet was resolubilised in 100 µl of DEPC water and stored at –80°C . The RNA was quantified spectrophotometrically.

3.14. Lipid analysis of myelin membranes and myelin lipid rafts (Schwarz et al., 1997) (van Echten-Deckert, 2000)

WT and KO brain myelin samples were washed twice with 5ml Hank's balanced salt solution (HBSS), homogenised in methanol and lipids were extracted by incubating the tissue for 4 hrs

in 4 ml of $CHCl_3/CH_3OH$ (2:1 v/v) at 60°C. The cells were centrifuged and the pellet was re-extracted for 2 hrs with 4 ml of $CHCl_3/CH_3OH$ (1:1v/v). The supernatants were evaporated under nitrogen gas at 60°C. The lipid trace on the walls of the pyrex tubes were dissolved in 2.5 ml of methanol and sonicated for 5 min. For degradation of phospholipids and 9-O-acetylated glycolipids, methanol solublised samples were incubated for 2 hrs at 37°C with 62.5 µl of 4M NaOH. The methanolic sodium hydroxide treatment was neutralised by adding 10 µl of 100% acetic acid. The sample was evaporated under nitrogen at 60°C. The pellet was dissolved in 1 ml of methanol with sonication for 5 min. To this, 1 ml of 300mM aqueous ammonium acetate was added. Subsequently, the samples were desalted by reverse phase (RP) chromatography on an RP-18 column (Schwarz et al., 1997) (van Echten-Deckert, 2000). Glycosphingolipids from samples after alkaline hydrolysis were separated by thin layer chromatography (TLC) using $CHCl_3/CH_3OH/H_2O$ (70:26:4 v/v/v) as the developing solvent. Lipids were visualised by cupric sulfate in aqueous sulphuric acid. Quantitative analysis was performed by employing a Camag automatic TLC sampler 4 and a Camag TLC scanner 3 (Berlin, Germany). For quantification of glycosphingolipids, all samples were compared to the same phospholipid levels.

3.15. Lipid analysis of Schwann cells

Schwann cells were seeded in 10 cm petri dishes and initially treated with 250 mU of ASA/ml for 2 days and maintained with 100mU ASA/ml for 5-6 days. Control cells were maintained without ASA. The cells cultured in proliferation medium were washed once with PBS, scraped using a rubber policeman in cold PBS, pelleted and freeze dried. Lipids were extracted and analysed as described above for the myelin.

3.16. TLC Immunoblotting

TLC plate with samples was dried after separation of bands and incubated with 0.3% Plexigum P28 (polyisobutylmethacrylate) for 2 times of 1 min each. The plate was dried at 55°C for 1 hr or overnight at 25°C. The plate was then incubated with 200mM Tris HCl (pH 7.5) for 1 hr at room temperature. The plate was shortly dried with hairdryer and incubated with 2% BSA in PBS with 0.05% Tween 20 for 1 hr at room temperature and later incubated with 1:50 diluted anti-sulfatide antibody for three hours at room temperature. The plate was washed five times with PBS for 15 min each with drying between each wash. The secondary

antibody incubation was done at a dilution of 1:250 in PBS containing 0.05% Tween 20. The plate was washed five times with PBS (similar to above) and developed with ECL solution. The bands were compared with the similarly developed TLC by cupric sulfate and sulphuric acid

3.17. Electro spray ionisation mass spectrophotometry (ESI-MS/MS) (Sandhoff et al., 2002)

For ESI-MS/MS analysis of DIG's, mass spectrometric standards for hexosylceramide (E.g. GlcCer[18:1,14:0], GlcCer[18:1,19:0] and GlcCer[18:1,25:0]) were synthesised from glucosylsphingosine (psychosine, Matreya, PA 16823, USA) with the corresponding fatty acids. Synthesis and standardisation occurred in analogy to the sulfatide standards as described else where (Sandhoff et al., 2002). All analyses were performed with a triple quadrupole instrument (VG micromass model Quattro II, Cheshire, UK) equipped with a nano-electrospray source as described by Roger Sandhoff and co-workers (Sandhoff et al., 2002). For the detection of sulfatides, the instrument was set to the negative precursor ion mode m/z –97 (corresponding to the fragment $[HSO_4]^-$) using a cone voltage of 95 V and a collision energy of 90 eV. For the detection of galactosylceramides, the instrument was set to the positive precursor ion mode m/z +264 (corresponding to the fragment [C18-sphingosine – $2H_2O + H^+]^+$) using a cone voltage of 28 V and a collision energy of 44 eV. Significance of differences between means was assessed by 1-way ANOVA. This experiment was done in association with Dr. Sandhoff. R (DKFZ, Heidelberg).

3.18. Cell culture

The cells used throughout this study (except SCs) were grown on polystyrene flasks, plates or dishes. They were maintained in DMEM with 10% fetal calf serum (FCS), penicillin/streptomycin (100U/ml), l-glutamine (2mM). Trypsinised (0.5g trypsin and 0.2g EDTA / 1000ml) and grown at 37^{o}C in a humidified 5% CO_2 incubator.

3.18.1. SC Culture

Animals which were used in this study to prepare SCs were derived from the same breed as previously described (Hess et al., 1996). Some modifications from the method of Brockes and his collaborators (Brockes et al., 1979) was used to prepare the SCs from the sciatic nerve.

Primary cells from the sciatic nerve of two week old ASA KO mice were prepared by chopping the excised sciatic nerve into 1mm pieces (Komiyama et al., 2003) in a small petri dish with HBSS. Chopped material was pelleted and dissociated by incubating with intermittent shaking for 50 min with trypsin solution (2.5%) without EDTA and Collagenase Type 1 (0.6%) at 37°C in a humidified 5% CO_2 incubator.

The cells and undissociated tissues were passed through a Pasteur pipette and 23G needle several times and the enzyme activity was neutralised using 10% FCS, 50 units/ml penicillin, 50 µg/ml streptomycin and 2mM L-glutamine containing Advanced Dulbecco's Modified Essential Medium (ADMEM). Cells were collected by centrifugation at 1800 x g for 10 min without brake. The pellet was suspended in proliferation medium (10% FCS containing ADMEM +100 µg/ml bovine pituitary extract (BPE) (Upstate Biotechnology, New York, USA) + 2 µM Forskolin) (Weiner and Chun, 1999) and seeded on 0.1% gelatine coated 6 well plates and incubated at 37°C in a humidified 5% CO_2 incubator for 5-6 days without any disturbance.

Initially, various methods to purify the SCs from the contaminating fibroblasts were attempted. To name some are complement lysis and cold jet (Jirsova et al., 1997). But they did not yield good results as expected. Hence, from the first stage of seeding, the supernatant with unattached cells was collected, centrifuged and the cell pellet was re-suspended in the proliferation medium and seeded similarly as above in two more wells of a 6 well plate (Komiyama et al., 2003) and incubated under the same conditions for further 3-4 days. In this so called second seed, almost 80% of pure SCs were obtained. Similarly unattached cells from the second seed were pelleted and plated in a fresh well of 6 well plate to get the third seed. Similarly fourth seeding was carried out which led up to 90% SC purity in these wells. Up to 80% of contaminating cells (unidentified cells apart form fibroblasts) were killed by holding the cells for 2 weeks in DMEM without FCS and replenishing with fresh medium once in 3-4 days (Komiyama et al., 2003). The cultures were passaged using accutase once in 5-8 weeks and the cells were maintained in culture for about 6 months until they spontaneously immortalised. Finally, the cells were purified by isolating clones using a cloning plate.(Greiner Labortechnik., No. 704160)

3.18.2. Renal epithelial cell culture

Primary cultures of kidney cells were prepared according to Pizzonia and co-workers (Pizzonia et al., 1991). Eighteen month old mice were killed under deep anaesthesia (intraperitoneal injection of tribromomethanol) by transcardial perfusion with phosphate buffered saline. Kidneys of 2-3 mice were dissected and placed in Optimem-I medium (Invitrogen, Karlsruhe, Germany) containing 100U/ml penicillin and 50µg/ml streptomycin, 0.1mM bumetanide and 0.1 mM chlorothiazide. Medullary tissue was isolated and minced using a single-edge razor. Tissue pieces were collected in Optimem-I containing 0.1% type 2-Collagenase (Worthington, Lakewood, NJ 08701, USA), 0.1mM furosemide and chlorothiazide and constantly bubbled with 95% O_2 : 5% CO_2 at room temperature. After combining minced tissue from all kidneys, medium was brought to 37°C and incubated for another 15min. During this incubation, tissue was mechanically digested by aspirating through successively smaller diameter Pasteur pipettes. After centrifugation at 800 x g for 5 min supernatant was discarded and cell pellet was washed with Optimem-I containing 10% FCS. Cell suspension was filtered through 200µm wire mesh, re-centrifuged and pellet was resuspended in Optimem I. Cells were plated on cover slips coated for 2h at 37°C with 100µg/ml Collagen I in PBS and analysed after 3-4 days in culture by light microscopy and immunofluorescence. This was done in association with Dr. Klein, University of Bonn).

3.18.3. Freezing of cells

To maintain long term stocks of cell lines, they must be kept frozen in liquid nitrogen. At least 10 vials of each cell type, frozen at an early passage should be kept in long term storage. All vials were labelled with the cell type, passage number and date. The positions, namely, tower and rack number of all vials in the liquid nitrogen tank were entered in the log book.

The cells were frozen when they were actively dividing, about 75-80% confluent. The cells were taken up in trypsin, protease activity was inhibited with FCS containing medium, counted, pelleted and resuspended in freezing medium (DMEM with 20% FCS and 10% DMSO) to give about 5 x 10^6 cells / ml. The cells were transferred to cryo vials, left at – 80^{o}C overnight and transferred to a liquid nitrogen container the next day.

3.18.4. Recovering cells from liquid nitrogen

It is important to thaw cells as quickly as possible and to remove the DMSO from the culture as soon as possible. The vial was removed from the liquid nitrogen tank and placed immediately in the 37°C water bath. 9 ml of prewarmed complete medium was placed (medium + serum + antibiotics) in a tissue culture flask or dish coated with appropriate substance. The cells were transferred from vials and swirled gently to disperse the cells. After 4-5 hrs, DMSO medium was removed, replaced with fresh growth medium and allowed to grow until confluence before passaging.

3.18.5. Genotyping of SCs from ASA knockout mice

Cells grown in a 10 cm dish were collected with accutase, washed with PBS and pelleted. The cells were resuspended in 500 µl of NET buffer (10mM NaCl; 25mM EDTA and 2mM Tris-HCl pH 7.5). To that, 50 µl of 2 mg/ml Proteinase K and 50 µl of 10% SDS were added, gently mixed and incubated with minimal shaking at 56°C overnight. This material was extracted for 1 min with minimal shaking with one volume of 1:1 phenol : chloroform, spun at 16,000 x g for 5 min. The upper aqueous layer was transferred to a fresh tube with 500 µl chloroform, spun and the aqueous layer was mixed with 2 volumes of 100% ethanol. DNA was collected from this mixture using a micropipette tip, centrifuged and the pellet was washed in 70% ethanol and air dried. Later, this was resuspended in 200 µl of TE buffer and quantified.

For PCR analysis of ASA KO cells, the following oligonucleotide primers were used. TGACCAAGGCCTTGTTCCCAT (ASA sense), TAGGGTGGAAGTTACCCTAGA (ASA antisense), GGAGAGGCTATTCGGCTATGAC (ASA neo). To amplify the sequence containing the ASA knockout gene, a PCR programme with initial denaturation at 94^{o}C for 5 min and 32 cycles of denaturation at 94^{o}C for 30 sec, annealing at 56^{o}C for 30 sec and extension at 72^{o}C for 60 sec was carried out. The PCR products were analysed on a 1% agarose gel containing 0.5µg/ml ethidium bromide.

3.18.6. Alcian blue staining

The 4% paraformaldehyde fixed cells were stained with alcian blue similar to the method described for tissue sections (Matzner et al., 2002; Scott and Dorling, 1965). In short, cells were incubated with 0.05% (w/v) alcian blue in 0.025M sodium acetate buffer (pH 5.7) containing 0.3 M $MgCl_2$ and 1% (w/v) paraformaldehyde for 2-3 weeks. These were then washed with physiological buffer and investigated microscopically.

3.18.7. Immunohistochemistry

Cells intended for immunohistochemistry were grown on 0.1% gelatine coated cover slips on a 24 well plate. All steps were performed at room temperature. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilised by incubation in 0.3% Triton X-100 in PBS for 5 min. Cellular auto fluorescence was reduced by incubation in freshly prepared 0.5% sodium borohydrate ($NaBH_4$) for 10 min. After saturation of unspecific binding sites with 2% normal goat serum (NGS) for 30 min, cover slips were incubated with primary antibodies diluted in PBS containing 0.2% NGS. After three washes with PBS, secondary antibodies were incubated for 1hr in PBS / NGS in the dark. After washing the secondary antibodies similarly as above, cell nuclei were counterstained with 4,6-Diamidino-2-phenylindole (DAPI) for 5 min. The cells were washed 2x in PBS and 1x with DD water and were mounted with 50% glycerol and analysed by epifluorescence microscopy [Axiovert M (Zeiss, Jena, Germany)].

For immunofluorescence against S100, the cells were post fixed after PFA treatment in methanol for 10 min at –20^{o}C. For p75, goat serum was replaced with 5% donkey serum for blocking.

3.18.8. Electron Microscopy

SCs grown on a 24 well plate were treated with 250mU ASA/ml for 4-5 days. The control untreated cells were fixed in 1% glutaraldehyde in 0.1M phosphate buffer and post fixed in 1% osmium tetroxide in 0.1M phosphate buffer and embedded in epon. The sections were stained with lead citrate and uranyl acetate and analysed. Electron microscopy pictures were taken by Prof. Büssow (Department of Anatomy, University of Bonn).

3.18.9. Transfection of cells

Transfection is the general process of introducing foreign DNA into cells and monitoring the expression of interest. DNA transfection is essential for the study of gene function and gene regulation. Common transfection techniques include calcium phosphate co-precipitation, electroporation and the use of viral vectors . These methods have produced variable results in a variety of cell types. Cationic liposome-mediated transfection methods (lipofection, cytofection) were an important addition to the previous methods. Additional classes of compounds known to mediate transfection include lipopolyamines and dendrimers .

3.18.9.1. Transfection with Exgen500

The transfection efficiency of ExGen 500 is due to its protonation profile, which increases from 20-45% between pH 7.0 and 5.0. Every third atom is an amino nitrogen that can be protonated, thus making the molecule a virtual “proton sponge. ExGen 500 and DNA charge-interact and form small (30-100nm), stable, highly diffusible particles that settle on the cell surface by gravity. The ExGen 500/DNA complex is then absorbed into the cell by endocytosis. The “proton sponge” effect of the complex buffers the acidic pH of the endosome. This facilitates rupture of the vesicles and release of the ExGen 500/DNA complex in the cytoplasm before lysosomal degradation of DNA occurs. The protected DNA is then translocated to the nucleus resulting in high transfection efficiency.

Procedure

Generally, the volume of the ExGen 500/ DNA mixture represents 1/10 of the total volume of the culture medium. 1 µg of DNA was diluted in 100 µl of 150mM NaCl. This was gently vortexed and spun briefly. To this, 3.3 µl of ExGen 500 was added and the solution was mixed with gentle vortexing for 10 sec. The complex was incubated for 10 min at room temperature. This complex was gently transferred to the cell monolayer and the plate was gently rocked back and forth and from side to side to achieve even distribution of the complexes. Incubated overnight and microscopically checked for gene expression.

3.18.9.2. Transfection with FuGENE

In a small sterile tube, sufficient serum-free medium to make up the final volume to 100 µl was added. To that, 3 to 6 µl of FuGENE 6 reagent was added and mixed by tapping gently. 1–2 µg DNA solution (0.02–2.0 µg/µl) was added to the prediluted FuGENE 6 reagent medium mix. The tube was gently tapped to mix the contents. The mixture was incubated for a minimum of 15 min at room temperature. The mix was added drop wise to the sub confluent cells in 3.5 cm dishes. The medium was swirled to ensure even dispersal. Incubated overnight and microscopically checked for gene expression.

3.18.9.3. Transfection with Lipofectamine

Approximately 2 x 10^5 cells were seeded per well of a 6 well plate or in 35 mm dishes containing DMEM with 10% FCS. When the cells reached partial confluency, the following were prepared in polystyrene tubes.

Solution A: For each transfection, 2 µg DNA (plasmid) was diluted in 375 µl serum-free DMEM
Solution B: For each transfection, 12 µl LIPOFECTAMINE reagent was diluted in 375 µl serum-free DMEM.

Solution A and B were combined, mixed gently and incubated at room temperature for 30 min. In the mean time, the cells were washed once with 2 ml serum-free DMEM. For each transfection, 750 µl serum-free DMEM was added to each tube containing the lipid-DNA complexes. This was mixed gently and the diluted complex solution was overlaid onto the washed cells. After 18 hrs, 20% FCS containing equal volume of medium was added to the cells and the transfection efficiency was checked after 48hrs.

3.18.9.4. Transfection with Transfectin

Cells were seeded at a density of 1 x 10^5 cells per well in a 24 well plate in 0.5 ml of the appropriate growth medium so that they are 70–80% confluent at the time of transfection. Optimum transfection is obtained when cells are transfected at high cell density. On the day of transfection, DNA (0.5–1 µg) and 50 µl of serum-free medium were mixed separately for each well of a 24 well plate. Transfectin lipid reagent (0.5 µl) was diluted to 50 µl in serum

free medium in a polystyrene tube. To this, the DNA was added and pipetted up and down for even mixing. This mixture was incubated for 20 min at room temperature to allow DNA-transfectin lipid reagent complex to be formed. In the mean time, 300 µl of serum containing medium was added to each well of cells in a 24 well plate. After incubation, the DNA-Transfectin lipid reagent complex (100 µl) was added directly to one well and mixed gently by rocking the plate. Cells were incubated at 37°C in a humidified 5% CO_2 incubator overnight. The next day, the medium was replaced with fresh serum containing medium.

3.18.9.5. Nucleofection

The cells were cultured until they reached confluence, washed briefly with PBS, detached with trypsin and neutralised with serum containing medium. 3 x 10^6 cells were pelleted. The supernatant was completely removed. DNA (3-5 µg) to be transfected was taken in 10 µl of Nucleofector solution. 500 µl of culture medium was prewarmed in an eppendorf and kept ready for use. 2 ml of serum containing medium was taken in each well to be seeded in a six well plate and kept ready for cell seeding. Pelleted cells were taken up in 90 µl of Nucleofector solution and the cells were transferred to the DNA solution by pipetting 3 times up and down and transferred to the electroporation cuvettes. The cuvettes were placed in the cuvette holder of the electroporation machine and appropriate programme specified for the cell type was selected. After the electrical impulse, the cells were collected in the 500 µl prewarmed medium and seeded in a 6 well plate by swirling with the pipette tip. This was incubated overnight at 37°C in a humidified CO_2 incubator. The next day, the medium was replaced with fresh serum containing medium.

3.19. Translipofection – A novel method to load sulfatide to SC

This is an indigenously developed system to load sulfatide on cultured cells. The plates and cover slips were coated with 0.1% gelatine for 30 min. SCs were seeded at a density of 25,000 cells/well in a 24 well plate containing 13 mm cover slips and 1,00,000 cells/well in a 6 well plate and allowed to grow until 80% confluency is reached.

0.4 and 1.5 µl of 5mM sulfatide and 0.5 µl and 1.5 µl of transfectin (BioRad) were used per well of a 24 well plate and 6 well plate respectively. Initially, sulfatide was taken in a polystyrene tube, to that, transfectin was added and mixed. To this mix, 100 µl (per well of 24

well plate) and 300µl (per well of a 6 well plate) of ADMEM without serum and growth factors was added, pipetted thoroughly 15 to 20 times and left at room temperature for 30 min for the formation of sulfatide transfectin complex.

In the mean time, the medium in the cells were replaced with 250 µl and 1.2 ml of proliferation medium for one well of a 24 well plate and one well of a 6 well plate respectively. After 30 min of incubation, the complex was pipetted gently up and down for a couple of times, added in drops to the cells containing medium and mixed by shaking the plate gently by hand. The plate was spun at 3200 x g for 4 min at 37°C in an eppendorf centrifuge and incubated for 48 hours before fixing the cells for microscopy or scraping the cells for lipid extraction.

3.20. ASA quantification and activity measurement

Depending on the approximately expected concentration of ASA, the material to be analysed was diluted accordingly with TBS (pH 7.0) . From this 5-50 µl was taken in a 2 ml eppendorf and just before warming at 37°C, 400µl of substrate (10mM p-nitro catechol sulfate in 10% (w/v) NaCl in 0.5M sodium acetate pH 5.0) was added to the ASA containing sample and the control and incubated for about 10-30 min depending upon the quantity of ASA. After incubation, the reaction was neutralised with 1250µl of 1N NaOH. The OD was measured at 515nm (12,400 cm^2 / mmol)

The amount of ASA was calculated using the following formula:

$$\text{mU/ml ASA} = \frac{\text{Total volume in ml} \times 1000}{\frac{\text{Molecular extinction coefficient (12.4)} \times \text{Volume of ASA/ml} \times \text{Incubation time(min)}}{1000}} \times \text{OD}$$

Specific activity of ASA = mU/ml ASA / mg/ml protein

3.21. Primers for SC markers and transcription factors

Details about the base pairs and gene bank accession numbers are illustrated in (Watabe et al., 2003) Primer sequences for PMP-22 and PO have been described in (Watabe et al., 1995; Watabe et al., 2001).

Table 8

Primers	Sequence(Position)
S100 sense	ATGGTTGCCCTCATTGATGTC
S100 antisense	GAACTCCTGGAAGTCACACTCC
MAL sense	TGACTTGCTCTTCGTTTGTGAG
MAL antisense	ACAAGATCATCAGGGAAGTGGT
PMP-22 sense	CCAACACTGCTACTCCTCATCA
PMP-22 antisense	AGGATTTGGAAGAATCCAGTGA
P0 sense	CTACAGTGACAATGGCACTTTC
P0 antisense	TCAGGTAGAAGAGCAACAGCAG
Pax-3 sense	AGTGAGCACCTTTGCCAGTA
Pax-3 antisense	AGAGGCCTGCCGTTGATAAA
Krox-20 sense	TCGAAAGTACCCTAACAGGC
Krox-20 antisense	TCAGCCAGAGCTTCATCTCA
SCIP sense	TCAAGCAACGACGCATCAAG
SCIP antisense	GCTTGGGACACTTGAGAAAG
Sox10 sense	CATGGCCGAGGAACAAGACC
Sox10 antisense	GCTCTGTCTTTGGGGTGGTTG
MASH1 sense	ATGGAGAGCTCTGGCAAGAT
MASH1 antisense	AGCGTCTCCACCTTGCTCAT
Egr-1 sense	GATGCAATTGATGTCTCCGC
Egr-1 antisense	CAGATAGTCAGGGATCATGG
c-Jun sense	CAATGGGCACATCACCACTA
c-Jun antisense	TCCTGAGACTCCATGTCGAT
CREB sense	TTTGACGCGGTGTGTTACGT
CREB antisense	CCCTGGTGCATCAGAAGATA
NFκb sense	ACCATGGACGATCTGTTTCC
NFκb antisense	GCTCCTCTATAGGAACGTGA

3.22. Primers for neurotrophic factors and receptors

Table 9

Primers	**Sequence**
TrkA sense	CTGGCCATGTCCCTACACTT
TrkA antisense	GGTGCAGACTCCAAAGAAGC
TrkB sense	CTGGCTGCTCCTAACCTCAC
TrkB antisense	ACAGTGAATGGAATGCACCA
TrkC sense	TGCCTGATGTGGACTGGATA
TrkC antisense	GTGGGCTTTTTGAAGAGCAG
p75 sense	CAACCAGACCGTGTGTGAAC
p75 antisense	GGAGAACACGAGTCCTGAGC
NT-3 sense	GGTTGCAGGGGGATTGAT
NT-3 antisense	TATTCGTATCCAGCGCCA
BDNF sense	TTGGATGCCGCAAACATGTC
BDNF antisense	CTGCCGCTGTGACCCACTC
NGF sense	GATCGGCGTACAGGCAGAA
NGF antisense	TGGGCTTCAGGGACAGAGTCT
GDNF sense	ATGAAGTTATGTTATGTCGTGGCTG
GDNF antisense	ACCGTTTAGCGGAATGCTTTCTTAG
GDNFR sense	TCGGGACTGATTGGCACAGTCATG
GDNFR antisense	GGCTGGAGGCACCAGCGAGACCAT
Ret sense	GGATGGAGAGGCCAGACAACTGCAGC
Ret antisense	CCATAGAGTTTGTTTTCAATCCATGTGG

Results

In the first part of the project, the analysis of brain, particularly the myelin of ASA deficient mice was carried out in comparison to the material of the wild type mice for significant alteration in lipid and protein composition. In the second part, an *in vitro* cell culture model was established from tissue of ASA KO mice to study the molecular aspects of MLD.

To get an overview about the protein composition of the ASA KO and WT mouse brain, whole brain extracts were prepared by homogenising the brain in HEPES buffer (pH 7.4) containing protease and phosphatase inhibitors. The homogenate was spun at 17,000 x g for 30 min. The upper part of the pellet was used for the preparation of myelin as described in materials and methods. The supernatant of this centrifugation was subjected to 2D SDS - PAGE and proteins were analysed by silver staining. No major difference in the pattern of protein distribution between the brains prepared from WT (Fig 6A) and ASA KO (Fig 6B) mice could be detected. Since the brain consists of tens of hundreds of proteins, it was realised that analysis of the whole brain material is not suitable to find any minor changes in protein expression. Therefore, myelin compartment of the brain was initially analysed.

A **B**

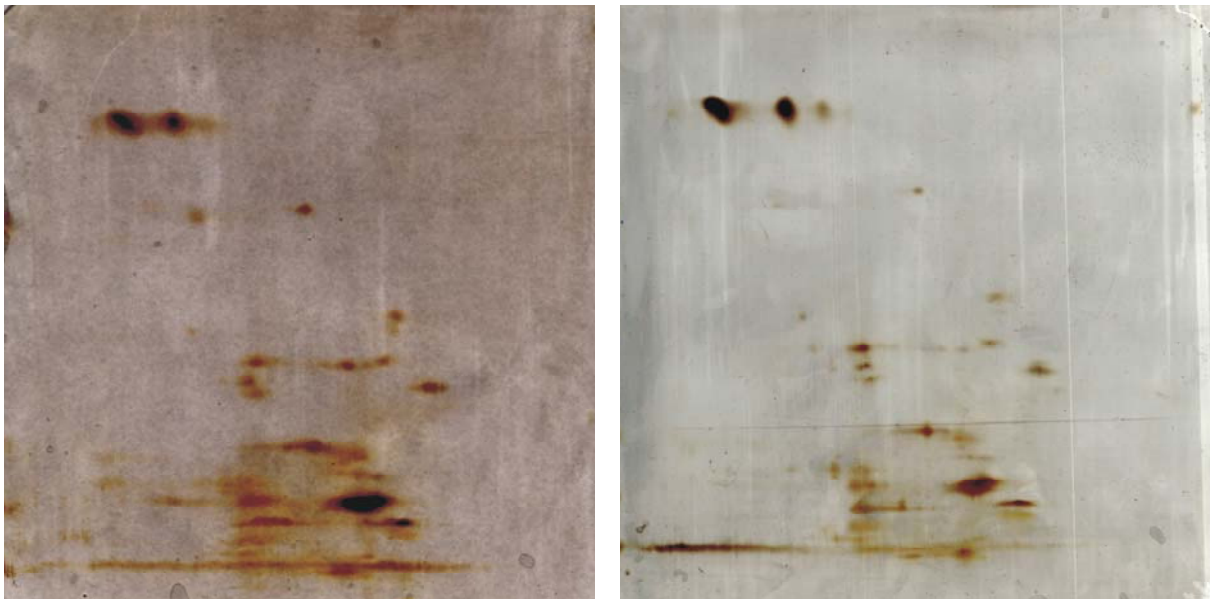


Fig: 6 Two dimension gel electrophoresis of ASA KO and WT brain proteins. Two dimension for the 17,000 x g supernatants of (A) WT and (B) ASA KO mice. Silver staining of the gel does not show any significant difference between the WT and the KO mice.

4.1 Analysis of myelin sulfatide content in ASA KO mice

Myelin is a multilayered membrane which surrounds nerve axons. This is the only example of a membranous structure where contact between extracellular surfaces of membrane from the same cell occurs. The two major glycosphingolipids of myelin are galactosylceramide and sulfatide (Boggs et al., 2004). Myelination is strongly dependent on the timely and correct expression of a series of myelin proteins by the oligodendrocytes and SCs (Frank et al., 1999). MLD patients show severe demyelination in the central and peripheral nervous system. There is a marked decrease in the white matter of the brain, in severe cases, grey or brown discoloration is observed. As observed in patients, storage of sulfatide occurs in myelin-forming oligodendrocytes and in some neurons in brains of two year old ASA KO mice (Gieselmann et al., 1998). Therefore, myelin sulfatide content of the ASA KO mice was investigated on first hand to find whether this sulfatide storage is restricted to the lysosomal compartment of the cell or elevated sulfatide levels can also be found in myelin, which may in turn cause some modifications in the myelin related proteins.

The galactosylceramide : sulfatide ratio in the normal human white matter is about 3. This is reduced in patients with late infantile MLD to less than 1. Hence the ratio of galactosylceramide to sulfatide is a sensitive indicator of alterations in lipid metabolism in MLD patients (von Figura et al., 2001). Therefore, lipids were extracted from myelin as well as from the whole brain. By thin layer chromatography (TLC), the glycosphingolipid content of the myelin and whole brain was examined. TLC analysis clearly showed that the levels of sulfatide increased about 1.4 fold in whole brain as well as in myelin of ASA KO mice (Fig. 7A+B), whereas the content of galactosylceramide decreased to about 85% of normal. The galactosylceramide : sulfatide ratio was decreased from 2.9 -1.7 in myelin and 1.9 to 1.2 in lipid preparations from brain (Fig 7C). With respect to the above changes, no significant differences in the proportions between the hydroxy and non-hydroxy fatty acid forms of sulfatide or galactosylceramide were observed.

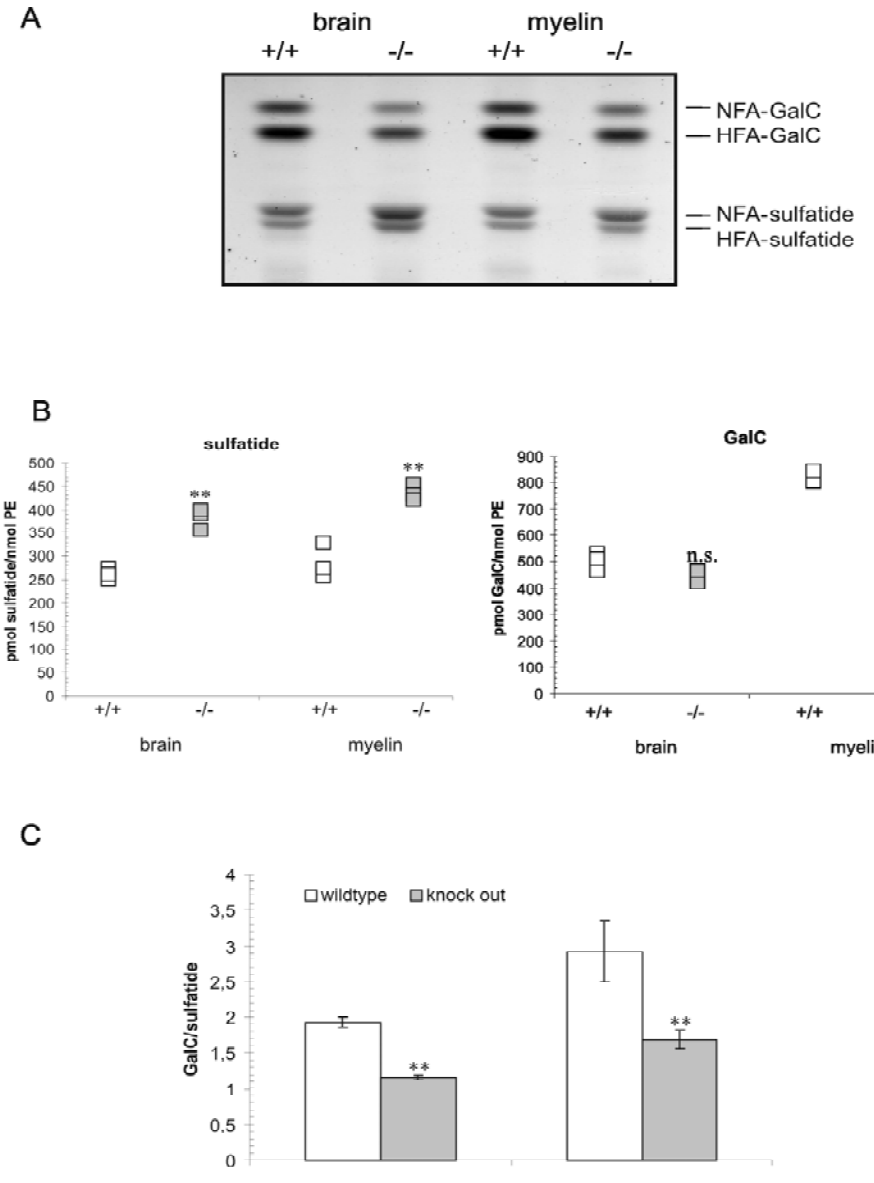


Fig 7: TLC analysis of brain and myelin lipids. (A) Brain and myelin lipids of 24 months old WT (+/+) and ASA KO (-/-) animals were extracted by methanol/chloroform and purified by reverse phase chromatography. The content of phospholipid is unchanged in ASA KO mice (data not shown). Therefore, the same amounts of lipids regarding to phospholipid content were separated on a TLC plate. (B) TLC bands of galactosylceramide and sulfatide were quantified using a Camag TLC scanner. Sulfatide and galactosylceramide amounts were examined from three animals of each genotype. (C) Shows the ratio of galactosylceramide to sulfatide, which is considered to be a sensitive indicator of lipid metabolism alterations in MLD. Values are means of SD of 3 independent experiments. Note that in ASA KO mice, the ratio between galactosylceramide and sulfatide is significantly diminished in whole-brain as well as myelin. NFA: non-hydroxy fatty acids; HFA: hydroxy fatty acids; GalC: galactosylceramide; PE: phosphoethanolamine. **P $\leq$ 0.01 ASA KO against WT by one-way ANOVA. n.s. : not significant.

4.2. Confirmation of myelin sulfatide content

Since myelin was prepared from the whole brain material, there is a possibility that the increase in sulfatide content of myelin may not be due to the accumulation of sulfatide in myelin, but could be due to lysosomal contamination from the brain material during myelin preparation. To confirm that the increase in the sulfatide content of myelin is not due to any contaminating lysosomal compartments during the myelin preparation, Western blot analysis of whole brain extracts and myelin with antibodies against myelin basic protein (MBP), myelin associated glycoprotein (MAG) and the lysosomal/late endosomal marker, lysosome-associated membrane protein-2 (LAMP-2) were carried out. If myelin preparation is free from any lysosomal contamination, the blot against LAMP-2 should be negative. The results clearly demonstrate that myelin prepared from ASA KO and WT mice was negative for the lysosomal marker LAMP-2. Whereas MBP and MAG are clearly enriched in the myelin membrane preparations compared to that of the whole brain. Therefore, the myelin is free of any lysosomal contamination (Fig. 8).

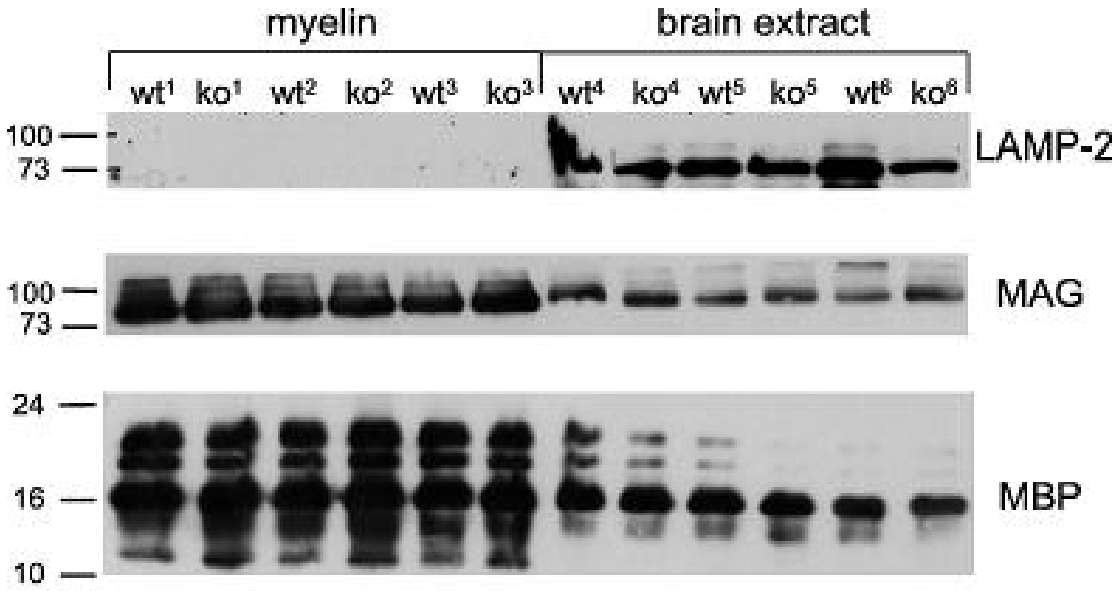


Fig 8 : Analysis of myelin preparation. Myelin membranes or whole brain extracts were prepared from six wild-type (wt^{1-6}) and six knockout (ko^{1-6}) mice. Fifty micrograms of protein from whole brain and five micrograms of myelin proteins were resolved by SDS-PAGE and transferred to nitrocellulose as described in materials and methods. Western blot analysis with the lysosomal/late endosomal marker LAMP-2 demonstrates that myelin prepared from ASA KO mice is not contaminated with membranes from this cellular compartment. Analysis with antibodies against MAG and MBP reveals their enrichment in myelin preparations. Antibody immunoreactivity was visualised using chemiluminescence. Bars on the left indicate the position of apparent molecular weight marker bands (in kDa).

4.3. ASA KO mice show reduction in MAL protein amount

The major proteins of central nervous system myelin are myelin proteolipid protein, myelin basic proteins and myelin-associated glycoprotein. To examine whether the increased sulfatide content of myelin has any effect on myelin protein composition, Western blot analysis was carried out to quantify various myelin proteins in the myelin prepared from 24 month old ASA KO and WT animals. The protein signals were quantified by AIDA 2.11 software.

Proteins separated by SDS-PAGE and transferred to nitrocellulose were processed for incubation with antibodies directed against various myelin proteins. As already evident in figure 8, there was no significant difference in MBP or MAG protein levels in myelin preparations from ASA KO mice. The amount of PLP, MOG, contactin/F3 and CNPase also remained unchanged when compared to WT mice (Fig. 9). Previous studies demonstrated that MAL mRNA is predominantly expressed in the highly myelinated regions of the nervous system such as spinal cord, brainstem and developing sciatic nerve. Lower expression levels were found in the cerebellum and cerebral cortex. In situ hybridization experiments showed the presence of MAL in the nervous system. MAL is exclusively expressed by oligodendrocytes and Schwann cells (Caduff et al., 2001). Therefore, the MAL protein was investigated in the preparations. The results indicated that the glycosphingolipid-associated protein MAL, a small proteolipid protein of 17kDa, was decreased between 40% and 75% in all myelin preparations analysed from ASA KO animals (four pairs of animals tested).

4.4. ASA KO mice show downregulation of MAL mRNA

To investigate whether the downregulation of MAL protein as detected by Western blot is caused by the transcriptional down regulation of MAL mRNA, Northern blot analysis was performed from brains of two year old ASA KO and WT animals. For comparison, MBP and PLP expression levels were also investigated in parallel, because these myelin proteins were not significantly affected in ASA KO mice (Fig. 8 and 9). The results indicate that there is no difference in the mRNA expression levels for both control proteins; however, MAL mRNA was decreased to about 50% in ASA KO when compared to WT animals (Fig.A1, see appendix).

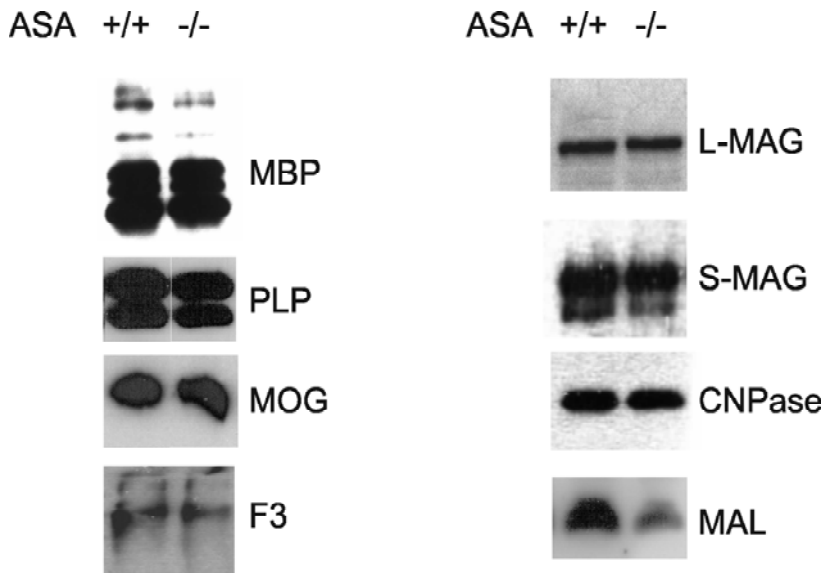


Fig 9 : Western blot analysis of myelin proteins. Equal amounts of myelin proteins (30µg) from wild-type (+/+) and ASA KO (−/−) animals were resolved on a 12.5% SDS PAGE gel and transferred to nitrocellulose. Membranes were incubated with antisera directed against the myelin proteins MBP, PLP, MOG, contactin/F3, L- and S-MAG, CNPase and MAL. Antibody immunoreactivity was visualised using chemiluminescence. It is significant that there is a strong reduction of MAL in ASA-deficient mice, whereas, all other myelin proteins investigated appeared normal.

With the help of transgenic animals, it becomes evident that the functional role of myelin is much more than only insulating axons for their signal transduction. In recent years, it also became apparent that the myelin structure is very sensitive to alterations of single constituents. MAL is a membrane protein expressed in central and peripheral myelin. The functional role of MAL in the apical sorting and transport mechanisms of polarized epithelial cells and MAL association with glycosphingolipids in myelin membranes suggest that MAL is involved in the formation, transport and/or maintenance of particular glycosphingolipid microdomains, the so called “rafts” of specialised plasma membranes.

4.5. Analysis of lipids in detergent soluble and insoluble fractions

Since MAL is a component of the detergent insoluble microdomain (otherwise mentioned as rafts or DIGs) and has shown significant downregulation on protein as well as mRNA level, it was investigated to find whether there are any alterations in lipid distribution between the detergent soluble and insoluble fractions and later to analyse the protein composition and distribution between the two compartments.

In order to determine whether alterations of sulfatide and galactosylceramide levels (as analysed by TLC) affect both soluble and insoluble fractions similarly, myelin was isolated, extracted with Triton X-100 at 4°C and subjected to sucrose density gradient centrifugation to separate detergent soluble and insoluble protein/lipid fractions. The lipids were quantified by ESI-MS/MS in both fractions. Figure A2 (see appendix) demonstrates the enrichment of sulfatide as well as galactosylceramide in the insoluble fractions of myelin DIGs (IS). In ASA KO animals, the concentration of sulfatide was increased in both the detergent soluble (S) as well as insoluble fractions (IS) of the sucrose gradient, whereas, galactosylceramide content was again decreased. The increase of sulfatide in ASA KO mice compared to WT was stronger in detergent insoluble fractions (2.33 fold of normal) than in the soluble compartment (1.7 fold of normal). In contrast, the decrease of galactosylceramide was more pronounced in the soluble fractions (0.54 of normal) than inside DIGs (0.86 of normal).

4.6. Distribution of membrane proteins between detergent soluble and insoluble compartments is not altered in myelin of ASA KO mice

Recent analysis of the protein composition in DIGs derived from primary cultured oligodendrocytes and from central myelin revealed a broad spectrum of proteins such as the GPI-anchored F3 and NCAM 120. The association of glycosphingolipids and DIG associated proteins may be crucial for the sorting of these components to the myelin sheath during development. Since sulfatide and MAL are known to be components of DIGs (Frank et al., 1998) and MAL being a putative element of the cellular sorting machinery into these membrane microdomains (Puertollano et al., 1999), it was investigated to find whether the alterations in MAL and sulfatide amount has any effect on the localisation of other myelin raft proteins.

DIGs were prepared from the brain myelin as described in materials and methods. Analysis of DIGs on a SDS PAGE gel by silver staining did not show any significant difference between the WT and the experimental ASA KO mice (Fig 10). Silver staining of proteins gives only a rough idea about protein composition and the quality of the preparation.

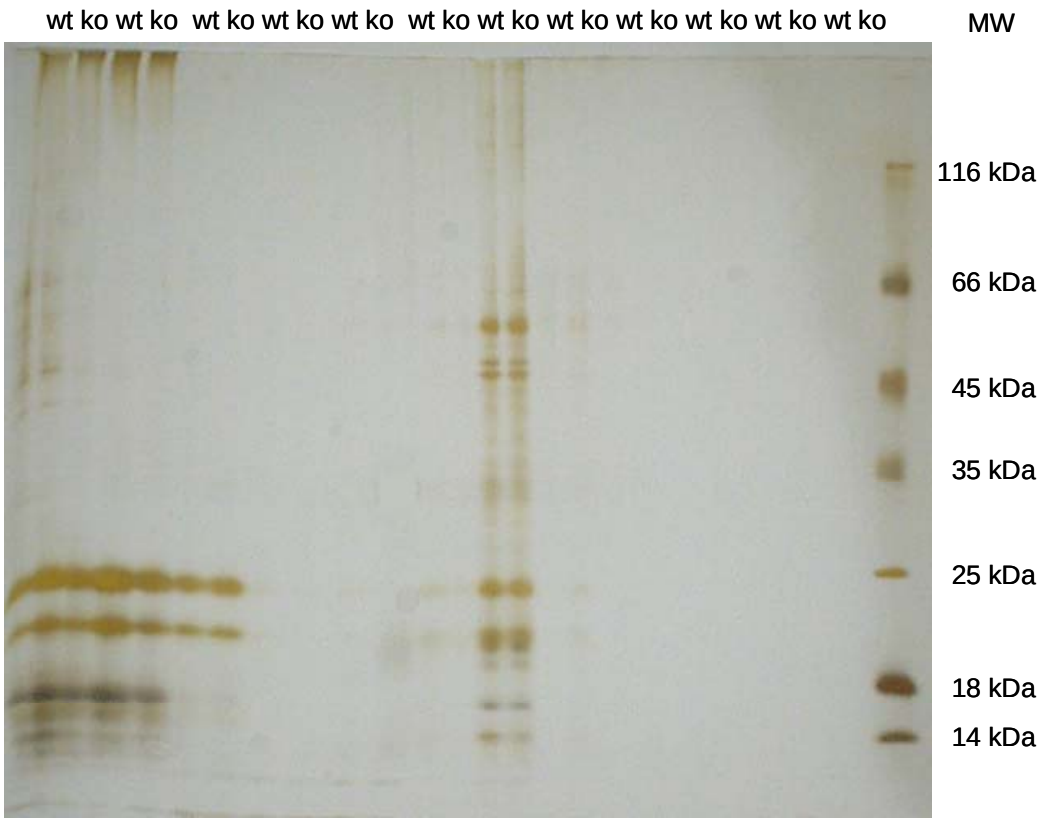


Fig 10: Silver staining of raft fractions. Silver staining of the DIGs and soluble fractions of myelin preparation shows equal amount of protein load in the ASA KO and WT. MW – molecular weight marker.

To examine possible consequences of the changed glycolipid and MAL content, the sucrose gradient fractions were analysed for proteins like PLP, CNPase, NCAM 120, contactin/F3 and MOG, which are known to be associated with myelin DIGs (Kramer et al., 1997) (Kim and Pfeiffer, 1999); (Kramer et al., 1999); (Kim and Pfeiffer, 2002). From 2 year old ASA KO and WT animals, myelin was prepared and was subjected to sucrose density gradient centrifugation and 1ml fractions were collected. Identical aliquots of each fraction were subjected to Western blot analysis with antibodies directed against various proteins (Fig.10). Blots were analysed densitometrically. The results of the densitometric analysis show that 50% of PLP as well as MOG were located in insoluble fractions. Antibody against F3 detected its antigen predominantly in the soluble fractions. CNPase, NCAM 120 and MAL were detected only in DIGs.

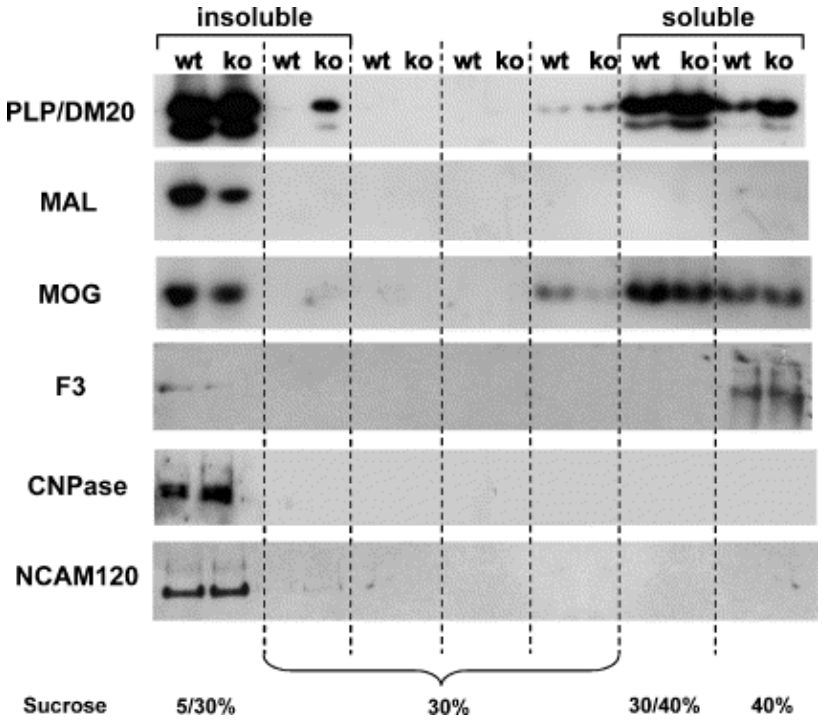


Fig 11: Western blot analysis of myelin DIGs and soluble proteins. Myelin of two year old WT and ASA KO mice was prepared, extracted with Triton X-100 and soluble and insoluble membrane material were separated by sucrose density centrifugation as described. Identical aliquots of each fraction were resolved by SDS-PAGE and transferred to nitrocellulose. Membranes were probed with different antibodies directed against the proteins indicated on the left. Antibody immunoreactivity was visualised using chemiluminescence. % - shows different percentages of sucrose concentrations in the gradient. In this experiment, Western blot against MAL was performed by Dr. N. Schaeren-Wiemers (University Hospital, Basel).

The discrepancy with the literature where higher amount of PLP, CNPase and NCAM 120 are usually found to be soluble in Triton-X 100 (Taylor et al., 2002) is probably caused by the investigation of 24 month-old animals. In this study using younger animals (3-6 month), it was also observed that most of the PLP protein to be soluble in Triton X-100 (Fig.11). Nevertheless, none of the myelin proteins investigated showed significant differences in their detergent solubility properties between myelin of normal and ASA KO mice (Fig.11). In some ASA KO animals, the amount of PLP protein in the soluble fractions was increased compared to WT animals (Fig. 12). This effect, however, was not consistent in all experiments and the difference was not significant upon statistical analysis. Slight differences in the amount of MOG were also frequently seen but without any detectable tendency between ASA KO and WT mice.

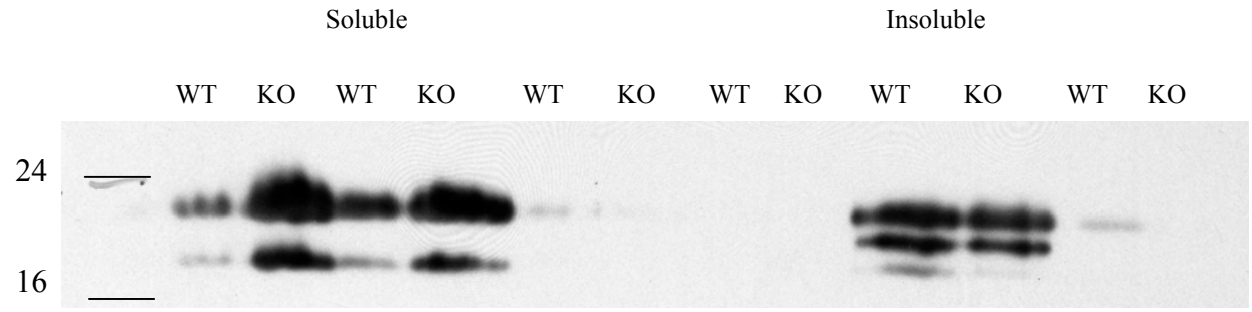


Fig 12: Western blot analysis of PLP expression. Western blot of myelin DIG and soluble fractions of ASA KO and WT mice with PLP antibody.

4.7. Fyn is downregulated in ASA KO myelin

Src family kinases have long been implicated in the regulation of cell growth and differentiation and the family members Fyn, Yes, Lyn and Lck are expressed in the nervous system. Fyn plays a unique role in CNS myelination (Sperber et al., 2001). Fyn is found in many brain areas, including glial cells in white matter tracts and in cultured oligodendrocytes and its activity in brain is highest during the developmental period approximately corresponding to the peak of myelination (Bare et al., 1993) (Osterhout et al., 1999). Fyn and lyn are major Src family kinases present in DIGs. Therefore it was investigated to find whether sulfatide storage has any effect on Src related signal transduction. Hence, lyn and fyn proteins of myelin from ASA KO and WT animals were quantified by Western blot. The results indicated that there is no significant change of lyn and fyn in 2-3 months old animals, whereas, in animals aged one year and above, fyn is down regulated by about 0.6 fold compared to WT of the same age group (Fig.13A). This effect is also visible in the DIGs prepared from myelin (Fig.13B), where a downregulation of fyn in the ASA KO compared to the WT can be observed. The results indicate that the sulfatide storage has some direct or indirect effect on the fyn kinase.

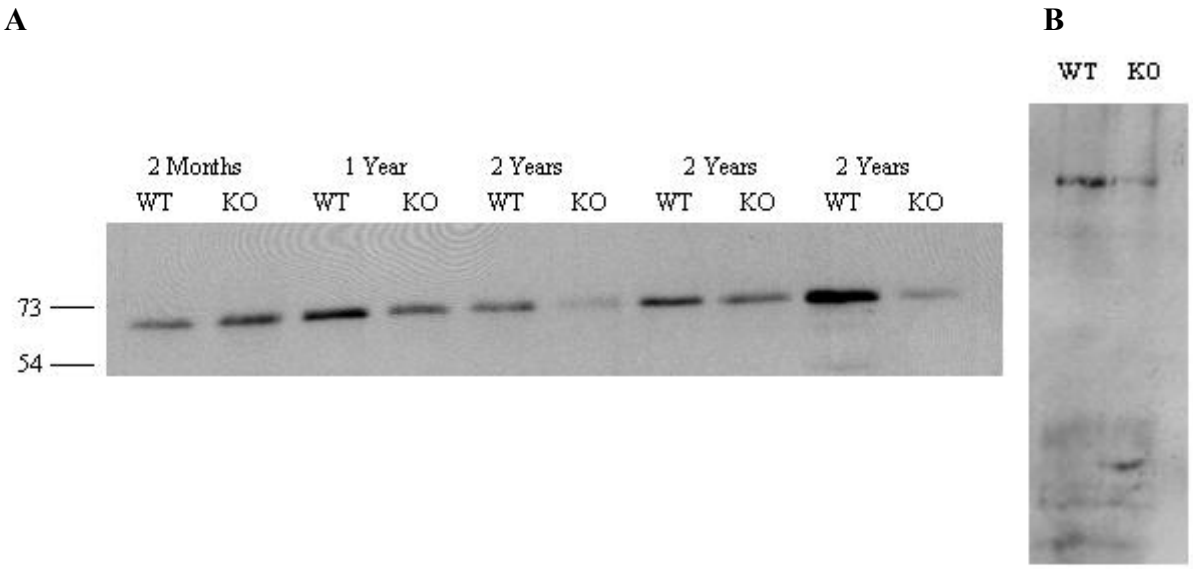


Fig 13: Western blot analysis of myelin and DIGs for fyn expression. (A) Investigation of brain myelin with fyn antibody. Three sets of 2 years, one 1 year and one 3 months old WT and ASA KO mice myelin (30µg protein equivalent) was loaded onto 12.5% SDS PAGE gel, blotted onto NCM and investigated with fyn antibody (1:400). (B) Investigation of myelin raft fractions (pooled) with fyn antibody shows downregulation of fyn in ASA KO compared to WT.

4.8. Examination of phosphorylated proteins in the myelin and whole brain material

Protein phosphorylation is a ubiquitous regulatory mechanism of signal transduction, where it plays an important role in controlling a large number of cellular processes. Reversible protein phosphorylation has been known for some time to control a wide range of biological functions and activities (Graves and Krebs, 1999). The primary players in phosphorylation are protein kinases that catalyse the transfer of the terminal phosphate group of adenosine triphosphate (ATP) to acceptor protein target(s) at serine, threonine and tyrosine residues. Over 500 kinases are present in cells and roughly one fifth of these are tyrosine kinases. Kinases are involved in most aspects of cell regulation, including cell growth, differentiation and metabolism. Protein phosphatases play opposite roles to protein kinases by dephosphorylating the target protein. As there is downregulation of fyn kinase in the ASA KO myelin and DIGs, the general phosphorylation state of the brain was analysed.

Whole brain extracts were prepared by homogenising the brain in HEPES buffer (pH 7.4) with protease and phosphatase inhibitors. This was spun at 17,000 x g for 30 min. The supernatant and the myelin prepared from the pellet were analysed by Western blot with antibody against phosphotyrosine (PY) (Fig.14A), phosphoserine (PS) (not shown) and

phosphothreonine (PT) (Fig.14B). The results indicate that there was no significant difference between WT and ASA KO preparations on Westerns with PS and PT, whereas, 2 unknown proteins phosphorylated at tyrosine residues with a molecular weight of 50kD and 25kD were up regulated in 60% of the ASA KO animals tested. There is no difference in the myelin associated proteins.

Since the whole brain extracts consists of tens of hundreds of proteins, it is difficult to analyse the molecular details in such extracts. To conduct studies on the alterations in MAL, down regulation of protein kinases and complicated patterns of cellular phosphorylation, it will be difficult to detect any minor changes by analysing the whole brain extract. Therefore cell culture system for sulfatide storing cells has to be established.

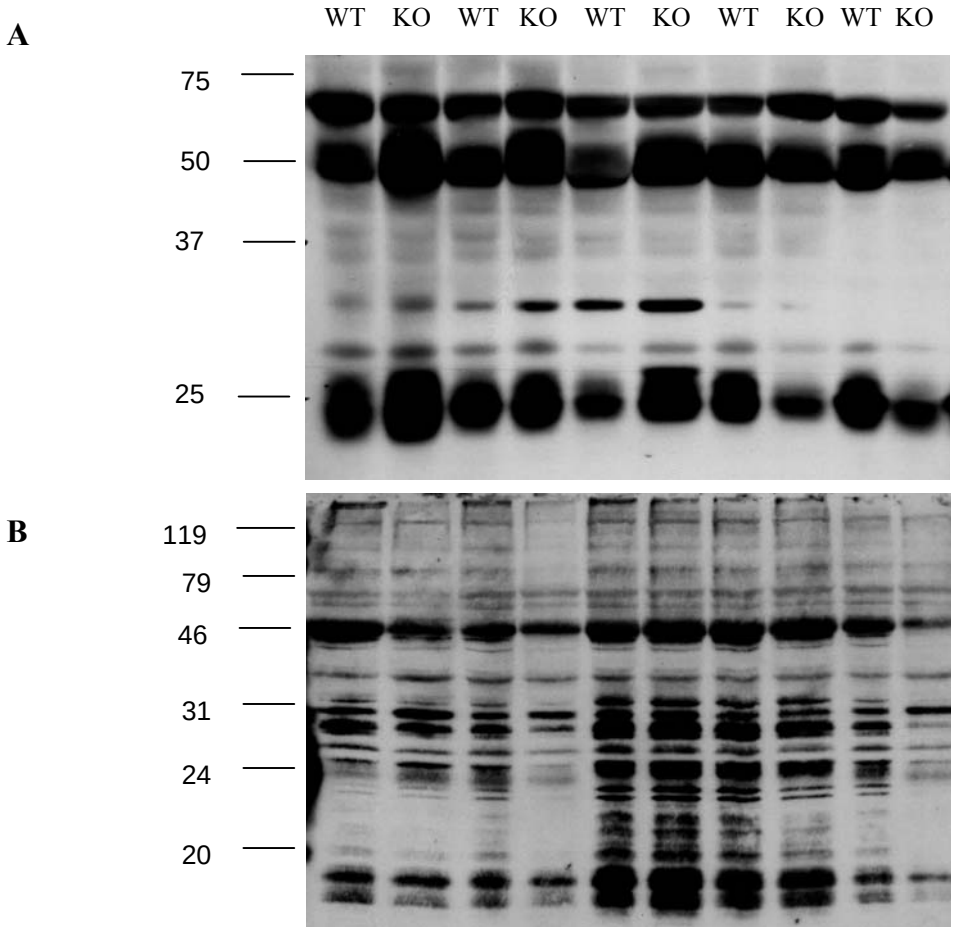


Fig 14: Western blot analysis of whole brain extracts to detect phosphorylated proteins. Whole brains of 5 sets of 2 year old ASA KO and WT mice were homogenised in 10.5% sucrose using potter homogeniser and spun at 17,000 x g for 50 min. Supernatant (50µg protein) was loaded onto 12.5% SDS PAGE gel, blotted on to NCM and investigated using (A) anti-phosphotyrosine, (B) anti-phosphotreonine and anti-phosphoserine (not shown) antibodies.

4.9. MAL is mistargeted into lysosomes of sulfatide storing kidney cells

Oligodendrocytes and astrocytes were isolated and investigated for the sulfatide content, but they did not store sulfatide in culture. At that time, the only cultured cells that were shown to accumulate a substantial amount of sulfatide were the renal distal tubule cells from the ASA KO animals (Hess et al., 1996); (Gieselmann et al., 1998; Lullmann-Rauch et al., 2001). It was evident from literature that these cells also express MAL in the apical membranes of these cells *in vivo* (Frank et al., 1998) and *in vitro* (Zacchetti et al., 1995). MAL has been shown to bind sulfatide (Frank, 2000). Therefore, these cells might serve as a model for examining the consequences of sulfatide storage on the sulfatide binding proteins like MAL. So, beside the observed decrease of MAL protein and mRNA, the storage of sulfatide in the lysosomal compartment of ASA KO mice might have consequences for the cellular distribution of MAL. Immunohistochemical analysis was carried out on cultured kidney cells to examine the distribution and co-localisation of sulfatide, MAL and LAMP-1, a late endosomal/lysosomal marker (Fig.15). Analysis of WT animals showed less sulfatide compared to the strong sulfatide staining observed in the ASA KO animals (Fig. 15 A+B). These cells show characteristic storage inclusions rich in sulfatide (Fig. 15 B arrow). In order to identify the sulfatide storing compartments, double staining with LAMP-1 was performed. This demonstrated the late endosomal/lysosomal location of these sulfatide inclusions (Fig.15 C).

Immunofluorescent staining of distinct plasma membrane areas was found on these cells when they were stained with affinity purified anti-MAL antiserum (Fig.15 D). These MAL positive areas were also rich in sulfatide (Fig. 15 E+F arrows). In most of the WT cells, MAL localised to plasma membranes as well as to vesicular intracellular compartments (Fig. 15 G). No co-localisation of MAL and LAMP-1 could be found in non-sulfatide storing wild type cells (Fig. 15 I). In contrast, sulfatide storing cells from ASA KO mice showed numerous intracellular inclusions with both MAL and LAMP-1 immunoreactivity (Fig. 15 J-L, arrows). Thus, in sulfatide storing cells, MAL and LAMP-1 co-localise to a substantial extent.

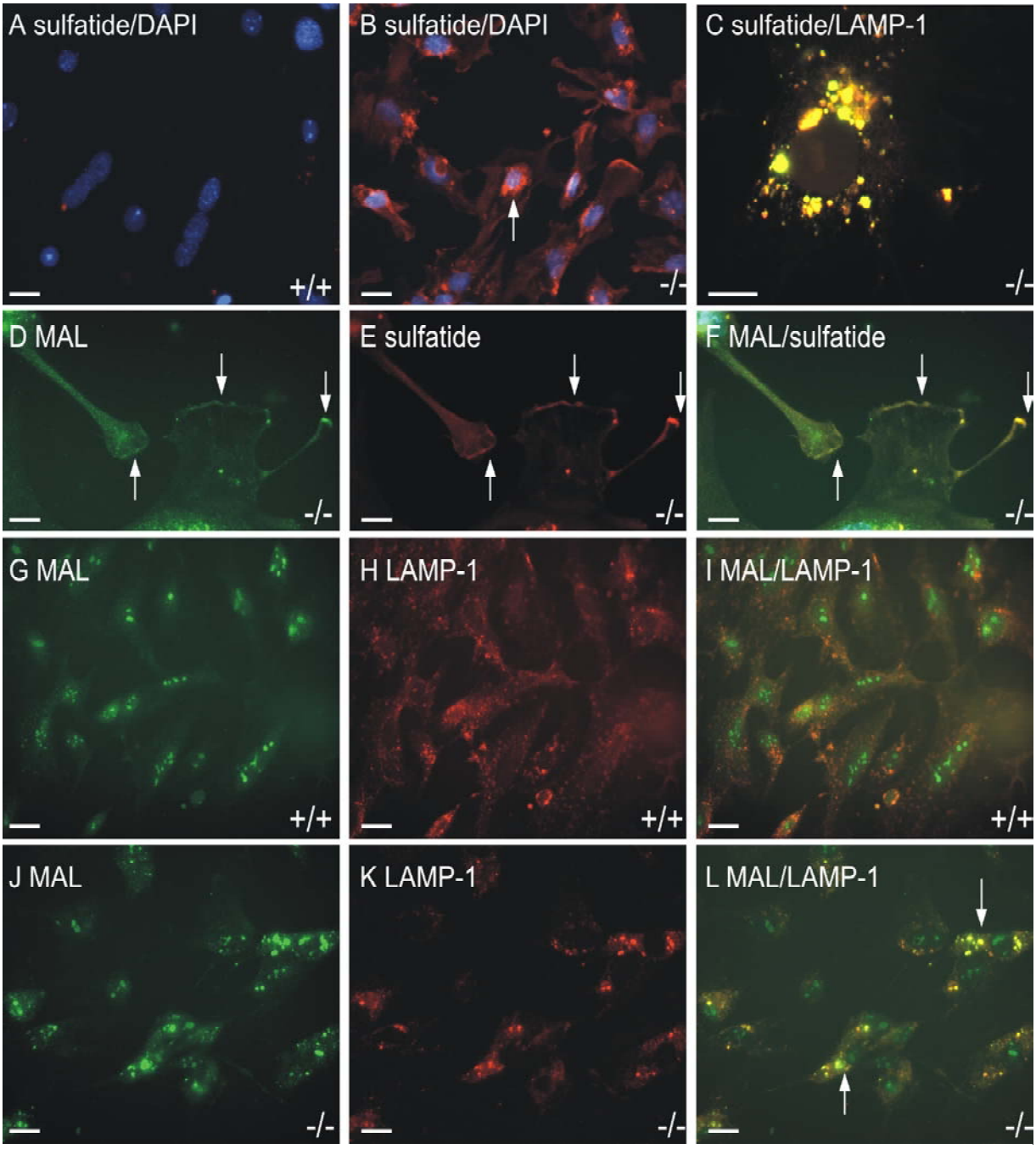


Fig 15: MAL and sulfatide colocalise in intracellular storage inclusions. Renal cells of WT (+/+) and ASA KO (−/−) mice were prepared and grown on glass coverslips. Comparison of WT (A) and ASA KO cells (B) shows the increase of sulfatide level in membranes as well as intracellular storage inclusions (arrow) of the latter. Co-localisation (yellow) of sulfatide (red) with LAMP-1 (green) demonstrates the lysosomal origin of these intracellular inclusions (C). Double staining of MAL and sulfatide demonstrates their colocalisation in distinct areas of the plasma membrane (D–F, arrows). WT cells show no colocalisation of MAL and LAMP-1 in intracellular structures (G–I), whereas cells heavily storing sulfatide contain MAL in LAMP-1 positive intracellular storage inclusions (J–L, arrows). Scale bar in A, B and G–L: 20µm and in C–F: 10µm. This experiment was performed by Dr. D.Klein, University of Bonn.

4.10. Establishing cell cultures of ASA deficient SCs

Studies carried out on sulfatide storing kidney cells (Klein et al., 2005) give some information about the cellular alterations with respect to sulfatide storage. The major disadvantage of this cell system is that this is of non-glial origin and does not mimic the glial cell phenotype. The patients as well as the ASA KO mice do not show any kidney phenotype. These cells loose their stored sulfatide after few passages and are extremely difficult to transfect. Therefore, a cell culture system using a cell type showing a pathological phenotype has to be established. To mention again, oligodendrocytes isolated from ASA KO mice have shown to be difficult to cultivate over longer time periods and didn't show substantial amounts of sulfatide storage in culture. Therefore, in this study, SCs, the other cell type responsible for the pathological phenotype in humans as well as ASA KO mouse model was used to establish a sulfatide storing cell culture model.

SCs have been studied previously for *in vitro* myelinating capability and can synthesise sulfatide *in vitro.* These cells express MAL and are of glial origin. The cells were prepared from WT as well as ASA KO mice and cultured as described under materials and methods. Majority of the cells had bipolar or triangular shape, which is characteristic for SCs (Jirsova et al., 1997). By repeated transfer and maintaining cells in serum free medium, more than 75% of contaminating cells were effectively removed which resulted in SC cultures with purities greater than 90%. Cells were further cultured by feeding them once a week with fresh proliferation medium and passaging them once a month for a period of 5-6 months. During this period they retained their spindle shaped morphology on 0.1% gelatine coated plates (Fig. 16 A) and were immunoreactive to polyclonal anti-S100 (Jessen et al., 1985; Komiyama et al., 2003; Watabe et al., 2003) (Fig. 16 B+C). During the first few months of culture, cells showed strict contact inhibition, whereas after 5-6 months in culture, spontaneously emerging colonies were observed (Fig. 16 D-F). Cells from these colonies tend to grow more rapidly than the primary SCs and were further purified by isolation via cloning plates to achieve 99-100% purity (Fig. 16G). One of the clone was designated as SC KO. After subculturing, they continued to show spindle shaped morphology, but were not contact inhibited and formed subcolonies when the culture reached confluency. These properties were also reported for SC lines established by other laboratories (Watabe et al., 2003). They were further passaged, stored and thawed. They also survived in culture for over 10 months (Fig. 16G). Similarly, wild type SCs (Fig. 17) were also prepared from sciatic nerve of 2 week old WT mice. These cells also spontaneously immortalised and were in culture for more than 10 months.

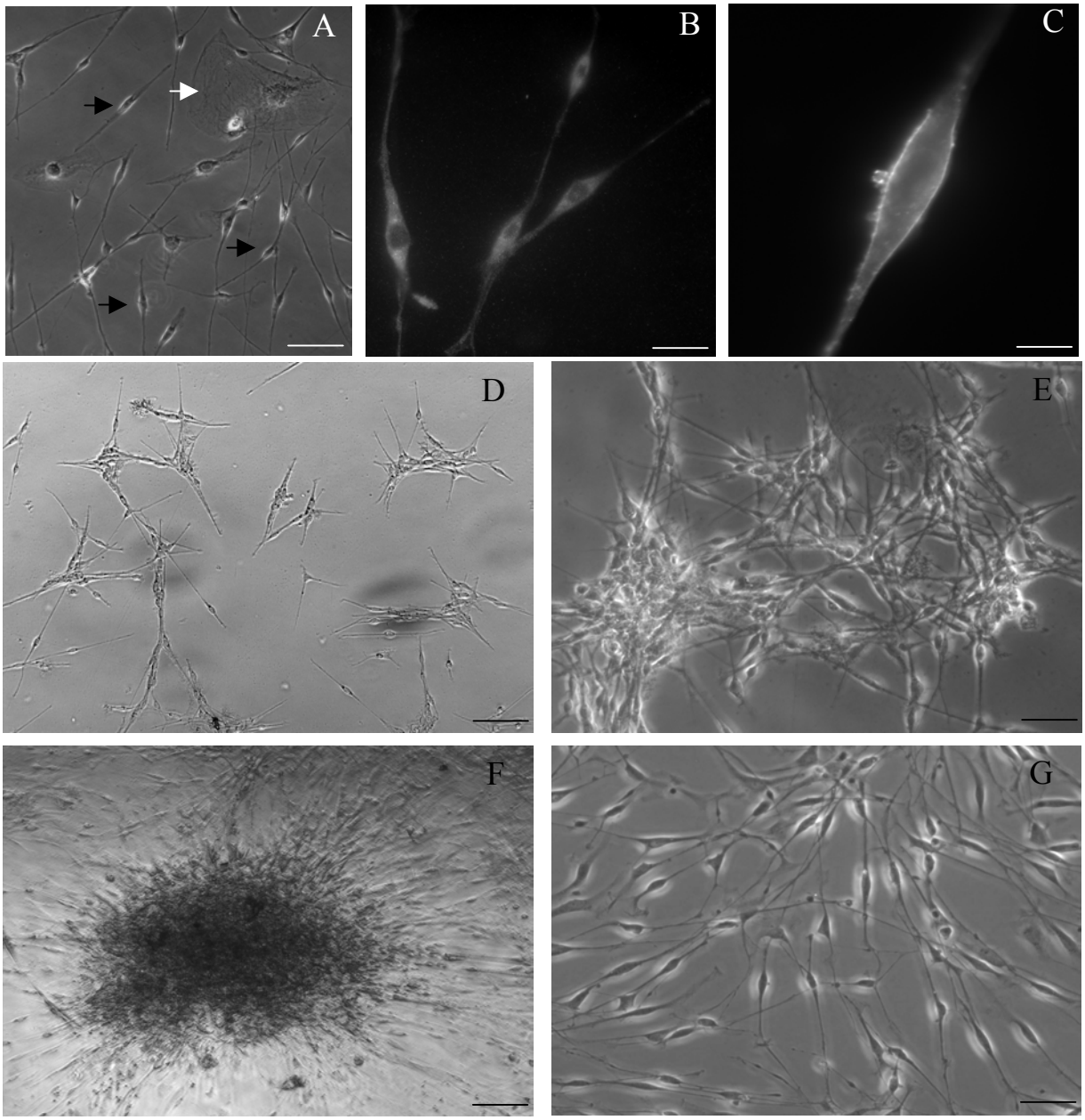


Fig 16: Schwann cell culture. Cells were maintained for 6 months by changing medium once in a week and passaging them once in a month into a new dish. (A) Mixed culture of SCs and fibroblasts at 2 month in culture. White and black arrows indicates fibroblast and SC respectively. (B) S100 Staining. In short, cells were fixed in 4% PFA and post fixed in 100% methanol at –20°C for 10 min. Incubated with 0.05% sodium borohydrate for 15 min at RT. Washed 3X with PBS and incubated with 0.3% Triton X-100 in PBS for 5 min. Washed 3X and blocked with 5% goat serum for 60 min. Incubated in anti-S100 antibody (1:200) in 0.5% blocking agent overnight at 4°C, rinsed and incubated in Cy2 labelled secondary antibody for 1 hr at RT. DAPI (1:500) staining was done for 5 min, rinsed and mounted in 50%w/v glycerol. (C) Immunofluoresence for p75 could differentiate between the positive Schwann cells and the negative fibroblasts. Similar to S100 staining, but the difference is that the cover slips were not post fixed with ethanol and the cells were blocked with 5% donkey serum. Incubated with anti-p75 antibody (1:500). (D), (E) and (F) are spontaneously emerging colonies in long term SC culture at 6 months *in vitro* (D) tiny colonies at 10X magnification. (E) Three days after the formation of clones and (F) SC clone. (G) Pure SC culture. Scale bar in A, D, E and F: 100 µm; B: 20 µm; C: 10 µm and in G: 50 µm.

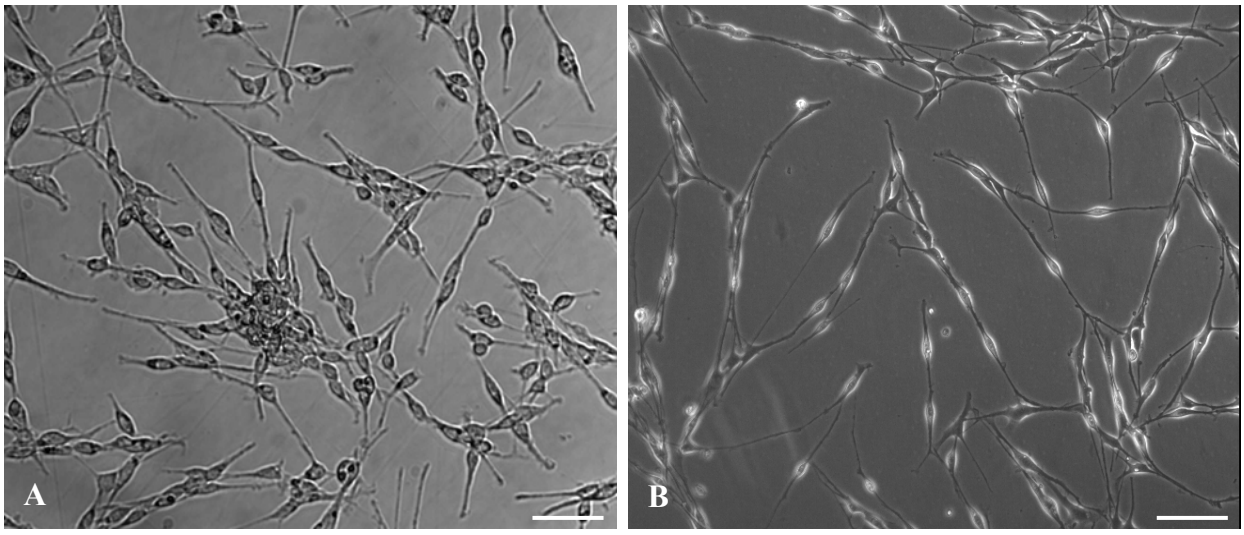


Fig 17: Schwann cell WT. Schwann cells were also prepared from WT control mice similar to SC KO. (A) Shows colony formation (B) Immortalised cells after long term in culture. Scale bar 100 μm.

4.11. Characterisation of SCs

SCs were initially characterised to prove their ASA deficiency. This was done by PCR analysis of the genomic DNA from SCs with appropriate oligonucleotide primers. A 1100 bp fragment for the ASA KO and 480 bp fragment for the WT was anticipated and visualized (Fig 18A). The 480bp fragment identifies the ASA wild type locus, the primer amplification of 1100 bp is possible only when the ASA deficient allele is present. This confirms that the cells were purely from ASA KO and WT origin, respectively.

S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells and are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. Staining with S100 for the identification of SCs has been widely used by various authors (Scarpini et al., 1988; Shen et al., 2002; Watabe et al., 2001). Immunoreactive S100 protein localises in the cytoplasm and nuclei of astrocytes, SCs, ependymomas and astrogliomas. SC KO cultures were positive for S100 staining (Fig 16B). To further confirm that the isolated cells are SCs, immunohistochemistry was done with these cells using an antibody against p75. The protein p75 plays an important role in many cellular activities, including cell survival (Davies et al., 1993), cell death (Rabizadeh et al., 1993) and SC migration (Anton et al., 1994). The immunohistochemistry results showed that the cells are positive for p75 (Fig. 16C). This staining was also previously used for staining SCs for their purification (Vroemen and Weidner, 2003).

To further prove the identity of SCs, RT-PCRs were performed for cDNA's of proteins that are known to be specific for SCs in cell culture. Figure 16B demonstrates that the isolated cells in addition to S100 and p75 are positive for the mRNA specific for myelin forming cells like MAL, PMP-22 and P0. This was previously shown by Watabe and his co-workers (Watabe et al., 2003) for SCs in culture.

Besides these characteristic myelin associated molecules, a set of transcription factors was reported to be expressed by SCs in culture (Watabe et al., 2003). In this study, SC KO was investigated by RT-PCR for transcription factors namely, Pax-3, Krox-20, SCIP, Sox10, MASH1, Egr-1, c-Jun, CREB and NF-kB. These are some of the transcription factors associated with SCs and were previously reported to be expressed by immortalised SC in culture (Watabe et al., 2003). The positive results (Fig. 18C) obtained for these transcription factors by RT-PCRs for the mRNAs of these factors also confirmed the character of the isolated clone SC KO. Together with immunohistochemistry and the cellular morphology, these data confirmed that the cells isolated from sciatic nerves of ASA KO mice have a SC phenotype. These cells were not investigated for the *in vitro* myelination capability until date, but they are capable of dividing indefinitely. Under favourable conditions, these cells may transform to myelinating SCs.

4.12. Lipid storage of SC KO

To serve as a cell culture model for metachromatic leukodystrophy, the immortalised SCs have to show two major characteristics beside their ASA deficiency. First, they should store sulfatide inside their lysosomal/late endosomal compartment similar to that found in tissue material from ASA deficient mice and MLD patients. Second, lipid storage should be reversible by treatment of the cells with recombinant ASA added to the cell culture medium. To investigate this, the following set of experiments were performed.

4.12.1. Alcian blue staining

Intracellular storage of sulfatide can be demonstrated by staining the cells with the cationic dye alcian blue (Lampert and Lewis, 1975; Schott et al., 2001). Alcian blue combines with sulfatide in the presence of 0.3M magnesium chloride in the tissue sections and cells from

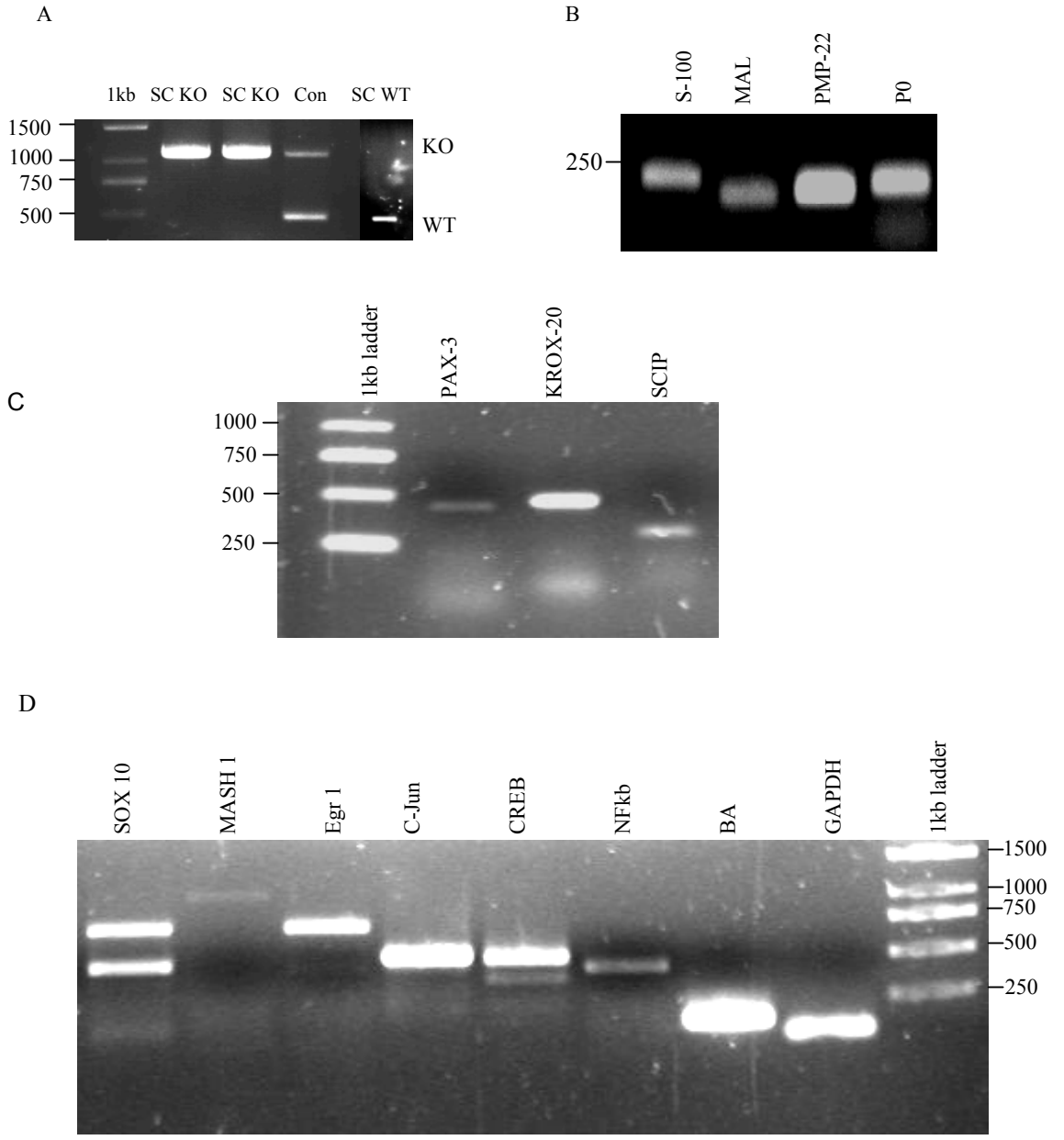


Fig 18: Genotyping and RT-PCR for SC markers and transcription factors. (A) Genotyping of Schwann cells. SC clones, SC KO and SC WT were subjected to PCR as described in materials and methods. KO indicates fragment specific for ASA KO and WT indicates fragment specific for controls. (B) Expression of mRNA transcripts for SC associated molecules. (C) & (D) Expression of transcription factors associated with SC. 1kb – 1 kilo base ladder (Fermentas) ; Con – heterozygous control ;

patients and animal models of MLD (Schott et al., 2001). This staining method resulted in the appearance of blue intracellular inclusions, most likely being sulfatide storage material (Fig. 19 arrows). Alcian blue staining at high salt concentrations is used as a routine method in the diagnosis of MLD (Lampert and Lewis, 1975).

4.12.2. Immunohistochemistry

To further improve the specificity of the examination, a monoclonal antibody for sulfatide was used to stain SC KO. This staining confirmed the result of the alcian blue staining. Double staining with antibody against sulfatide and lysosomal/late endosomal marker LAMP-2 demonstrated that most, but not all of the material localises within these cellular compartments (Fig. 19 B-D).

4.12.3. ASA treatment significantly reduces the sulfatide storage

When SCs were treated initially with 250mU / ml ASA and maintained with 100mU / ml ASA for further 6 days, a significant decrease in the amount of intracellular storage material was observed (Fig 19 E-F). The nature of the storage material in ASA deficiency has been described earlier for tissue as well as cell culture material by electron microscopy (Klein et al., 2005; Wittke et al., 2004).

Therefore, the storage material of cells from clone SC KO was examined by this method. As described earlier for material from other sources, sulfatide storing compartments show onion like shaped, lamellated, acinophilic and tuff stone like bulky inclusions (Fig. 20 A-C). No similar intracellular structures could be detected in cells from clone SC-KO that had been treated with recombinant ASA prior to electron microscopy (Fig. 20 D-F).

The presence of sulfatide in SC KO was also confirmed by TLC immunoblot (Fig. 21 D). To receive more quantitative data on sulfatide storage and its reduction by ASA treatment, thin layer chromatography experiments were performed. Lipids from treated and untreated cells were extracted and processed by TLC as described earlier (Fig. 21 A). The sulfatide levels before and after treatment were determined by densitometry and compared to the phospholipid content of the samples (Fig. 21 B) because there is no alteration in the phospholipid content. From these data a 2.4 fold reduction in the amount of sulfatide after

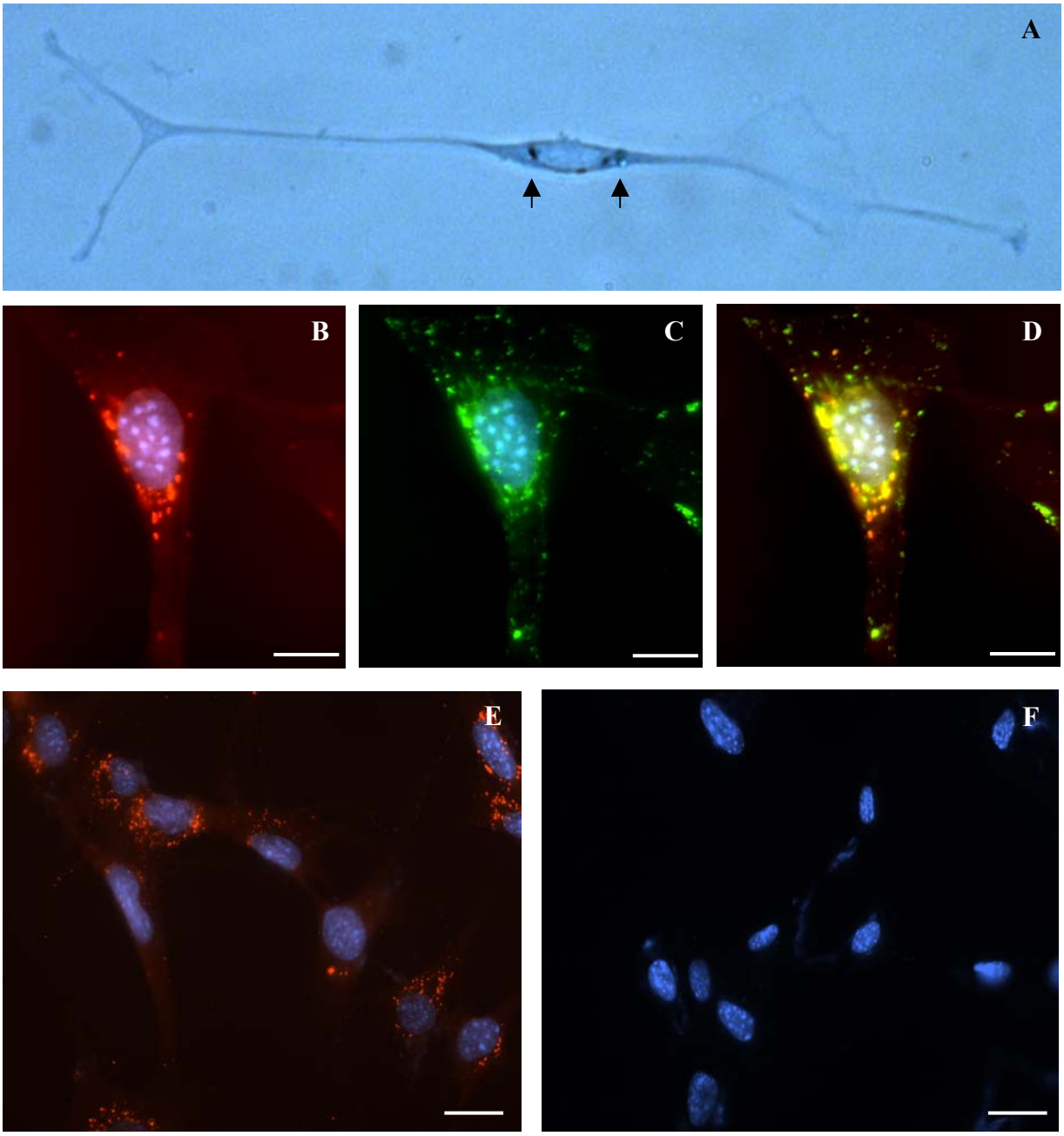


Fig 19: Sulfatide storage and arylsulfatase A treatment. (A) Alcian blue staining. Cells were fixed in 4% PFA, incubated with 0.05%(w/v) alcian blue in 0.025M sodium acetate buffer, pH5.7, containing 0.3 M $MgCl_2$ and 1%(w/v) PFA. The dark spots seen on the cells are the alcian blue positive storage granules. **(**B) (C) (D) Sulfatide and LAMP co-staining. Staining for sulfatide (Red) LAMP (Green) and overlap (Yellow). Sulfatide and LAMP co-localise in intracellular storage inclusions. Co-localisation of sulfatide with LAMP-1 demonstrates the lysosomal nature of these intracellular inclusions. (E) and (F) Sulfatide staining on ASA treated and untreated cells. (E) Untreated cells, (F) Treated with 250mU ASA/ml on 1st day and maintained with addition of 100mU/ml for 6 days. Cells were fixed in 4% PFA and incubated with 0.05% sodium borohydrate for 5 min. Washed 3x with PBS and incubated with 0.3% Triton X-100 in PBS for 5 min. Washed 3X and blocked with 5% goat serum for 60 min. Incubated with anti-sulfatide antibody (1:100) in 0.5% blocking agent at RT for 60min, washed and incubated in Cy3 (1:500) labelled secondary antibody. DAPI (1:500) staining was done for 5 min, washed and mounted in 50% w/v glycerol. Scale bar in B, C and D: 10µm and in E, F: 20µm.

ASA treatment of the cells was observed. Beside the amount of sulfatide storage the ratio between the amount of galactosylceramide and sulfatide is considered to be the most sensitive indicator for alterations of lipid metabolism in MLD. Therefore, galactosylceramide level was determined before and after ASA treatment of SC KO and the galactosylceramide : sulfatide ratio between treated and untreated cells was compared (Fig. 21 C). Remarkably, this ratio changed from 0.4 in untreated to 1.6 in treated cells.

Storage of sulfatide in the lysosomal/late endosomal compartments might interfere with the intracellular transport processes. COPs are required for the formation of Golgi-derived vesicles and for membrane transport from the intermediate endoplasmic reticulum compartment to the Golgi complex. Beta COP (COP I) and COP II are involved in the transport processes in the early stages of biosynthetic pathways. Therefore, these proteins can serve as markers for intracellular transport. In the ASA KO sulfatide storing kidney cells, COP I, a protein involved in intra-Golgi and retrograde Golgi to endoplasmic reticulum transport shows a more diffuse distribution. Whereas, there is no difference in the pattern of distribution in case of COP II, which is a marker for the transport vesicles from endoplasmic reticulum to Golgi and endoplasmic reticulum-Golgi-intermediate compartment (ERGIC-53)(unpublished data from Dr. Klein). Therefore, the distribution pattern of beta COP, COP II and ERGIC in the ASA treated and untreated SC KO was investigated. The results indicate that there is no major difference in the distribution of proteins as described above in these cells (Fig. 22).

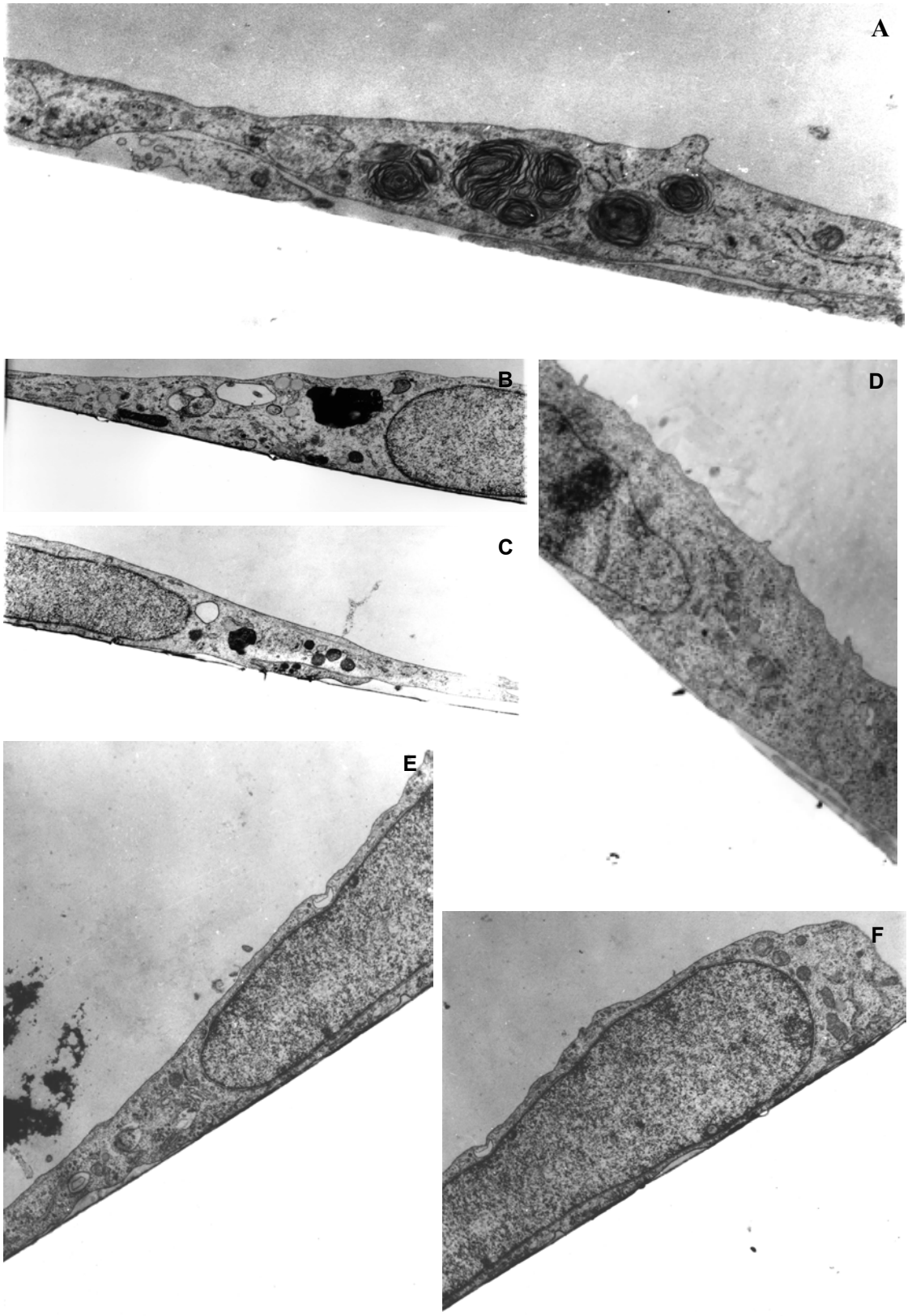


Fig 20 : Electron microscopy of ASA treated and untreated SCs. Electron microscopy of (A, B &C) untreated and (C, D & F) ASA treated cells. Cells were incubated with 250mU ASA/ml on 1st day and maintained with addition of 100mU/ml for 6days before fixing for microscopy. (Pictures were taken by Prof. Büssow, Department of Anatomy, University of Bonn).

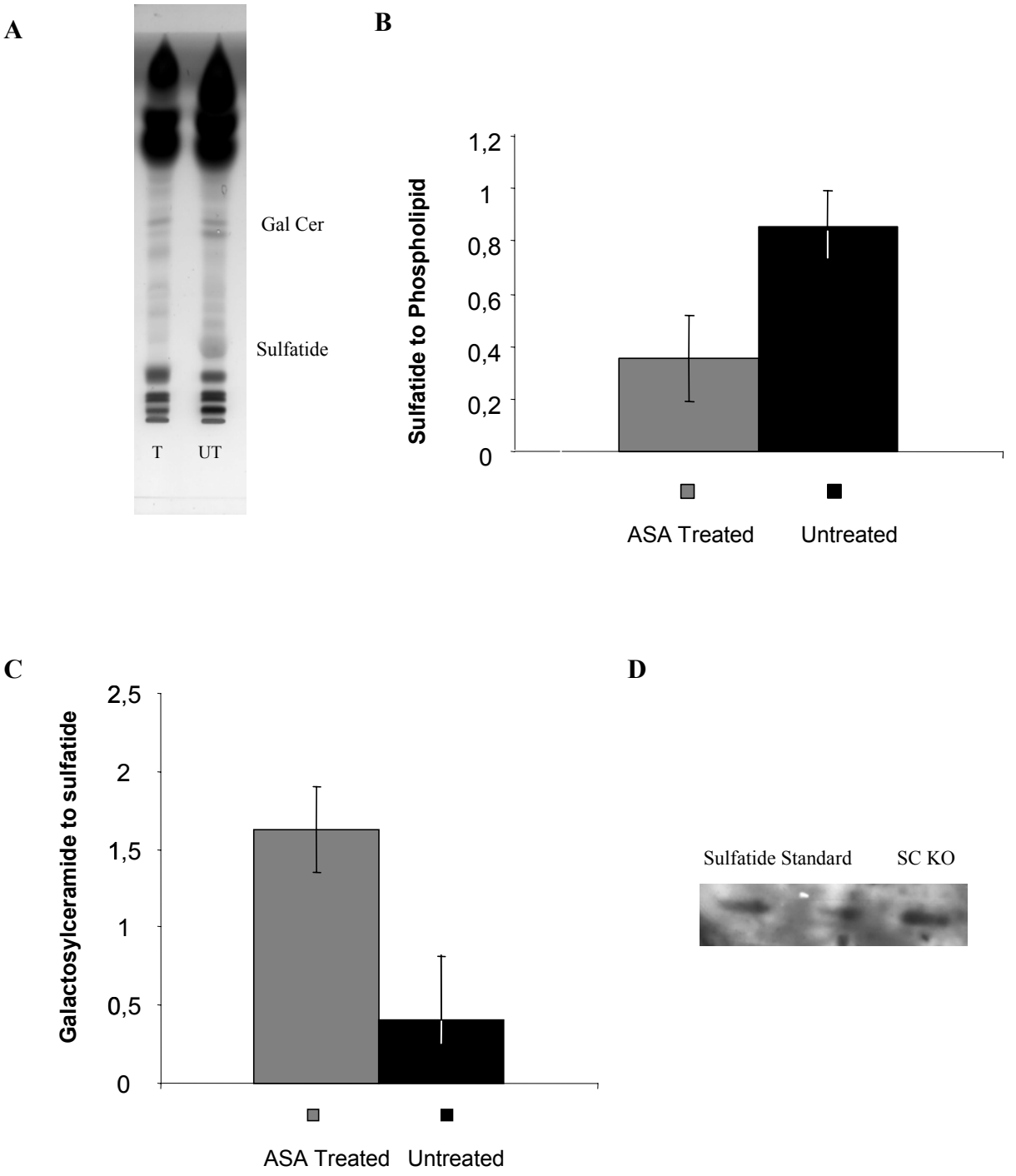


Fig 21: TLC of ASA treated and untreated SC KO. Cells grown in 10cm dishes were treated with 250mU ASA/ml on 1st day and maintained with addition of 100mU/ml for 6 days. Lipids were extracted and seperated on a HPTLC plate. (A) TLC of ASA untreated (UT) and ASA treated (T) cells. (B) and (C) are quantitative determination (AIDA 2.11) of the ratio of signal intensity between sulfatide/phospholipid and galactosylceramide/sulfatide respectively. (D) TLC immunoblot using antisulfatide antibody to confirm the sulfatide band. $P < 0.05$ ASA treated against untreated by one-way ANOVA.

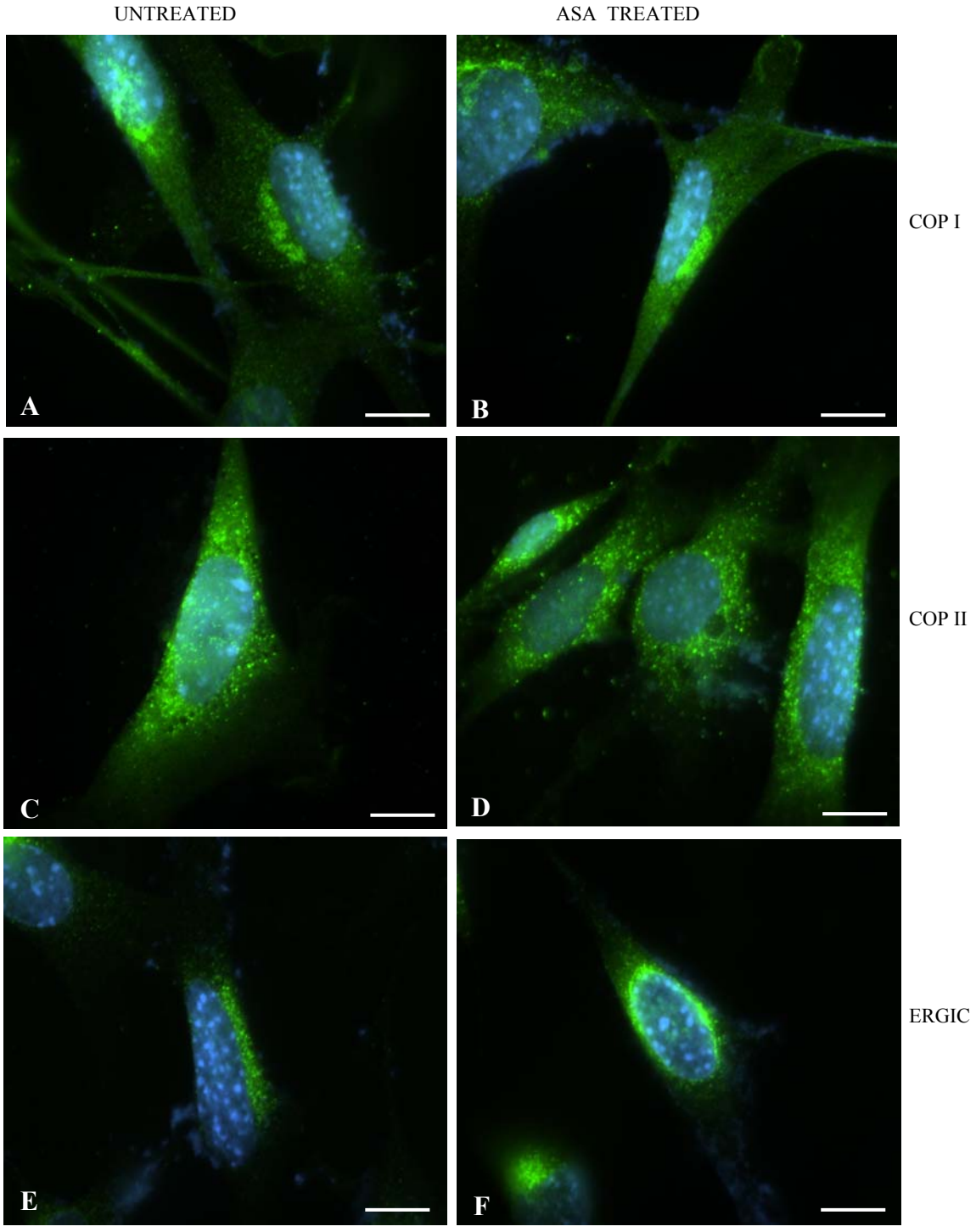


Fig 22: Distribution of COP I, COP II and ERGIC in SC KO. SC KO COP I (A+B), Beta COPs(C+D) and ERGIC (E+F). B, D and F were treated with 250mU of ASA/ml. A, C and E are ASA untreated cells. Scale bar in A - F: 10µm

4.13. MAL is downregulated at RNA level in SC KO

Since there is a downregulation of MAL in myelin of the ASA KO mice, it was investigated to find whether it is possible to detect any difference in the cell culture model of this disease. But, the expression of MAL protein in SCs could not be detected by the sensitivity of the Western blot. Therefore, RT PCR was carried out for mRNA isolated from ASA treated and untreated SC KO to analyse the MAL mRNA expression. To perform this experiment, SC KO were treated with 200mU/ml ASA on the first day and maintained with addition of 100mU/ml of ASA for six days. From these cells, mRNA and subsequently cDNA was prepared. The cDNA was subjected to PCR analysis using primers for MAL mRNA and compared with the untreated cells. The results showed that there is an upregulation of MAL mRNA in the ASA treated cells compared to untreated counterparts. Quantification of the signal revealed that the ASA treatment increases MAL mRNA by 1.5 fold compared to the untreated cells (Fig. 23). There is no difference in the expression of other SC markers like S100, PMP and P0 (not shown).

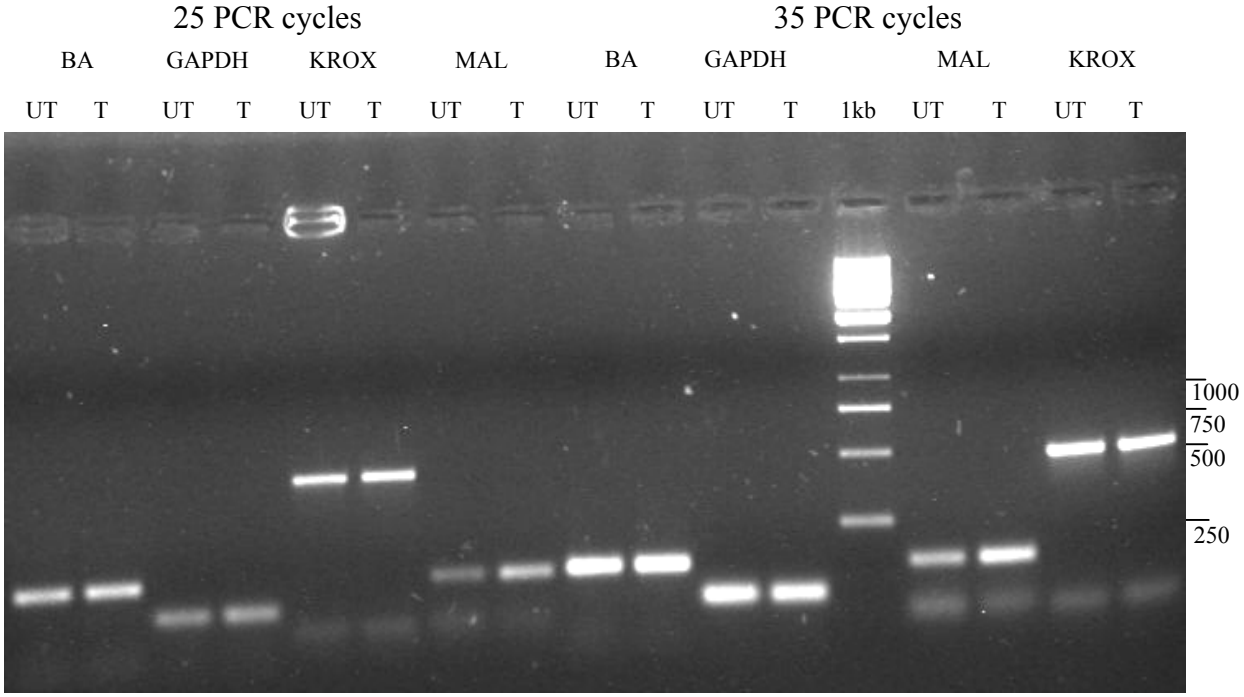


Fig 23: RT-PCR of MAL with ASA treated and ASA untreated SC KO cDNA. Comparison of ASA treated and untreated SC KO by RT-PCR with 3 different cycles (15; 25 and 35 cycles) for MAL, KROX, beta-actin and GAPDH. As there is no difference in KROX expression, it was also used for comparison. PCR signal could not be detected at 15cycles (gel not shown) for any of the above markers and MAL. UT: ASA untreated; T: ASA treated; BA: beta-actin. Numbers on the right side indicate the size of the standard fragments in bp.

4.14. Expression of neurotrophic factors in ASA treated and untreated SC KO

In patients suffering from MLD, extensive loss of myelin and myelinating cells are observed. Due to the impairment of lipid composition because of sulfatide storage, there is a possibility that there could be a difference in the ligand receptor system of the neurotrophic factors and its receptors. As previously mentioned, culturing of sulfatide storing oligodendrocytes *in vitro* is very difficult. Therefore, the sulfatide storing SCs were used to study alterations in the expression of neurotrophic receptors and the endogenous neurotrophins due to sulfatide storage.

SC KO were cultured in the presence and absence of ASA similar to the investigation of MAL mRNA. RT-PCR analysis was done for the neurotrophic factors and their receptors which were previously shown to be expressed by SCs in culture. These are NGF, GDNF, BDNF, TrkA, TrkB, TrkC, NT3, P75, GDNFR and RET (Watabe et al., 2003). The results revealed that there is no major difference in the neurotrophic factors or its receptors analysed except for TrkA. Quantification revealed that ASA treatment significantly reduces the TrkA expression by 2.2 fold in these cells (Fig. 24 A and B). This alteration in TrkA led to investigate whether this difference could also be observed at the mRNA level from the sciatic nerve of ASA KO mice when compared to the WT mice. The fragment that is amplified by primers specific for TrkB is not consistent in both ASA treated and untreated mRNA.

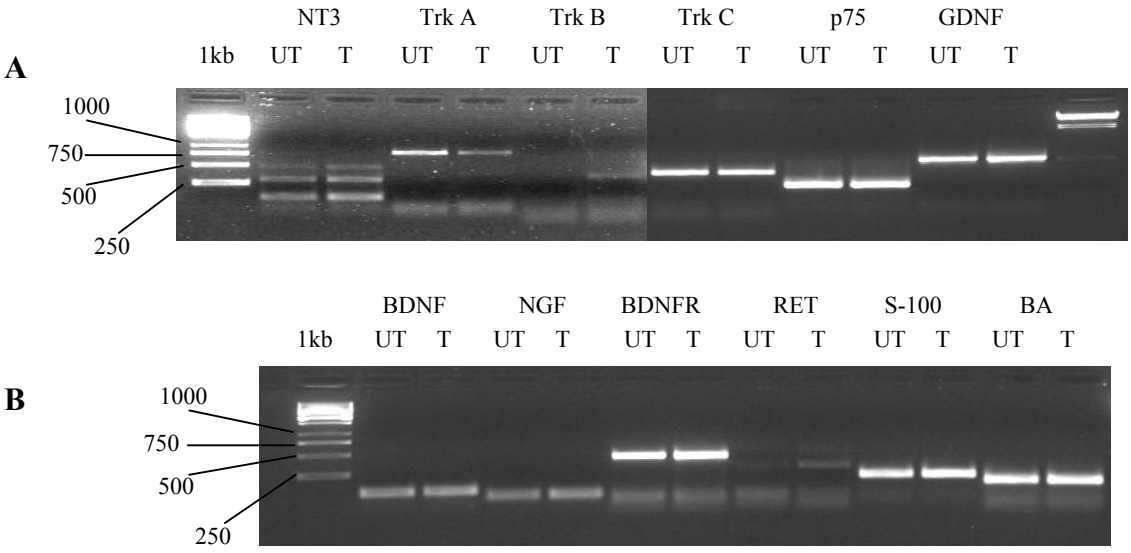


Fig 24: Expression of neurotrophic factors and receptors by ASA treated and untreated SC KO. (A) and (B) mRNA was extracted from ASA treated (T) and untreated (UT) SC KO and subjected to RT-PCR with specific primers for neurotrophic factors and their receptors expressed by SCs in culture. NT3 – Neurotrophin 3; Trk: Tropomycin-related kinase; GDNF: Glial cell derived neurotrophic factor; BDNF: Brain derived neurotrophic factor; NGF: Nerve growth factor; BDNFR: Brain derived neurotrophic factor receptor; BA: β-actin. All experiments were done three times independently.

4.15. Neurotrophic receptors are upregulated in the sciatic nerve of ASA KO mice

The previous experiment shows that ASA treatment significantly reduces the expression of TrkA. If the difference observed in that experiment is comparable with RNA prepared from sciatic nerve of ASA KO and WT mice, it can prove that the addition of ASA reverts the phenotype of sulfatide storing cells to normal cells at the level of cellular signaling.

Therefore, RNA was prepared from the sciatic nerve of one year old SC KO and WT mice as described in the materials and methods. RT PCR for a set of neurotrophic factors and their receptors revealed that the receptors TrkA, TrkB, Trk C and p75 were upregulated in the ASA KO mice compared to the WT. The neurotrophic factors like BDNF and NGF were downregulated in the ASA KO mice (Fig. 25 A and B). Expression of MAL is also downregulated in ASA KO sciatic nerve. As shown in previous experiments, MAL is down-regulated in the brain and myelin of ASA KO mice (Appendix A1 and Fig. 9). These results prove that this downregulation can also be observed in the sciatic nerve and this down-regulation of MAL observed here also acts as a marker for the RT-PCR to detect substantial alterations in the expression of other molecules.

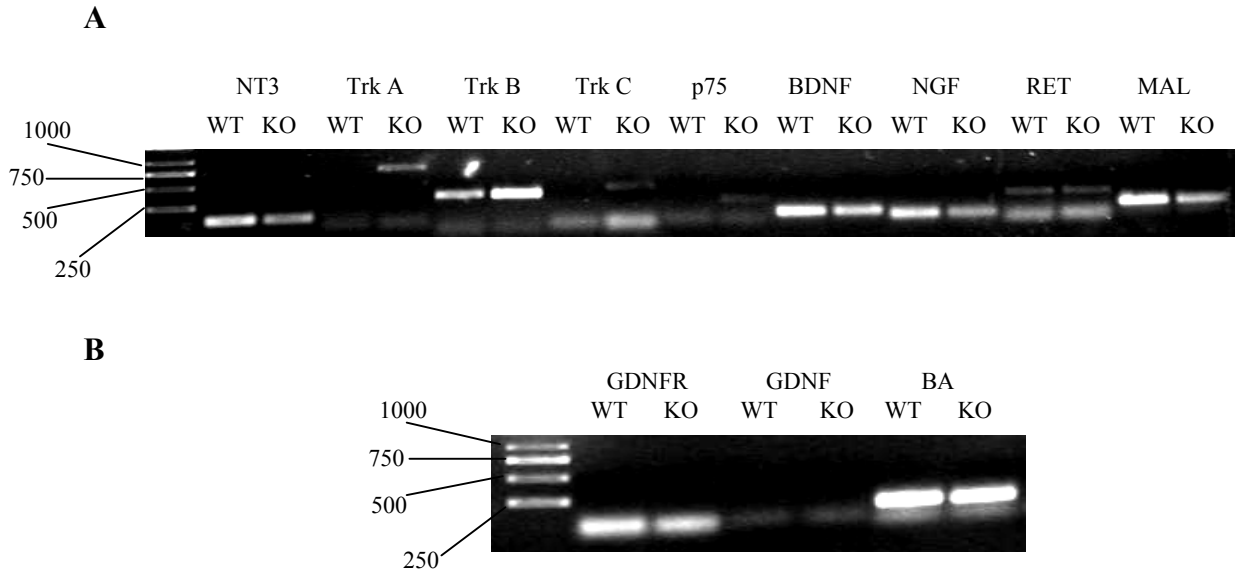


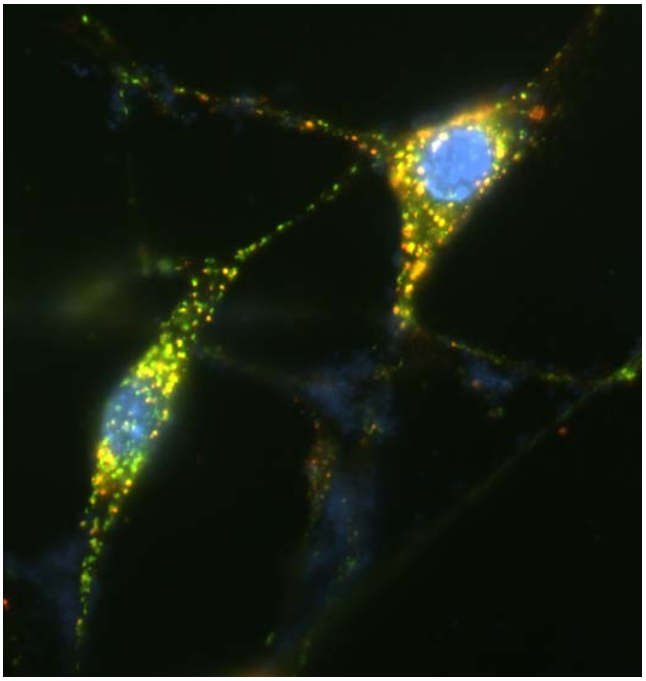
Fig 25: Expression of neurotrophic factors and receptors by sciatic nerve of ASA KO and WT mice. (A) and (B) mRNA was extracted from sciatic nerve of wild type (WT) and ASA knockout (KO) mouse and subjected to RT-PCR with specific primers for neurotrophic factors and their receptors. As the preparation of RNA from one pair of sciatic nerve is very difficult, the RT-PCR was repeated three times from the same RNA. NT3 – Neurotrophin 3; Trk – Tyrosine kinase; GDNF – Glial cell derived neurotrophic factor; BDNF – Brain derived neurotrophic factor; NGF – Nerve growth factor; BDNFR – Brain derived neurotrophic factor receptor; BA – β–actin.

4.16. Translipofection of sulfatide in SC KO NS

When SC KO were cultured for more than a year, the sulfatide storage in these cells reduced substantially. These clones were named as SC KO NS (SC KO Non Storing). Although the SC KO is currently the only available sulfatide storing *in vitro* glial cell culture system to study the molecular aspects of MLD and it gives valuable information on the effects of sulfatide storage at the cellular level, the sulfatide storage in these cells may not be in par with the storage observed in cells of the MLD patients. The diseased cells from MLD patients may show comparatively high amounts of sulfatide. Therefore, if it is possible to increase the sulfatide amount in long term cultured SC KO NS by any means, it may aid to magnify the mild alterations that are observed in the naturally sulfatide storing SC KO.

Therefore, as described in materials and methods, treatment of SC KO NS with sulfatide by a process similar to transfection using the Bio-Rad reagent transfectin® was carried out. This method was termed 'Translipofection' because it transports lipid through the cell membrane. This method has significantly increased the intracellular sulfatide level in the SC KO NS. Immunofluoresence using sulfatide antibody has shown that all the cells in culture after treatment take up sulfatide (Fig. 26 A). Co-staining with LAMP antibody revealed that the sulfatide is localised in the late endosomal/lysosomal compartments (Fig. 26 A). The accumulation of sulfatide in these cells was also confirmed by TLC analysis which was compared with the neat sulfatide treatment control and transfectin treatment control (Fig. 26 B). As estimated by TLC, translipofection with sulfatide increases the sulfatide storage by 6.2 fold compared to the transfectin treated control cells and by 3.8 fold when compared to neat sulfatide treated cells. These sulfatide accumulating cells could be of further value to study the cellular signaling in the pathology of MLD. In addition to the endogenously sulfatide storing SC KO, these *in vitro* sulfatide loaded cells can also act as a perfect cell culture system to study the molecular aspects of MLD. This system can also be used to compare the changes that are observed in the naturally sulfatide storing SC KO.

A



B

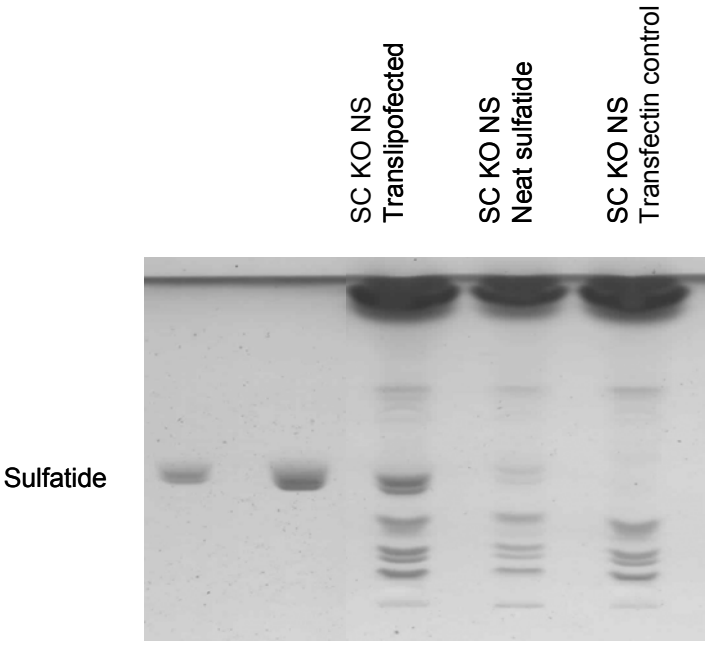


Fig 26: Immunohistochemical and TLC analysis of sulfatide translipofected cells. (A) Sulfatide and LAMP-II co-staining of SC KO NS cells. This shows that most of the internalised sulfatide is targeted to the LAMP positive compartments. (B)TLC of SC KO NS cells translipofected with sulfatide. The bands were compared with the same amount of neat sulfatide treated cells and untreated cells.

5. Discussion

Lysosomal storage diseases are clinically heterogeneous with respect to their age of onset, progression of symptoms and particular organs involved. Varying levels of residual enzyme activity, associated with different defective alleles that cause the respective diseases are responsible in part for this clinical heterogeneity. In general, the higher the residual enzyme activity, the milder the phenotype (Gieselmann, 2005). ASA is a lysosomal enzyme involved in the degradation of sulfatide, a polar glycolipid that is mainly found as a component of myelin sheaths of the nervous system. Genetic deficiency of ASA leads to lysosomal accumulation of the sulfatide which leads to progressive demyelination leading to lethal neurological symptoms which is characterised by a disease called MLD.

Until the past decade, research on MLD was mainly focussed on the genetic aspects. Very little is known about how sulfatide storage affects the cell function. As there is no naturally occurring animal model for this disease, development of a ASA deficient animal model (Hess et al., 1996) had opened a new way to study and understand the disease under cellular and molecular level. The ASA knockout mice are unable to degrade sulfatide; therefore, they store the lipid intra lysosomally. The pattern of lipid storage in neuronal and non-neuronal tissues resembles as described for the patients. Several studies previously conducted on this animal model have revealed lipid storage in oligodendrocytes, astrocytes and some neurons. These animals display astrogliosis and a decreased average axonal diameter. Purkinje cells and Bergmann glia of the cerebellum are morphologically aberrant. Demyelination is seen in the acoustic ganglion and occurs between the ages of 6 and 12 months. The animals are deaf at this age and display various neuromotor abnormalities. However, compared to humans, the mice have a surprisingly mild phenotype, since they have a normal life span and do not develop widespread demyelination (Gieselmann et al., 1998). Sulfatide histochemistry and ultrastructure of the inner ears of ASA KO mice at 0.5 - 26 months of age has shown lysosomal accumulation of sulfatide in various cell types such as SCs that maintain the myelin sheaths around the spiral and vestibular ganglion cells, periaxonal SCs, macrophages and spiral and vestibular ganglion cell perikarya (Coenen et al., 2001). Investigation of visceral organs of ASA KO mice showed that the kidneys are particularly affected (Lullmann-Rauch et al., 2001) with abundant storage of lipid. Apart from that, sulfolipid storage was also found in gall bladder, intrahepatic bile ducts, exocrine pancreatic ducts, respiratory epithelium and with low degree in testicular Sertoli cells (Schott et al., 2001).

Since previous studies were done on detecting sulfatide storage in various parts of the ASA KO mice, the present work was aimed at investigation of the myelin for sulfatide storage which led to investigate the lipid raft related lipid and protein composition of myelin. Cell membrane contains a variety of lipid species that differ in their physico-chemical properties. Lipid-lipid immiscibility gives rise to lateral heterogeneities in the membrane plane, a subset of which is termed as rafts (Rajendran and Simons, 2005). Since myelin membrane is rich in cholesterol, galactosylceramide and sulfatide, it is an interesting model membrane from the DIG perspective (Taylor et al., 2002). DIGs have been described to be functionally important in different processes like lipid and protein transport and signal transduction (Simons and Gruenberg, 2000; Simons and Ikonen, 1997). Since sphingolipids are important constituents of DIGs in plasma membranes, their accumulation may interfere with sorting and signal transduction processes (Simons and Ehehalt, 2002; Simons and Gruenberg, 2000). During the last years, increasing attention has been drawn to the connection of DIGs and various diseases (Simons et al., 2002). For lipid storage diseases, it has been proposed that alterations in the DIG compartment may contribute substantially to pathogenesis (Kobayashi and Hirabayashi, 2000; Simons and Gruenberg, 2000).

Sulfatide and galactosylceramide are two major components of DIGs in myelin membranes and they have been proposed to play crucial roles in myelin formation, maintenance and function (Kim and Pfeiffer, 1999; Simons and Ikonen, 1997; Taylor et al., 2002). In Krabbe disease and MLD, the degradation of these two major myelin glycolipids, galactosylceramide and sulfatide respectively, are impaired leading to severe dys- or demyelination. However, the pathogenic molecular mechanisms between lipid storage and oligodendrocyte dysfunction remain unknown (von Figura et al., 2001; Wenger et al., 2001).

Elevation of sulfatide was observed in the myelin of humans (Eto et al., 1976; Malone et al., 1975; Poduslo et al., 1982). In the experiments conducted, it was observed that the deficiency of ASA leads to elevation of sulfatide not only in lysosomes but also in the myelin of ASA KO mice. This suggests that lipid storage is also pathogenically relevant at the level of plasma membranes. Investigation of the myelin detergent soluble and insoluble fractions by TLC shows that the sulfatide accumulates in the detergent soluble and insoluble membrane domains to a different extent compared to the WT. In the soluble fraction, the increase was only 1.7 fold, whereas a 2.33 fold accumulation was observed in DIGs isolated from myelin of ASA KO mice.

Administration of excess amounts of gangliosides to the cells can displace DIG components to detergent soluble compartment of membranes (Simons et al., 1999). In addition, there have been reports that mice which lack galactosylceramide due to UDP-galactose:ceramide-galactosyltransferase deficiency show a higher amount of PLP in the detergent soluble compartment of myelin (Coetzee et al., 1996). Therefore, alterations in the distribution of DIG proteins might be possible by the lower amount of galactosylceramide in myelin of ASA KO mice. Hence, protein distribution between the detergent soluble and insoluble fractions was investigated in the ASA KO and WT mice. The results did not reveal any significant differences in the distribution of myelin proteins between these two fractions. As previously mentioned, it is known that these ASA KO mice store less sulfatide in the brain than MLD patients and they do not show demyelination. Thus, upon further increase of sulfatide accumulation, the balance between DIG and non-DIG fractions may finally result in altered protein distribution and respective pathogenic consequences.

It was found that the increase in sulfatide level was accompanied by varying degrees of reduction of galactosylceramide in all lipid fractions. The reason for this is unknown, but there is a possibility that sulfatide accumulation leads to feedback inhibition of galactosylceramide synthesis. A decrease of galactosylceramide was also reported for MLD patients (von Figura et al., 2001). About 30% of decrease in galactosylceramide in human was reported by (Kolodny, 1989). In case of ASA KO mice the decrease was about 85% of normal.

Since sulfatide and galactosylceramide levels are altered in brains of MLD patients, the ratio of these lipids is considered the most sensitive indicator of alterations of lipid metabolism in MLD (von Figura et al., 2001). In humans, the ratio of galactosylceramide to sulfatide drops from 4 in normal white matter to about 0.5 in MLD patients (Norton and Poduslo, 1982). In mice, this ratio is about 2.9 in control animals and drops to only 1.7 in ASA KO mice. The difference which is seen in the lipid ratio between the human and the mice may be a reason for the lack of demyelination in the ASA KO mice. Comparison of these ratios between humans and mice reveals that the lipid metabolism in mice is comparatively less affected than observed in humans. Therefore, the mouse model only reflects the very early stage of the disease.

Myelination, the spiral wrapping of oligodendrocytes and SC processes around an axon segment is strongly dependent on the timely and correct expression of a series of myelin proteins by these cells. Even though the CNS and the PNS myelin have almost same lipid composition, their protein composition differs. The myelin proteolipid protein MAL has four transmembrane domains which tightly binds glycosphingolipids, particularly sulfatide. It is developmentally regulated in differentiated cultured oligodendrocytes and its expression level peaks in the nervous system during myelin formation (Frank et al., 1998; Kim et al., 1995; Schaeren-Wiemers et al., 1995b). MAL appears at the same time as sulfatide in the SCs and it is a component of myelin rafts and oligodendrocytes (Frank, 2000). Galactosylceramide sulfotransferase as well as MAL can be regulated by thyroid hormone, therefore MAL and sulfatide metabolism appears to be tightly linked. (Ferret-Sena et al., 1990; Koper et al., 1986; Pombo et al., 1998). Outside the nervous system, MAL is expressed in apical plasma membranes of epithelial cells (Frank et al., 1998; Schaeren-Wiemers et al., 1995a) and is involved in transport processes to these membrane compartments in polarised cells like MDCK cells or Fischer rat thyroid cells (Cheong et al., 1999; Martin-Belmonte et al., 2000; Puertollano and Alonso, 1999).

MAL expression parallels myelin formation and MAL is predominantly localised in compact myelin. MAL co-purifies with these glycosphingolipids in detergent insoluble domains, indicating a close association and possible functional interactions of MAL with glycosphingolipids in these tissues. (Frank, 2000). Because of its localisation in Schmidt-Lanterman incisures, it is assumed to be involved in myelin turnover and stability (Erne et al., 2002). Investigation of myelin associated proteins revealed that the sulfatide storage does not have effect on any other proteins other than MAL. Analysis of mRNA revealed that the reduction of MAL synthesis may be due to the downregulation of mRNA. There is a possibility that this effect could be a consequence of transcriptional downregulation. But, it is also possible that there is destabilisation of MAL mRNA. Of the investigated myelin proteins, MAL is the only protein affected due to sulfatide storage and the data supports a close linkage between sulfatide and MAL metabolism. The data also suggests that the amount of sulfatide in oligodendrocytes has a negative feedback on MAL synthesis. The capacity of MAL to bind sulfatide may be the basis of glycosphingolipid transport to the myelin membrane. Thus, MAL mediated sulfatide transport may be one determinant of myelin sulfatide content. In this scenario, it is reasonable to assume that the downregulation of MAL mRNA reflects a cellular

reaction to decrease the further transport of sulfatide to the myelin membrane in ASA KO animals.

The mechanisms which ensure a well-balanced synthesis of lipids and proteins are so far unknown. The enormous synthesis of myelin lipids must be coordinated with the synthesis of myelin proteins in oligodendrocytes. The specific effect on MAL protein levels in response to sulfatide storage proves that regulatory loops indeed exist and that lipid storage diseases are a tool to reveal the underlying mechanisms.

Sulfatide is normally degraded in lysosomes and accumulates in these organelles during ASA-deficiency. Since MAL binds sulfatide, it was investigated to find whether lipid storage has also an effect on intracellular MAL distribution. Because cultured oligodendrocytes from ASA-deficient mice do not store sulfatide, the only cells that were shown to accumulate substantial amount of sulfatide is the renal distal tubule cells from the ASA KO animals (Hess et al., 1996); (Gieselmann et al., 1998; Lullmann-Rauch et al., 2001). It was evident from literature that these cells also expresses MAL in the apical membranes of these cells *in vivo* (Frank et al., 1998) and *in vitro* (Zacchetti et al., 1995). MAL has been shown to bind sulfatide (Frank, 2000). Therefore these cells might serve as a model for examining the consequences of sulfatide storage on the sulfatide binding proteins like MAL. In cultures prepared from ASA KO animals, lysosomal sulfatide storage was demonstrated and these cells represent a suitable cell culture system to examine the effects of intracellular sulfatide storage. The clear sulfatide staining of cells from ASA KO mice allowed to show for the first time the *in vivo* co-localisation of MAL and sulfatide in plasma membranes. In addition, MAL co-localised with LAMP-1, a late endosomal/lysosomal marker, in intracellular structures positive for sulfatide storing cells. No co-localisation was seen in normal kidney cells. Thus, sulfatide storage diverts MAL to the late endosomal/lysosomal compartment. Whether lysosomal redistribution of MAL leads to increased degradation could not be examined due to the limited sensitivity of the MAL antiserum in Western blots of extracts or whole membrane preparations of renal epithelia cells. However, the overall intensity of MAL immunofluorescent staining appeared comparable between WT and sulfatide storing cells. This argues for a redistribution of MAL into LAMP-1 positive compartments possibly without a concomitant proteolytic decrease. Such an intracellular MAL mistargeting could be another reason for the observed decrease of MAL in the myelin membrane (in addition to mRNA downregulation). To test this hypothesis, the amount of MAL must also be determined

in whole brain homogenates. Unfortunately, due to insensitivity of the MAL antiserum, MAL could not be detected in western blots on whole brain extracts.

To investigate whether sulfatide storage has any effect on raft related tyrosine kinases, expression of lyn and fyn were investigated in the ASA KO animal model. Fyn is one of the nine known members of the Src family protein tyrosine kinases. Fyn and the other Src family members possess protein tyrosine kinase activity that plays a prominent role in their function as signaling molecules. Fyn is involved in the initial events of myelination especially essential for normal forebrain myelination. Fyn -/- mice revealed myelin deficiency right from P14 (Sperber et al., 2001). Fyn is present in myelin-forming cells and is activated through stimulation of cell surface receptors such as large myelin-associated glycoprotein (L-MAG). The Fyn-deficient mice had thinner, more irregular myelin than the WT mice. (Umemori et al., 1999). In the ASA KO mice, about 0.6 fold downregulation of fyn was observed. The reason for this downregulation is not clear. There is a possibility that due to the mild down-regulation of fyn, the mice shows the early stages of the pathology of the disease.

The general phosphorylated state of the brain was analysed as there was a downregulation of fyn kinase. Protein phosphorylation/dephosphorylation on Ser/Thr/Tyr is involved in an enormous range of cellular processes. Protein phosphorylation catalyzed by protein kinases plays a critical role in cellular signaling. The whole brain extract and myelin from ASA KO and WT mice was investigated for any modification in protein phosphorylation. The reason for the up- regulation of these tyrosine phosphorylated proteins is unknown in ASA KO mice. Since performing further studies by analysing the whole brain or the myelin to evaluate the minor alterations was difficult, an *in vitro* cell culture system to mimic the sulfatide storage as seen in the MLD patients was absolutely needed.

To develop an in *vitro cell* culture system of sulfatide storing cells, spontaneously immortalized mouse SC line was established from the peripheral nerve of two weeks old ASA KO mice. The accumulation of sulfatide was demonstrated in these *in vitro* cultured SCs to confirm the MLD phenotype in them. The phenotype of the disease could be reversed in these cells by treating them with ASA. As of now, this cell culture system is the only available appropriate *in vitro* tool to study the molecular aspects of this disease.

To serve as a cell culture model for Metachromatic leukodystrophy, the isolated cells have to show two major characteristics beside their ASA deficiency. First, they should store sulfatide inside their lysosomal/late endosomal compartment similar to that found in tissue material from ASA KO mice and MLD patients. Second, lipid storage should be reversible by treatment of the cells with recombinant ASA added to the cell culture medium. The only available kidney cell culture (Klein et al., 2005) is of non-neural origin and it cannot mimic the phenotype of the neural cells. These cells are quite difficult to transfect and they loose the sulfatide storage in a couple of passages. The other possibility is to culture oligodendrocytes, but the difficulty in isolating these cells and maintaining them as long term cultures makes it quite difficult to have it as an *in vitro* model for the disease. The ASA KO animal model older than 18 months also show degeneration of up to 20% of fibres in the peripheral nerves. (Gieselmann, 2003). This encouraged venturing into the isolation and long term culturing of SCs.

Several methods for the preparation of a pure population of neonatal SCs have been described and are in use in many laboratories. In this study, SCs were purified and characterised using a combination of various published methods (Brockes et al., 1979; Jirsova et al., 1997; Komiyama et al., 2003; Shen et al., 2002; Tanaka and Webster, 1993; Watabe et al., 1995; Watabe et al., 1994; Watabe et al., 2001). Although the detailed mechanism of spontaneous immortalisation of SC lines in still unknown, it is likely that the spontaneous immortalisation of long term cultured SCs is a general phenomenon in both wild type and mutated mice (Watabe et al., 2003). SC KO spontaneously immortalised after 5 months in culture.

The ASA KO and WT SCs established in this study morphologically resemble the cells reported by (Watabe et al., 2003). This cell line was initially genotyped to confirm its origin. SC KO show distinct SC morphology and expression of S100 and p75 as demonstrated by immunofluorescence and RT PCR analysis. These SCs are quiescent and grow very slowly even in FCS. Forskolin synergises with glial growth factor for enhancing their growth *in vitro*. Electron microscopically, varieties of cytoplasmic inclusions were demonstrated in this cell line. The results indicated that SC KO sufficiently represents the *in vivo* pathological feature of the mouse, namely sulfatide storage. These cells expressed a set of SC markers and transcription factors which are crucial for SC development. Similar cell culture models are also available for Niemann-Pick disease type C (Watabe et al., 2001). To date, more than fifteen SC lines have been established by spontaneous immortalisation or transfection with

oncogenes (Bolin et al., 1992; Boutry et al., 1992; Chen et al., 1987; Goda et al., 1991; Porter et al., 1987; Ridley et al., 1988; Tennekoon et al., 1987; Thi et al., 1998; Toda et al., 1994; Watabe et al., 1995; Watabe et al., 1990). The degree of phenotype expression and differentiation differ from each other (Hai et al., 2002). SCs obtained in this present study had distinct phenotype and were maintained for over 10 months without any alterations. Many studies have attempted to reduce the fibroblasts contamination and raise SC purity. In this study, it was observed that few fibroblasts in culture enhances the SC proliferation and support their survival when compared to a completely pure SC culture. This is similar to the results of (Komiyama et al., 2003).

Both in human MLD and ASA KO mice, the intensity of sulfatide storage vary greatly from one cell type to another. Due to polyanionic character of sulfatide aggregates, staining with cationic dyes is most appropriate for their histochemical detection. Alcian blue gives specific staining for polysulfated compounds (Schott et al., 2001). Hence, the cells were stained initially for sulfatide storage by alcian blue, which showed small loosely arranged circular aggregates of alcianophilic material in the cell body and the extensions. To confirm this, sulfatide storage was proven in these cells by immunostaining with an antibody specific for sulfatide, the late endosomal/lysosomal location of it, it was further confirmed by electron microscopy and TLC. The ultrastructure shows bulky inclusions, globular and bizarre shaped inclusions and also a collection of lamellated material present in small circular aggregates similar to those reported by (Lullmann-Rauch et al., 2001).

The perfect control to study the cellular alterations for sulfatide storing cells is the ASA treated counterparts of the same culture, because they are derived from the same culture. Otherwise clonal differences between cell lines derived from normal and ASA KO mice could influence results substantially. To show that ASA actively reduces sulfatide accumulation, the cells were incubated with recombinant hASA polypeptide (Matzner et al., 2000). Biochemical determination of the sulfatide content in the cells revealed that ASA treatment reduces the lipid storage by 2.4 fold compared to the untreated cells. In brain and tissues of MLD patients, sulfatide elevation is accompanied by a decrease in galactosylceramide. Therefore, the ratio of galactosylceramide to sulfatide is a sensitive indicator for abnormalities of lipid metabolism. The ratio of these two lipids changed from 0.4 in untreated to 1.6 in treated cells. (Mirsky et al., 1980; Watabe et al., 1990) have reported that galactosylceramide is not detectable in SC after several days in culture. But, in this study, it was possible to detect

galactosylceramide in SC KO even after the cells were maintained for 10 months in culture and the amount increased after ASA treatment.

As discussed earlier regarding the presence of MAL in microdomains and its assumption to be involved in myelin turnover and stability, the upregulation of MAL mRNA in the ASA treated SC KO cells further proves that there is a relationship between MAL and sulfatide accumulation. The mechanism behind this is unknown.

Transcription factors also act as markers to identify different cell types. Transcription factors associated with SCs were investigated in the ASA treated and untreated SCs. No difference in their expression was detected. But all these transcription factors are expressed by SC KO and they are required for various functions of the cell. In the pathogenesis leading to demyelination, myelin is degraded and myelinating SCs disappear to be replaced by Pax-3 (paired box protein) expressing non-myelinating SCs. Krox 20 plays major roles in differentiation and myelination of SCs. Krox-20 expression provides cells with strong protection against apoptotic death (Parkinson et al., 2004). SCIP is strongly and rapidly up-regulated in cultured SCs by agents that elevate intracellular cAMP (E.g., forskolin, cAMP analogs), a manipulation that in many respects yields cultured cells with the molecular phenotype of promyelinating cells *in vivo* (Zorick et al., 1999). Foskolin must have induced SCIP in SC KO culture. Sox10 modulates the function of Pax3 and Krox 20 and two other transcription factors involved in SC development (Scherer et al., 1994). Basic helix-loop-helix (bHLH) transcription factor gene is *Mash1*, which is expressed in both the CNS and PNS and is essential for the development of both olfactory and peripheral autonomic neurons. MASH 1 is essential for autonomic neurogenesis (Lo et al., 1997) (Lo et al., 1998). Egr-1 is a member of the immediate-early transcription factors that are rapidly and transiently induced by growth factors and other signals. Several of its target genes are involved in the control of cell responses to changes in extra cellular environment and in the complex molecular processes leading to proliferation and/or differentiation (Pines et al., 2003). cJun exerts its function as a component of several dimeric protein complexes. Cyclic AMP responsive element binding proteins (CREB) are transcription factors which bind to certain sequences called CRE elements in DNA and thereby increase or decrease the transcription of certain genes. NF-kB was highly up-regulated in premyelinating SCs. NF-kB is an essential signal for the progression of axon-associated SCs into a myelinating phenotype (Nickols et al., 2003). The

expression of all the above discussed transcription factors by these cells defines the nature of these isolated cells and are crucial for SC development and peripheral myelin formation.

In patients suffering from MLD, it was observed that there is extensive loss of myelination and myelinating cells. It could be due to the fact that the oligodendrocytes are subject to sensitive changes due to alterations in trophic signals (McTigue et al., 1998) (Dougherty et al., 2000). As previously mentioned, culturing of sulfatide storing oligodendrocytes *in vitro* is very difficult, therefore SCs were used to study any alterations in the neurotrophic receptors and the endogenous neurotrophins due to sulfatide storage.

During development, glial cells and neurons depend on each other for survival and differentiation. Among the most dramatic but least understood of these cell-cell interactions are those initiating the formation of myelin. Various positive and negative signals from nerve growth factors and axons regulate myelination program of glia. In particular, it has been previously shown that the p75 neurotrophin receptor positively regulates myelination by SCs in the PNS (Hempstead and Salzer, 2002). The neurotrophins support different neuronal populations and there is a therapeutic potential for the neurotrophins in the nervous system in case of Alzheimer disease, Parkinson disease and Huntington disease as well as peripheral neuropathies.

Neurotrophins bind to specific Trk receptors. In neurons, TrkA is primarily a receptor for NGF, TrkB for BDNF and NT4/5 and TrkC for NT3. All four neurotrophins also bind to the low affinity receptor, p75 that may assist retrograde transport of the bound neurotrophins or increase the affinity between Trk receptors and their respective ligands. Binding of neurotrophins induces receptor dimerisation at the cell surface followed by phosphorylation of receptor kinase residues. The phosphorylated tyrosine then recruits intracellular proteins involved in signal transduction. These factors initiate dramatic changes such as survival, proliferation and differentiation in their target cells.

Tissue culture studies have shown that agents like NGF, progesterone, insulin-like growth factor-1, BDNF and GDNF can sustain or increase the myelination of small axons *in vitro* by SCs (Hoke et al., 2003). Expression of functional TrkA, TrkB and TrkC receptors on oligodendrocytes and their progenitors indicates that a direct action of neurotrophins may be possible on these cells. Indeed, the survival of purified mature oligodendrocytes in culture

was enhanced by neurotrophin-3 (NT-3) or to a lesser degree, ciliary neurotrophic factor (CNTF) in the absence of other growth factors (McTigue et al., 1998).

The TrkA neurotrophin receptor has been linked to human diseases. The TrkA gene was originally described as an oncogene in colon cancer (Martin-Zanca et al., 1986) and its translocations are common in papillary thyroid carcinoma (Bongarzone et al., 1989). Recently, a mutation in the TrkA gene was found to cause congenital insensitivity to pain with anhidrosis (CIPA) syndrome (Indo et al., 1996) that closely resembles the phenotype of the TrkA deficient mice. No disease associations have been described either for the Trk B gene, or the genes for $p75^{NTR}$ or any of the neurotrophins.

The downregulation of neurotrophin receptor TrkA in ASA treated SC KO suggested to investigate its expression in the sciatic nerve as a whole. The upregulation of the receptors TrkA and TrkB in the sciatic nerve shows that the molecular changes observed in the animal model is reflected to some extent in the cell culture system also. BDNF protein was considered to be involved in proliferation, maturation and survival of neurons (Baquet et al., 2005). BDNF and NT-3, but not NGF, bind to full-length and truncated forms of a receptor-like tyrosine kinase, TrkB. Endogenous BDNF and neurotrophin-3 (NT3) were shown to modulate the myelination program of the peripheral nervous system. BDNF levels correlated with active myelin formation, whereas NT3 expression was initially high and then down-regulated throughout the proliferation and premyelination periods. Addition of exogenous BDNF enhanced myelination, whereas the removal of the endogenous BDNF by using the BDNF receptor TrkB-Fc fusion protein inhibited the formation of mature myelin internodes (Chan et al., 2001).

NGF interacts with two structurally unrelated receptors, the TrkA receptor-tyrosine kinase and p75, a tumor necrosis factor receptor family member. Each receptor binds independently to NGF with predominantly low affinity, but they produce high affinity binding sites upon receptor co-expression (Esposito et al., 2001). Almost all BDNF mRNA expressing neurons co-expressed TrkA, a high affinity NGF receptor mRNA (Fukuoka et al., 2001). NGF has potent effects on both central and peripheral myelination and these effects are mediated by changes to the axonal signals that control myelination rather by direct action on myelinating glia. The continued addition of NGF strongly enhanced the amount of myelin in co-cultures of SCs. Depletion of NGF by NGF scavengers reduced expression of myelin proteins *in vivo*

(Chan et al., 2004). Therefore, there is a possibility that the demyelination observed in the peripheral nervous system of the ASA KO mice may be due to the synergestic effect of the downregulation of NGF and BDNF in the sciatic nerve. The downregulation of these ligands might have stimulated the upregulation of the receptors for maintaining the normal activity of the cell.

The neurotrophins are currently under investigation as therapeutic agents for the treatment of neurodegenerative disorders. (Ebadi et al., 1997) (Tuszynski et al., 2002) (Longo and Massa, 2004). In response to neurotrophins, the neurotrophin cell surface receptors are activated, the ligand receptor complex is internalised which in turn activates multiple signal transduction pathways. Till date, various strategies were adopted for the treatment of MLD. The main problem with the treatment with ASA in enzyme replacement therapy is that the ASA does not cross the blood brain barrier and there is no enzyme activity in the brain after ASA treatment (Matzner et al., 2005). If the alteration in NGF and BDNF could be one of the reasons for the demyelination, an alternative therapy could be to administer these neurotrophins to prevent myelin loss associated with MLD.

The recently innovated method (translipofection) to load non sulfatide storing SCs with sulfatide will be a suitable comparative system to the naturally sulfatide storing SC KO. Since the sulfatide storing capacity by the sulfatide loaded cells through this method is higher than the SC KO, these cells may show a phenotype similar to that observed in humans. Further, studies on these SC KO and sulfatide loaded cells will help to elucidate the disease further at a molecular level. Hence, further investigation using these cells may elucidate the sulfatide storage, myelin gene expression, constitution of plasma membrane regarding the lipid and protein content, *in vitro* survival and death of cells and to check any progressive dysfunction in signal transduction mechanism. Studies in these aspects may help to explore new therapeutic approaches in the patients.

6. Summary

The ASA KO mouse is a murine model of the human metachromatic leukodystrophy (MLD), a disease that is caused by a genetic defect in the ASA gene. Deficiency of ASA causes accumulation of sulfatide in the central and peripheral nervous systems and in the visceral organs which subsequently causes demyelination in these areas. The first part of the work deals with investigation of myelin and rafts prepared from myelin of the ASA KO mice and compare it with the wild type mice for any abnormalities in lipids and proteins associated with them. It was found that accumulation of sulfatide is not restricted to the lysosomal compartment of cells but also occurs in myelin itself in ASA KO mice.

Since MAL binds sulfatide, it was investigated whether lipid storage has also an effect on intracellular MAL distribution. Although this sulfatide storage did not affect the overall composition of most myelin proteins, it specifically caused a severe reduction in MAL. This demonstrates a regulatory link between sulfatide accumulation and MAL expression and indicates the existence of regulatory mechanisms between lipid and myelin protein synthesis in oligodendrocytes.

Because sulfatide and MAL are known to be components of DIGs and MAL is a putative element of the cellular sorting machinery in these membrane microdomains, it was investigated whether the alterations in MAL and sulfatide amount affect the localisation of known myelin raft markers. Densitometric analysis showed that in the preparation, 50% of PLP as well as MOG were located in the insoluble fractions, whereas, F3 protein was predominantly found in the soluble fractions. CNPase, NCAM 120 and MAL were detected only in DIGs. The discrepancy with the literature where higher amounts of PLP, CNPase and NCAM 120 are usually found to be soluble in Triton-X 100 are probably caused by the investigation of 24 month-old animals. In this study with younger animals (3-6 months), most of the PLP protein was also found to be soluble in Triton X -100. Nevertheless, none of the myelin proteins investigated showed significant differences in their detergent soluble properties between myelin of normal and ASA KO mice.

MAL has been shown to bind sulfatide. Therefore, possible consequence of sulfatide storage in cellular distribution of MAL in sulfatide storing kidney cells was investigated. Double staining with LAMP-1 demonstrates the late endosomal/lysosomal location of these sulfatide

inclusions. No co-localisation of MAL with LAMP was found in WT cells, whereas in case of ASA KO cells, there was a partial co-localisation of MAL and sulfatide on LAMP positive compartments, which indicates that MAL is mistargeted to lysosomal compartments in sulfatide storing cells. Even though the reason for the downregulation of fyn is unknown, alteration of this kinase may have some impact on the cell signaling related to myelination.

The second part of this work was to establish an *in vitro* sulfatide storing cell culture system to study the molecular aspects of MLD. Hence, SCs from the sciatic nerve of 2-week-old ASA KO and WT mice were prepared and designated as SC KO and SC WT respectively.. The perfect control to study the cellular alterations in sulfatide storing cells are its ASA treated counterparts, otherwise clonal differences would influence the results substantially. Therefore, SC KO was thoroughly studied.

SC KO cells were initially characterised to determine their origin by PCR and tested for SC markers namely, S100 and p75 by immunostaining. The cells were also investigated for a set of transcription factors and neurotrophic factors by RT PCR which were expected to be expressed by these cells. Initially the cells were analysed for sulfatide storage by alcian blue staining, which showed positive results. Later, they were immunolabelled with antibodies specific for sulfatide and LAMP, which demonstrated that sulfatide is present in late endosomal/lysosomal compartments of these cells. Interestingly, ASA treatment reduced the sulfatide accumulation in these cells. This was further confirmed by electron microscopy of the ASA treated and untreated cells. Biochemical investigation of lipids by TLC showed the presence of sulfatide, which was also proved by TLC immunoblot. ASA treatment shows significant reduction of sulfatide in these cells. The upregulation of MAL in the ASA treated cells correlates with the previous experiments using myelin and brain of the ASA KO and WT mice. This further strengthens the hypothesis that there is a regulatory link between sulfatide accumulation and MAL downregulation. The TrkA and TrkB receptor upregulation and downregulation of NGF, BDNF and NT3 in the ASA KO sciatic nerve shows that the sulfatide storage has some effect on the ligand receptor interaction which in turn could also be one of the reason for the demyelination observed in the peripheral nervous system of the 2-year-old ASA KO mice. The sulfatide loading that was carried out during the last days of this project will be a suitable comparative system to the naturally sulfatide storing SC KO cells. Due to its enormous sulfatide storing capability, these cells can also be used to compare the

phenotype that is observed in humans and to proceed with further studies to explore the disease at a molecular level.

In conclusion, apart from these changes in glycosphingolipid content of the myelin membrane, it is the first report of a specific downregulation of a myelin protein in response to the accumulation of a lipid. MAL is believed to play a crucial role in myelin turnover and stability. Therefore, the observed changes in mice myelin might reflect only the onset of the pathological events in MLD. In humans, these events are then followed by more severe changes in myelin and myelin lipid rafts. A spontaneously immortalised SC line was developed from ASA KO mice that exhibit distinct MLD phenotypes. As there is no proper i*n vitro* system available to study MLD extensively, these sulfatide storing SCs derived from ASA KO mouse could be a valuable tool to investigate the disease further at a cellular level. This is the first report to show that there is an alteration in the expression of neurotrophic factors and receptors in this mouse model of the disease. This is also the first report to show that the SC sulfatide content can be manipulated by external treatment of sulfatide using a transfection reagent. It is possible to assume that short term sulfatide storage in the cells and the impact in the molecular changes may be very mild to identify and evaluate. Therefore, long term treatment of cells with sulfatide is essential. Further investigation using these cells may elucidate the sulfatide storage, myelin gene expression, lipid constitution and protein content of the plasma membrane and their distribution, *in vitro* survival and death of cells and to analyse any progressive dysfunction in signal transduction mechanism. Alternative therapy with neurotrophins to prevent loss of myelin is another suggestion that comes out of this study. Hence, studies in these aspects may also help to explore new therapeutic approaches in the patients.

7. References

Anderson, T.J., A. Schneider, J.A. Barrie, M. Klugmann, M.C. McCulloch, D. Kirkham, E. Kyriakides, K.A. Nave, and I.R. Griffiths. 1998. Late-onset neurodegeneration in mice with increased dosage of the proteolipid protein gene. J Comp Neurol. 394:506-19.

Anton, E.S., G. Weskamp, L.F. Reichardt, and W.D. Matthew. 1994. Nerve growth factor and its low-affinity receptor promote Schwann cell migration. Proc Natl Acad Sci U S A. 91:2795-9.

Arvanitis, D., M. Dumas, and S. Szuchet. 1992. Myelin palingenesis. 2. Immunocytochemical localization of myelin/oligodendrocyte glycolipids in multilamellar structures. Dev Neurosci. 14:328-35.

Baquet, Z.C., P.C. Bickford, and K.R. Jones. 2005. Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. J Neurosci. 25:6251-9.

Bare, D.J., J.M. Lauder, M.B. Wilkie, and P.F. Maness. 1993. p59fyn in rat brain is localized in developing axonal tracts and subpopulations of adult neurons and glia. Oncogene. 8:1429-36.

Bassam, B.J., G. Caetano-Anolles, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem. 196:80-3.

Boggs, J.M., H. Wang, W. Gao, D.N. Arvanitis, Y. Gong, and W. Min. 2004. A glycosynapse in myelin? Glycoconj J. 21:97-110.

Bolin, L.M., T.P. Iismaa, and E.M. Shooter. 1992. Isolation of activated adult Schwann cells and a spontaneously immortal Schwann cell clone. J Neurosci Res. 33:231-8.

Bongarzone, I., M.A. Pierotti, N. Monzini, P. Mondellini, G. Manenti, R. Donghi, S. Pilotti, M. Grieco, M. Santoro, A. Fusco, and et al. 1989. High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. Oncogene. 4:1457-62.

Bosio, A., E. Binczek, and W. Stoffel. 1996. Functional breakdown of the lipid bilayer of the myelin membrane in central and peripheral nervous system by disrupted galactocerebroside synthesis. Proc Natl Acad Sci U S A. 93:13280-5.

Boutry, J.M., J.J. Hauw, A. Gansmuller, N. Di-Bert, M. Pouchelet, and A. Baron-Van Evercooren. 1992. Establishment and characterization of a mouse Schwann cell line which produces myelin in vivo. J Neurosci Res. 32:15-26.

Braulke, T., S. Tippmer, H.J. Chao, and K. von Figura. 1990. Regulation of mannose 6-phosphate/insulin-like growth factor II receptor distribution by activators and inhibitors of protein kinase C. Eur J Biochem. 189:609-16.

Brockes, J.P., K.L. Fields, and M.C. Raff. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res. 165:105-18.

Butt, A.M., M. Ibrahim, N. Gregson, and M. Berry. 1998. Differential expression of the L- and S-isoforms of myelin associated glycoprotein (MAG) in oligodendrocyte unit phenotypes in the adult rat anterior medullary velum. J Neurocytol. 27:271-80.

Caduff, J., S. Sansano, A. Bonnet, U. Suter, and N. Schaeren-Wiemers. 2001. Characterization of GFP-MAL expression and incorporation in rafts. Microsc Res Tech. 52:645-55.

Caroni, P., and M.E. Schwab. 1988. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J Cell Biol. 106:1281-8.

Chan, J.R., J.M. Cosgaya, Y.J. Wu, and E.M. Shooter. 2001. Neurotrophins are key mediators of the myelination program in the peripheral nervous system. Proc Natl Acad Sci U S A. 98:14661-8.

Chan, J.R., T.A. Watkins, J.M. Cosgaya, C. Zhang, L. Chen, L.F. Reichardt, E.M. Shooter, and B.A. Barres. 2004. NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. Neuron. 43:183-91.

Chen, G.L., N.L. Halligan, N.F. Lue, and W.W. Chen. 1987. Biosynthesis of myelin-associated proteins in simian virus 40 (SV40)-transformed rat Schwann cell lines. Brain Res. 414:35-48.

Cheong, K.H., D. Zacchetti, E.E. Schneeberger, and K. Simons. 1999. VIP17/MAL, a lipid raft-associated protein, is involved in apical transport in MDCK cells. Proc Natl Acad Sci U S A. 96:6241-8.

Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18:5294-9.

Coenen, R., V. Gieselmann, and R. Lullmann-Rauch. 2001. Morphological alterations in the inner ear of the arylsulfatase A-deficient mouse. Acta Neuropathol (Berl). 101:491-8.

Coetzee, T., N. Fujita, J. Dupree, R. Shi, A. Blight, K. Suzuki, and B. Popko. 1996. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. Cell. 86:209-19.

Coulter-Mackie, M.B., D.A. Applegarth, J.R. Toone, L. Gagnier, A.R. Anzarut, and G. Hendson. 2002. Isolated peripheral neuropathy in atypical metachromatic leukodystrophy: a recurrent mutation. Can J Neurol Sci. 29:159-63.

Davies, A.M., K.F. Lee, and R. Jaenisch. 1993. p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron. 11:565-74.

Davis, J.B., and P. Stroobant. 1990. Platelet-derived growth factors and fibroblast growth factors are mitogens for rat Schwann cells. J Cell Biol. 110:1353-60.

D'Hooge, R., D. Hartmann, J. Manil, F. Colin, V. Gieselmann, and P.P. De Deyn. 1999. Neuromotor alterations and cerebellar deficits in aged arylsulfatase A-deficient transgenic mice. Neurosci Lett. 273:93-6.

D'Hooge, R., D. Van Dam, F. Franck, V. Gieselmann, and P.P. De Deyn. 2001. Hyperactivity, neuromotor defects, and impaired learning and memory in a mouse model for metachromatic leukodystrophy. Brain Res. 907:35-43.

Dougherty, K.D., C.F. Dreyfus, and I.B. Black. 2000. Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiol Dis. 7:574-85.

Ebadi, M., R.M. Bashir, M.L. Heidrick, F.M. Hamada, H.E. Refaey, A. Hamed, G. Helal, M.D. Baxi, D.R. Cerutis, and N.K. Lassi. 1997. Neurotrophins and their receptors in nerve injury and repair. Neurochem Int. 30:347-74.

Eccleston, P.A., K.R. Jessen, and R. Mirsky. 1989. Transforming growth factor-beta and gamma-interferon have dual effects on growth of peripheral glia. J Neurosci Res. 24:524-30.

Eccleston, P.A., E.J. Collarini, K.R. Jessen, R. Mirsky, and W.D. Richardson. 1990. Schwann Cells Secrete a PDGF-like Factor: Evidence for an Autocrine Growth Mechanism involving PDGF. Eur J Neurosci. 2:985-992.

Eccleston, P.A., R. Mirsky, and K.R. Jessen. 1991. Spontaneous immortalisation of Schwann cells in culture: short-term cultured Schwann cells secrete growth inhibitory activity. Development. 112:33-42.

Erb, M., Steck, A..J., Nave, K. A., Schaeren-Wiemers, N. 2003. Differential expression of L- and S-MAG upon cAMP stimulated differentiation in oligodendroglial cells. J Neurosci Res. 71:326-37.

Erne, B., S. Sansano, M. Frank, and N. Schaeren-Wiemers. 2002. Rafts in adult peripheral nerve myelin contain major structural myelin proteins and myelin and lymphocyte protein (MAL) and CD59 as specific markers. J Neurochem. 82:550-62.

Esposito, D., P. Patel, R.M. Stephens, P. Perez, M.V. Chao, D.R. Kaplan, and B.L. Hempstead. 2001. The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. J Biol Chem. 276:32687-95.

Eto, Y., C. Meier, and N.N. Herschkowitz. 1976. Chemical compositions of brain and myelin in two patients with multiple sulphatase deficiency (a variant form of metachromatic leukodystrophy). J Neurochem. 27:1071-6.

Farrell, D.F. 1974. Enzymatic sulphation of some galactose- containing sphingolipids in developing rat brain. J Neurochem. 23:219-25.

Ferret-Sena, V., A. Sena, F. Besnard, C. Fressinaud, G. Rebel, and L.L. Sarlieve. 1990. Comparison of the mechanisms of action of insulin and triiodothyronine on the synthesis of cerebroside sulfotransferase in cultures of cells dissociated from brains of embryonic mice. Dev Neurosci. 12:89-105.

Frank, M., M.E. van der Haar, N. Schaeren-Wiemers, and M.E. Schwab. 1998. rMAL is a glycosphingolipid-associated protein of myelin and apical membranes of epithelial cells in kidney and stomach. J Neurosci. 18:4901-13.

Frank, M., N. Schaeren-Wiemers, R. Schneider, and M.E. Schwab. 1999. Developmental expression pattern of the myelin proteolipid MAL indicates different functions of MAL for immature Schwann cells and in a late step of CNS myelinogenesis. J Neurochem. 73:587-97.

Frank, M. 2000. MAL, a proteolipid in glycosphingolipid enriched domains: functional implications in myelin and beyond. Prog Neurobiol. 60:531-44.

Franken, S., U. Junghans, V. Rosslenbroich, S.L. Baader, R. Hoffmann, V. Gieselmann, C. Viebahn, and J. Kappler. 2003. Collapsin response mediator proteins of neonatal rat brain interact with chondroitin sulfate. J Biol Chem. 278:3241-50.

Fredman, P., L. Mattsson, K. Andersson, P. Davidsson, I. Ishizuka, S. Jeansson, J.E. Mansson, and L. Svennerholm. 1988. Characterization of the binding epitope of a monoclonal antibody to sulphatide. Biochem J. 251:17-22.

Fressinaud, C., J.M. Vallat, M. Masson, M.O. Jauberteau, N. Baumann, and J. Hugon. 1992. Adult-onset metachromatic leukodystrophy presenting as isolated peripheral neuropathy. Neurology. 42:1396-8.

Fukuoka, T., E. Kondo, Y. Dai, N. Hashimoto, and K. Noguchi. 2001. Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. J Neurosci. 21:4891-900.

Gieselmann, V., J. Zlotogora, A. Harris, D.A. Wenger, and C.P. Morris. 1994. Molecular genetics of metachromatic leukodystrophy. Hum Mutat. 4:233-42.

Gieselmann, V., U. Matzner, B. Hess, R. Lullmann-Rauch, R. Coenen, D. Hartmann, R. D'Hooge, P. DeDeyn, and G. Nagels. 1998. Metachromatic leukodystrophy: molecular genetics and an animal model. J Inherit Metab Dis. 21:564-74.

Gieselmann, V. 2003. Metachromatic leukodystrophy: recent research developments. J Child Neurol. 18:591-4.

Gieselmann, V., S. Franken, D. Klein, J.E. Mansson, R. Sandhoff, R. Lullmann Rauch, D. Hartmann, K. Saravanan, P.P. De Deyn, R. D'Hooge, A.M. Van Der Linden, and N. Schaeren-Wiemers. 2003. Metachromatic leukodystrophy: consequences of sulphatide accumulation. Acta Paediatr Suppl. 92:74-9; discussion 45.

Gieselmann, V. 2005. What can cell biology tell us about heterogeneity in lysosomal storage diseases? Acta Paediatr Suppl. 94:80-6; discussion 79.

Goda, S., J. Hammer, D. Kobiler, and R.H. Quarles. 1991. Expression of the myelin-associated glycoprotein in cultures of immortalized Schwann cells. J Neurochem. 56:1354-61.

Graves, J.D., and E.G. Krebs. 1999. Protein phosphorylation and signal transduction. Pharmacol Ther. 82:111-21.

Guenard, V., L.A. Gwynn, and P.M. Wood. 1994. Astrocytes inhibit Schwann cell proliferation and myelination of dorsal root ganglion neurons in vitro. J Neurosci. 14:2980-92.

Gustavson, K.H., and B. Hagberg. 1971. The incidence and genetics of metachromatic leucodystrophy in northern Sweden. Acta Paediatr Scand. 60:585-90.

Hai, M., N. Muja, G.H. DeVries, R.H. Quarles, and P.I. Patel. 2002. Comparative analysis of Schwann cell lines as model systems for myelin gene transcription studies. J Neurosci Res. 69:497-508.

Hansen, L.M., O. Kristensen, and M.L. Friis. 1994. [Neuropathy in adult metachromatic leukodystrophy]. Ugeskr Laeger. 156:2252-3.

Harzer, K., and B. Kustermann-Kuhn. 1987. Brain galactolipid content in a patient with pseudoarylsulfatase A deficiency and coincidental diffuse disseminated sclerosis, and in patients with metachromatic, adreno-, and other leukodystrophies. J Neurochem. 48:62-6.

Hempstead, B.L., and J.L. Salzer. 2002. Neurobiology. A glial spin on neurotrophins. Science. 298:1184-6.

Hess, B., P. Saftig, D. Hartmann, R. Coenen, R. Lullmann-Rauch, H.H. Goebel, M. Evers, K. von Figura, R. D'Hooge, G. Nagels, P. De Deyn, C. Peters, and V. Gieselmann. 1996. Phenotype of arylsulfatase A-deficient mice: relationship to human metachromatic leukodystrophy. Proc Natl Acad Sci U S A. 93:14821-6.

Hirn, M., M. Pierres, H. Deagostini-Bazin, M. Hirsch, and C. Goridis. 1981. Monoclonal antibody against cell surface glycoprotein of neurons. Brain Res. 214:433-9.

Hoke, A., T. Ho, T.O. Crawford, C. LeBel, D. Hilt, and J.W. Griffin. 2003. Glial cell line-derived neurotrophic factor alters axon schwann cell units and promotes myelination in unmyelinated nerve fibers. J Neurosci. 23:561-7.

Honke, K., Y. Hirahara, J. Dupree, K. Suzuki, B. Popko, K. Fukushima, J. Fukushima, T. Nagasawa, N. Yoshida, Y. Wada, and N. Taniguchi. 2002. Paranodal junction formation and spermatogenesis require sulfoglycolipids. Proc Natl Acad Sci U S A. 99:4227-32.

Indo, Y., M. Tsuruta, Y. Hayashida, M.A. Karim, K. Ohta, T. Kawano, H. Mitsubuchi, H. Tonoki, Y. Awaya, and I. Matsuda. 1996. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet. 13:485-8.

Jessen, K.R., L. Morgan, M. Brammer, and R. Mirsky. 1985. Galactocerebroside is expressed by non-myelin-forming Schwann cells in situ. J Cell Biol. 101:1135-43.

Jirsova, K., P. Sodaar, V. Mandys, and P.R. Bar. 1997. Cold jet: a method to obtain pure Schwann cell cultures without the need for cytotoxic, apoptosis-inducing drug treatment. J Neurosci Methods. 78:133-7.

Jung, M., I. Sommer, M. Schachner, and K.A. Nave. 1996. Monoclonal antibody O10 defines a conformationally sensitive cell-surface epitope of proteolipid protein (PLP): evidence that PLP misfolding underlies dysmyelination in mutant mice. J Neurosci. 16:7920-9.

Kelly, B.M., C.Z. Yu, and P.L. Chang. 1989. Presence of a lysosomal enzyme, arylsulfatase-A, in the prelysosome-endosome compartments of human cultured fibroblasts. Eur J Cell Biol. 48:71-8.

Kim, T., K. Fiedler, D.L. Madison, W.H. Krueger, and S.E. Pfeiffer. 1995. Cloning and characterization of MVP17: a developmentally regulated myelin protein in oligodendrocytes. J Neurosci Res. 42:413-22.

Kim, T., and S.E. Pfeiffer. 1999. Myelin glycosphingolipid/cholesterol-enriched microdomains selectively sequester the non-compact myelin proteins CNP and MOG. J Neurocytol. 28:281-93.

Kim, T., and S.E. Pfeiffer. 2002. Subcellular localization and detergent solubility of MVP17/rMAL, a lipid raft-associated protein in oligodendrocytes and myelin. J Neurosci Res. 69:217-26.

Klein, D., H. Bussow, S.N. Fewou, and V. Gieselmann. 2005. Exocytosis of storage material in a lysosomal disorder. Biochem Biophys Res Commun. 327:663-7.

Kobayashi, T., and Y. Hirabayashi. 2000. Lipid membrane domains in cell surface and vacuolar systems. Glycoconj J. 17:163-71.

Koch, T., T. Brugger, A. Bach, G. Gennarini, and J. Trotter. 1997. Expression of the immunoglobulin superfamily cell adhesion molecule F3 by oligodendrocyte-lineage cells. Glia. 19:199-212.

Kolodny, E.H. 1989. Metachromatic leukodystrophy and multiple sulfatase deficiency: Sulfatide lipidosis. In The metabolic bases of inherited disease. Vol. 2. C.R. Scriver, Beaudet, A.L., Sly, W.S., Valle D., editor. McGraw-Hill, New York. 1721-1750.

Komiyama, T., Y. Nakao, Y. Toyama, H. Asou, C.A. Vacanti, and M.P. Vacanti. 2003. A novel technique to isolate adult Schwann cells for an artificial nerve conduit. J Neurosci Methods. 122:195-200.

Koper, J.W., R.C. Hoeben, F.M. Hochstenbach, L.M. van Golde, and M. Lopes-Cardozo. 1986. Effects of triiodothyronine on the synthesis of sulfolipids by oligodendrocyte-enriched glial cultures. Biochim Biophys Acta. 887:327-34.

Kramer, E.M., T. Koch, A. Niehaus, and J. Trotter. 1997. Oligodendrocytes direct glycosyl phosphatidylinositol-anchored proteins to the myelin sheath in glycosphingolipid-rich complexes. J Biol Chem. 272:8937-45.

Kramer, E.M., C. Klein, T. Koch, M. Boytinck, and J. Trotter. 1999. Compartmentation of Fyn kinase with glycosylphosphatidylinositol-anchored molecules in oligodendrocytes facilitates kinase activation during myelination. J Biol Chem. 274:29042-9.

Krendel, D.A., L.A. Shutter, and P.J. Holt. 1994. Fiber type disproportion in metachromatic leukodystrophy. Muscle Nerve. 17:1352-3.

Kreysing, J., K. von Figura, and V. Gieselmann. 1990. Structure of the arylsulfatase A gene. Eur J Biochem. 191:627-31.

Lampert, I.A., and P.D. Lewis. 1975. Staining of sulphatides in metachromatic leukodystrophy with Alcian blue at high salt concentrations. Histochemistry. 43:269-73.

Li, R.H., M.X. Sliwkowski, J. Lo, and J.P. Mather. 1996. Establishment of Schwann cell lines from normal adult and embryonic rat dorsal root ganglia. J Neurosci Methods. 67:57-69.

Lo, L., L. Sommer, and D.J. Anderson. 1997. MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. Curr Biol. 7:440-50.

Lo, L., M.C. Tiveron, and D.J. Anderson. 1998. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development. 125:609-20.

Longo, F.M., and S.M. Massa. 2004. Neurotrophin-based strategies for neuroprotection. J Alzheimers Dis. 6:S13-7.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem. 193:265-75.

Lullmann-Rauch, R., U. Matzner, S. Franken, D. Hartmann, and V. Gieselmann. 2001. Lysosomal sulfoglycolipid storage in the kidneys of mice deficient for arylsulfatase A (ASA) and of double-knockout mice deficient for ASA and galactosylceramide synthase. Histochem Cell Biol. 116:161-9.

Maertens, S.R. 1994. Serum activity induces Schwann cell proliferation in vitro. Glia. 10:142-8.

Malone, M.J., and P. Stoffyn. 1966. A comparative study of brain and kidney glycolipids in metachromatic leucodystrophy. J Neurochem. 13:1037-45.

Malone, M.J., N. Sakuragawa, and M. Szoke. 1975. A comparative study of myelin fractions from metachromatic and globoid leukodystrophies. Neurology. 25:827-32.

Martin-Belmonte, F., R. Puertollano, J. Millan, and M.A. Alonso. 2000. The MAL proteolipid is necessary for the overall apical delivery of membrane proteins in the polarized epithelial Madin-Darby canine kidney and fischer rat thyroid cell lines. Mol Biol Cell. 11:2033-45.

Martinez, A.C., M.T. Ferrer, E. Fueyo, and L. Galdos. 1975. Peripheral neuropathy detected on electrophysiological study as first manifestation of metachromatic leucodystrophy in infancy. J Neurol Neurosurg Psychiatry. 38:169-74.

Martin-Zanca, D., G. Mitra, L.K. Long, and M. Barbacid. 1986. Molecular characterization of the human trk oncogene. Cold Spring Harb Symp Quant Biol. 51 Pt 2:983-92.

Mathon, N.F., D.S. Malcolm, M.C. Harrisingh, L. Cheng, and A.C. Lloyd. 2001. Lack of replicative senescence in normal rodent glia. Science. 291:872-5.

Matzner, U., M. Habetha, and V. Gieselmann. 2000. Retrovirally expressed human arylsulfatase A corrects the metabolic defect of arylsulfatase A-deficient mouse cells. Gene Ther. 7:805-12.

Matzner, U., D. Hartmann, R. Lullmann-Rauch, R. Coenen, F. Rothert, J.E. Mansson, P. Fredman, R. D'Hooge, P.P. De Deyn, and V. Gieselmann. 2002. Bone marrow stem cell-based gene transfer in a mouse model for metachromatic leukodystrophy: effects on visceral and nervous system disease manifestations. Gene Ther. 9:53-63.

Matzner, U., E. Herbst, K.K. Hedayati, R. Lullmann-Rauch, C. Wessig, S. Schroder, C. Eistrup, C. Moller, J. Fogh, and V. Gieselmann. 2005. Enzyme replacement improves nervous system pathology and function in a mouse model for metachromatic leukodystrophy. Hum Mol Genet. 14:1139-52.

McTigue, D.M., P.J. Horner, B.T. Stokes, and F.H. Gage. 1998. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. J Neurosci. 18:5354-65.

Mehl, E., and H. Jatzkewitz. 1965. Evidence for the Genetic Block in Metachromatic Leucodystrophy (Ml). Biochem Biophys Res Commun. 19:407-11.

Mirsky, R., J. Winter, E.R. Abney, R.M. Pruss, J. Gavrilovic, and M.C. Raff. 1980. Myelin-specific proteins and glycolipids in rat Schwann cells and oligodendrocytes in culture. J Cell Biol. 84:483-94.

Nave, K.A. 1994. Neurological mouse mutants and the genes of myelin. J Neurosci Res. 38:607-12.

Neufeld, E.F. 1991. Lysosomal storage diseases. Annu Rev Biochem. 60:257-80.

Nickols, J.C., W. Valentine, S. Kanwal, and B.D. Carter. 2003. Activation of the transcription factor NF-kappaB in Schwann cells is required for peripheral myelin formation. Nat Neurosci. 6:161-7.

Norton, W.T., and S.E. Poduslo. 1973. Myelination in rat brain: changes in myelin composition during brain maturation. J Neurochem. 21:759-73.

Norton, W.T., and S.E. Poduslo. 1982. Biochemical studies of metachromatic leukodystrophy in three siblings. Acta Neuropathol (Berl). 57:188-96.

Osterhout, D.J., A. Wolven, R.M. Wolf, M.D. Resh, and M.V. Chao. 1999. Morphological differentiation of oligodendrocytes requires activation of Fyn tyrosine kinase. J Cell Biol. 145:1209-18.

Parkinson, D.B., A. Bhaskaran, A. Droggiti, S. Dickinson, M. D'Antonio, R. Mirsky, and K.R. Jessen. 2004. Krox-20 inhibits Jun-NH2-terminal kinase/c-Jun to control Schwann cell proliferation and death. J Cell Biol. 164:385-94.

Pines, A., M. Romanello, L. Cesaratto, G. Damante, L. Moro, P. D'Andrea, and G. Tell. 2003. Extracellular ATP stimulates the early growth response protein 1 (Egr-1) via a protein kinase C-dependent pathway in the human osteoblastic HOBIT cell line. Biochem J. 373:815-24.

Pizzonia, J.H., F.A. Gesek, S.M. Kennedy, B.A. Coutermarsh, B.J. Bacskai, and P.A. Friedman. 1991. Immunomagnetic separation, primary culture, and characterization of cortical thick ascending limb plus distal convoluted tubule cells from mouse kidney. In Vitro Cell Dev Biol. 27A:409-16.

Poduslo, S.E., K. Miller, and Y. Jang. 1982. Biochemical studies of the late infantile form of metachromatic leukodystrophy. Acta Neuropathol (Berl). 57:13-22.

Pombo, P.M., N. Ibarrola, M.A. Alonso, and A. Rodriguez-Pena. 1998. Thyroid hormone regulates the expression of the MAL proteolipid, a component of glycolipid-enriched membranes, in neonatal rat brain. J Neurosci Res. 52:584-90.

Porter, S., L. Glaser, and R.P. Bunge. 1987. Release of autocrine growth factor by primary and immortalized Schwann cells. Proc Natl Acad Sci U S A. 84:7768-72.

Puertollano, R., and M.A. Alonso. 1999. MAL, an integral element of the apical sorting machinery, is an itinerant protein that cycles between the trans-Golgi network and the plasma membrane. Mol Biol Cell. 10:3435-47.

Puertollano, R., F. Martin-Belmonte, J. Millan, M.C. de Marco, J.P. Albar, L. Kremer, and M.A. Alonso. 1999. The MAL proteolipid is necessary for normal apical transport and accurate sorting of the influenza virus hemagglutinin in Madin-Darby canine kidney cells. J Cell Biol. 145:141-51.

Rabizadeh, S., J. Oh, L.T. Zhong, J. Yang, C.M. Bitler, L.L. Butcher, and D.E. Bredesen. 1993. Induction of apoptosis by the low-affinity NGF receptor. Science. 261:345-8.

Rajendran, L., and K. Simons. 2005. Lipid rafts and membrane dynamics. J Cell Sci. 118:1099-102.

Ridley, A.J., H.F. Paterson, M. Noble, and H. Land. 1988. Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. Embo J. 7:1635-45.

Ridley, A.J., J.B. Davis, P. Stroobant, and H. Land. 1989. Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells. J Cell Biol. 109:3419-24.

Rintoul, D.A., and R. Welti. 1989. Thermotropic behavior of mixtures of glycosphingolipids and phosphatidylcholine: effect of monovalent cations on sulfatide and galactosylceramide. Biochemistry. 28:26-31.

Sandhoff, R., S.T. Hepbildikler, R. Jennemann, R. Geyer, V. Gieselmann, R.L. Proia, H. Wiegandt, and H.J. Grone. 2002. Kidney sulfatides in mouse models of inherited glycosphingolipid disorders: determination by nano-electrospray ionization tandem mass spectrometry. J Biol Chem. 277:20386-98.

Scarpini, E., B.Q. Kreider, R.P. Lisak, and D.E. Pleasure. 1988. Establishment of Schwann cell cultures from adult rat peripheral nerves. Exp Neurol. 102:167-76.

Schaeren-Wiemers, N., D.M. Valenzuela, M. Frank, and M.E. Schwab. 1995a. Characterization of a rat gene, rMAL, encoding a protein with four hydrophobic domains in central and peripheral myelin. J Neurosci. 15:5753-64.

Schaeren-Wiemers, N., C. Schaefer, D.M. Valenzuela, G.D. Yancopoulos, and M.E. Schwab. 1995b. Identification of new oligodendrocyte- and myelin-specific genes by a differential screening approach. J Neurochem. 65:10-22.

Scherer, S.S., D.Y. Wang, R. Kuhn, G. Lemke, L. Wrabetz, and J. Kamholz. 1994. Axons regulate Schwann cell expression of the POU transcription factor SCIP. J Neurosci. 14:1930-42.

Schluff, P., B. Flott-Rahmel, V. Gieselmann, P. Zimmer, A. Das, and K. Ullrich. 1998. Localization of receptors for endocytosis of lysosomal enzymes on different brain cells. J Inherit Metab Dis. 21:313-7.

Schott, I., D. Hartmann, V. Gieselmann, and R. Lullmann-Rauch. 2001. Sulfatide storage in visceral organs of arylsulfatase A-deficient mice. Virchows Arch. 439:90-6.

Schumacher, M., I. Jung-Testas, P. Robel, and E.E. Baulieu. 1993. Insulin-like growth factor I: a mitogen for rat Schwann cells in the presence of elevated levels of cyclic AMP. Glia. 8:232-40.

Schwarz, A., G.C. Terstappen, and A.H. Futerman. 1997. Isolation of gangliosides by cloud-point extraction with a nonionic detergent. Anal Biochem. 254:221-5.

Scott, J.E., and J. Dorling. 1965. Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. Histochemie. 5:221-33.

Shen, J.S., K. Watabe, X.L. Meng, H. Ida, T. Ohashi, and Y. Eto. 2002. Establishment and characterization of spontaneously immortalized Schwann cells from murine model of globoid cell leukodystrophy (twitcher). J Neurosci Res. 68:588-94.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. Nature. 387:569-72.

Simons, K., and J. Gruenberg. 2000. Jamming the endosomal system: lipid rafts and lysosomal storage diseases. Trends Cell Biol. 10:459-62.

Simons, K., and R. Ehehalt. 2002. Cholesterol, lipid rafts, and disease. J Clin Invest. 110:597-603.

Simons, M., T. Friedrichson, J.B. Schulz, M. Pitto, M. Masserini, and T.V. Kurzchalia. 1999. Exogenous administration of gangliosides displaces GPI-anchored proteins from lipid microdomains in living cells. Mol Biol Cell. 10:3187-96.

Simons, M., E.-M. Krämer, P. Macchi, S. Rathke-Hartlieb, J. Trotter, K.-A. Nave, and J.B. Schulz. 2002. Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus-Merzbacher disease.

Sperber, B.R., E.A. Boyle-Walsh, M.J. Engleka, P. Gadue, A.C. Peterson, P.L. Stein, S.S. Scherer, and F.A. McMorris. 2001. A unique role for Fyn in CNS myelination. J Neurosci. 21:2039-47.

Stewart, K., O.A. Brown, A.E. Morelli, L.J. Fairbairn, L.S. Lashford, A. Cooper, C.E. Hatton, T.M. Dexter, M.G. Castro, and P.R. Lowenstein. 1997. Uptake of alpha-(L)-iduronidase produced by retrovirally transduced fibroblasts into neuronal and glial cells in vitro. Gene Ther. 4:63-75.

Stoffyn, P., and A. Stoffyn. 1963. Structure of sulfatides. Biochim Biophys Acta. 70:218-20.

Stoffyn, P.J. 1966. The structure and chemistry of sulfatides. J Am Oil Chem Soc. 43:69-74.

Suzuki, K. 1995. The twitcher mouse: a model for Krabbe disease and for experimental therapies. Brain Pathol. 5:249-58.

Suzuki, K. 2003. Globoid cell leukodystrophy (Krabbe's disease): update. J Child Neurol. 18:595-603.

Tanaka, K., and H.D. Webster. 1993. Effects of psychosine (galactosylsphingosine) on the survival and the fine structure of cultured Schwann cells. J Neuropathol Exp Neurol. 52:490-8.

Taylor, C.M., T. Coetzee, and S.E. Pfeiffer. 2002. Detergent-insoluble glycosphingolipid/cholesterol microdomains of the myelin membrane. J Neurochem. 81:993-1004.

Tennekoon, G.I., J. Yoshino, K.W. Peden, J. Bigbee, J.L. Rutkowski, Y. Kishimoto, G.H. DeVries, and G.M. McKhann. 1987. Transfection of neonatal rat Schwann cells with SV-40 large T antigen gene under control of the metallothionein promoter. J Cell Biol. 105:2315-25.

Thi, A.D., C. Evrard, and P. Rouget. 1998. Proliferation and differentiation properties of permanent Schwann cell lines immortalized with a temperature-sensitive oncogene. J Exp Biol. 201 (Pt 6):851-60.

Toda, K., J.A. Small, S. Goda, and R.H. Quarles. 1994. Biochemical and cellular properties of three immortalized Schwann cell lines expressing different levels of the myelin-associated glycoprotein. J Neurochem. 63:1646-57.

Tuszynski, M.H., J. Conner, A. Blesch, D. Smith, D.A. Merrill, and H.L. Vahlsing. 2002. New strategies in neural repair. Prog Brain Res. 138:401-9.

Umemori, H., Y. Kadowaki, K. Hirosawa, Y. Yoshida, K. Hironaka, H. Okano, and T. Yamamoto. 1999. Stimulation of myelin basic protein gene transcription by Fyn tyrosine kinase for myelination. J Neurosci. 19:1393-7.

Vacher, M., M. Waks, and C. Nicot. 1989. Myelin proteins in reverse micelles: tight lipid association required for insertion of the Folch-Pi proteolipid into a membrane-mimetic system. J Neurochem. 52:117-23.

van Echten-Deckert, G. 2000. Sphingolipid extraction and analysis by thin-layer chromatography. Methods Enzymol. 312:64-79.

von Figura, K., V. Gieselmann, and J. Jaeken. 2001. Metachromatic leukodystrophy. In The metabolic and molecular bases of inherited disease. Vol. 3. C.R. Scriver, Beaudet, A.L., Sly, W.S., Valle, D., editor. McGraw-Hill, New York. 3695-3724.

Vroemen, M., and N. Weidner. 2003. Purification of Schwann cells by selection of p75 low affinity nerve growth factor receptor expressing cells from adult peripheral nerve. J Neurosci Methods. 124:135-43.

Watabe, K., M. Yamada, T. Kawamura, and S.U. Kim. 1990. Transfection and stable transformation of adult mouse Schwann cells with SV-40 large T antigen gene. J Neuropathol Exp Neurol. 49:455-67.

Watabe, K., T. Fukuda, J. Tanaka, K. Toyohara, and O. Sakai. 1994. Mitogenic effects of platelet-derived growth factor, fibroblast growth factor, transforming growth factor-beta, and heparin-binding serum factor for adult mouse Schwann cells. J Neurosci Res. 39:525-34.

Watabe, K., T. Fukuda, J. Tanaka, H. Honda, K. Toyohara, and O. Sakai. 1995. Spontaneously immortalized adult mouse Schwann cells secrete autocrine and paracrine growth-promoting activities. J Neurosci Res. 41:279-90.

Watabe, K., H. Ida, K. Uehara, K. Oyanagi, T. Sakamoto, J. Tanaka, W.S. Garver, S. Miyawaki, K. Ohno, and Y. Eto. 2001. Establishment and characterization of immortalized Schwann cells from murine model of Niemann-Pick disease type C (spm/spm). J Peripher Nerv Syst. 6:85-94.

Watabe, K., T. Sakamoto, Y. Kawazoe, M. Michikawa, K. Miyamoto, T. Yamamura, H. Saya, and N. Araki. 2003. Tissue culture methods to study neurological disorders: Establishment of immortalized Schwann cells from murine disease models. Neuropathology. 23:68-78.

Weiner, J.A., and J. Chun. 1999. Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. Proc Natl Acad Sci U S A. 96:5233-8.

Weinmaster, G., and G. Lemke. 1990. Cell-specific cyclic AMP-mediated induction of the PDGF receptor. Embo J. 9:915-20.

Wenger, D.A., K. Suzuki, Y. Suzuki, and K. Suzuki. 2001. Galactosylceramide Lipidosis: Globoid Cell Leukodystrophy (Krabbe disease). In The metabolic and molecular bases of inherited disease. Vol. 3. B.A. Scriver CR, Sly WS, Valle D, editor. McGraw-Hill Professional Publishing, New York. 3669-3694.

Werner, H., M. Jung, M. Klugmann, M. Sereda, I.R. Griffiths, and K.A. Nave. 1998. Mouse models of myelin diseases. Brain Pathol. 8:771-93.

Wittke, D., D. Hartmann, V. Gieselmann, and R. Lullmann-Rauch. 2004. Lysosomal sulfatide storage in the brain of arylsulfatase A-deficient mice: cellular alterations and topographic distribution. Acta Neuropathol (Berl). 108:261-71.

Yaghootfam, A., F. Schestag, T. Dierks, and V. Gieselmann. 2003. Recognition of arylsulfatase A and B by the UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-phosphotransferase. J Biol Chem. 278:32653-61.

Yoshimura, T., T. Kobayashi, S. Goda, and I. Goto. 1994. Inhibition of the proliferation of cultured immortalized Schwann cells by forskolin with a decreased basal level of diacylglycerol. Neurochem Res. 19:735-41.

Zacchetti, D., J. Peranen, M. Murata, K. Fiedler, and K. Simons. 1995. VIP17/MAL, a proteolipid in apical transport vesicles. FEBS Lett. 377:465-9.

Zorick, T.S., D.E. Syroid, A. Brown, T. Gridley, and G. Lemke. 1999. Krox-20 controls SCIP expression, cell cycle exit and susceptibility to apoptosis in developing myelinating Schwann cells. Development. 126:1397-406.

8. Appendix

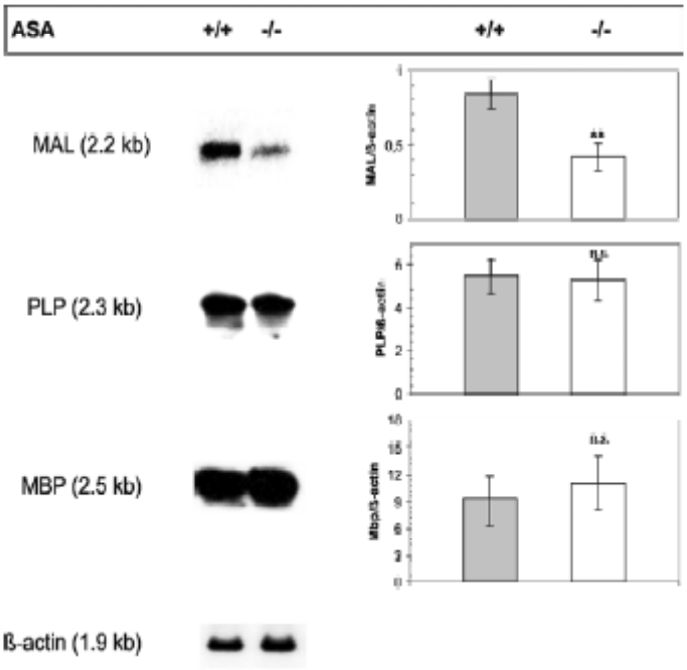


Fig A1: Northern blot analysis of MAL mRNA. Total RNA was prepared from brains of WT and ASA KO mice, resolved on a denaturing agarose gel and transferred to a nylon membrane. The membrane was hybridised sequentially with radioactively-labelled cDNA probes as indicated. Left panel shows film exposure for the different probes of one animal pair. Densitometric quantification (right panel) was made on three animal pairs using a phosphoimager. A reduction by 50% for MAL mRNA can be detected whereas the levels of PLP and MBP mRNA are not significantly reduced in brains of ASA KO mice. Values are means ± SD. $^{**}P \leq 0.01$ ASA KO against WT by one-way ANOVA. n.s. : not significant. This experiment was performed by Dr. A. Yaghootfam.

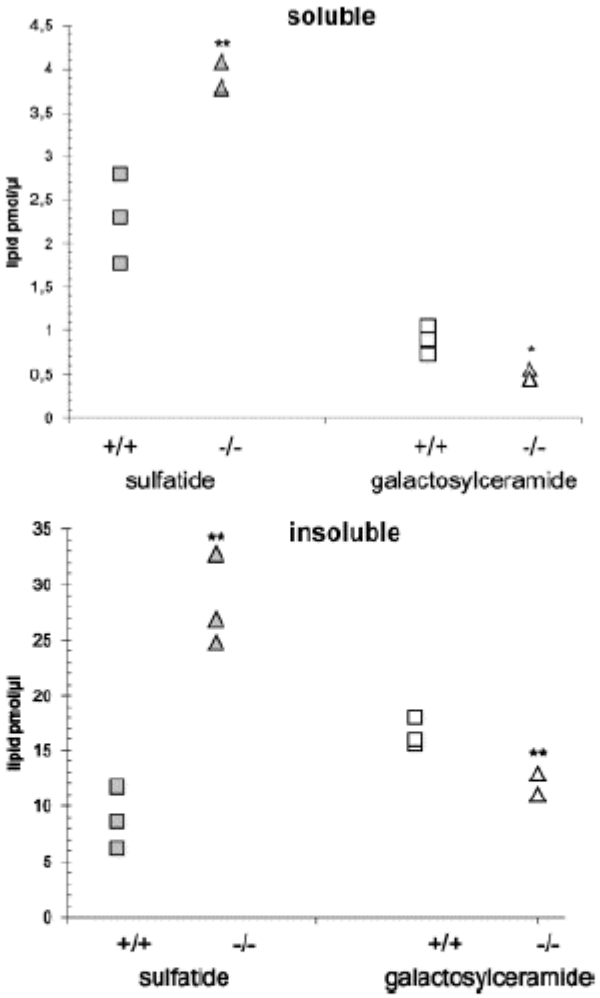


Fig A2: ESI-MS/MS analysis of galactosylceramide and sulfatide in detergent-soluble and detergent-insoluble membrane fractions. Myelin was prepared from the brains of 24-month-old normal and ASA KO mice and protein content was quantified. Subsequently, myelin containing the comparable amounts of protein was extracted with Triton X-100 at 4°C and subjected to sucrose density centrifugation. Twelve fractions per gradient were collected; for analysis, fractions 1 and 2 (containing detergent-soluble membrane material) and fractions 6 and 7 (containing detergent-insoluble membrane material) were pooled. Lipid concentrations are given for the pooled fractions. Three animals of each genotype were analysed. $*P < 0.05$ and $**P \leq 0.01$ ASA KO against WT by one-way ANOVA. As ESI-MS/MS facility is not available in the laboratory, samples were prepared and sent to Dr. R. Sandhoff (Deutsches Krebsforschungszentrum, Heidelberg) to perform this experiment.

9. CURRICULUM VITAE

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EDUCATIONAL QUALIFICATION

- **(Doctor of Philosophy)**, under the supervision of Prof. Dr. Volkmar Gieselmann, Institute for Physiological Chemistry, University of Bonn, GERMANY. Since *September 2001 till date.*
- **Masters in Pharmacy (Pharmaceutical Biotechnology)**, The Tamil Nadu Dr. M.G.R Medical University, Chennai, INDIA. *1995-1997 - with Distinction (76.67%)*
- **Bachelors in Pharmacy,** The Tamil Nadu Dr. M.G.R Medical University, Chennai, INDIA. *1991-1995 with First Class (66%)*
- **Higher Secondary school**, Thiru Kamaraj Municipal Higher Secondary School, Villupuram, INDIA, 1*987-1990*

- **High School,** Thiru Kamaraj Municipal Higher Secondary School, Villupuram, INDIA, *1982-1987*

- **Secondary and Primary School,** Sacred Heart Convent, Villupuram, INDIA, *1976-1982*

PROJECT PROFILES

- **Establishment and Characterization of a Spontaneously Immortalized Schwann Cell Culture to Study the Molecular Aspects of Metachromatic Leukodystrophy**
  Institute for Physiological Chemistry, Nussallee 11, 53115 Bonn, GERMANY, June 2003-Present
  Guide: Prof. Dr.V.Gieselmann

- **Specific downregulation and mistargeting of the lipid raft-associated protein MAL in a glycolipid storage disorder**
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  Guide: Prof.Dr.V.Gieselmann

- **Cytotoxicity And Antiviral Screening For Some Quinazolone Derivatives**
  J. S. S College of Pharmacy, Ooty, INDIA , September 1996 – August 1997
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PUBLICATIONS

- **K.Saravanan**, V.Gieselmann, H. Bussow and S.Franken, "Establishment and Characterization of a Spontaneously Immortalized Schwann Cell Culture to Study the Molecular Aspects of Metachromatic Leukodystrophy"- ***To be submitted***

- **K. Saravanan,** N. Schaeren-Wiemers, D. Klein, R. Sandhoff, A. Schwarz, A. Yaghootfam, V. Gieselmann and S. Franken, "Specific downregulation and mistargeting of the lipid raft-associated protein MAL in a glycolipid storage disorder", **Neurobiology of disease**, 2004 Jul;16(2):396-406

- Gieselmann V, Franken S, Klein D, Mansson JE, Sandhoff R, Lullmann Rauch R, Hartmann D, **Saravanan K**, De Deyn PP, D'Hooge R, Van Der Linden AM, Schaeren-Wiemers N, "Metachromatic leukodystrophy: consequences of sulphatide accumulation", **Acta Paediatrica. Supplement**. 2003 Dec; 92 (443):74-9; discussion 45

- Murugan V, Sahreef.H, Rama Sharma G.V.S, Ramanathan M, **Saravanan K** and Suresh.B, "Antiinflammatory and Antibacterial Activity of stem bark of Alangium Salviifolium(Linn.F) Wang". **Indian Drugs**, 38(1) January 2001.

PROFESSIONAL EXPERIENCE

- **J.S.S College of Pharmacy, Ooty, INDIA**
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- **J.S.S Centre for Advanced Drug Research and Testing (CADRAT), Ooty, INDIA**
  Developed protocols for sterility and microbial evaluation of various pharmaceutical formulations. Established a tissue culture laboratory and standardized protocols for *in-vitro* cytotoxicity and anti-viral screening of various metabolites. Screened various pharmaceutical formulations for microbial analysis. Maintained the sterility of the tissue culture facility and microbiological laboratory at standards above those recommended for bio safety level, *April 1999 – August 2001*

- **Christian Medical College and Hospital, Vellore, INDIA**
  Worked as a senior pharmacist / lecturer. Supervised the purchase, storage and distribution of drugs and surgicals for the hospital and its associated pharmacies. Supervised supply of raw materials, manufacturing and quality control in the in-house manufacturing facility. Lectured to Diploma in Pharmacy students on subjects related to clinical and hospital pharmacy, *January 1998 – March 1999*