

Pharmacological Approaches for Lysosomal Storage Diseases



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Abstract

Niemann–Pick disease Type C (NPC) is a neurodegenerative lysosomal storage disorder that arises from defects in lysosomal proteins involved in intracellular lipid trafficking. Cellular pathology leads to clinical neurological manifestations, including ataxia. The anti-vertigo drug N-Acetyl-DL-Leucine (ADLL) has been found to be beneficial for treating vestibular anomalies and cerebellar ataxia. ADLL has also been found to ameliorate cerebellar symptoms in 12 NPC patients treated with this drug in observational clinical studies. However, the mode of action of this drug requires further study. This project has investigated the effects of ADLL and other leucine amino acid derivatives in *in vitro* and *in vivo* models of NPC. We found that ADLL, and its individual enantiomers acetyl-l-leucine (ALL) and acetyl-d-leucine (ADL) improve symptoms of ataxia in a mouse model of NPC (*Npc1*^{-/-}). However, when ADLL and ALL were administered pre-symptomatically to *Npc1*^{-/-} mice they were neuroprotective and ADLL synergised with the standard of care drug, miglustat. Altered energy metabolism was implicated as the mechanism of action in *Npc1*^{-/-} mice. A neuroprotective effect of ADLL was also demonstrated in a mouse model of GM2 gangliosidosis (Sandhoff disease mouse) with differential effect on metabolism. We also explored the efficacy of natural, non-acetylated forms of leucine including DL-leucine, L-leucine, D-leucine and an ethyl ester form of l-leucine and found that N-acetylation of either L-leucine or D-leucine led to increased efficacy *in vivo* in *Npc1*^{-/-} mice. Interestingly, esterification provided neuroprotective effect to L-leucine, which was not seen with N-acetylation. NPC1 null CHO cells responded to all leucine isomers in terms of correction of mitochondrial volume and mitochondrial reactive oxygen species defects characteristic of NPC but also reduced lysosomal volume and lipid storage. Finally, we performed discovery proteomics to identify ALL interacting proteins in NPC1 cells and found 9 potential interactors that could represent new therapeutic targets.

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Abbreviations

AAV	Adeno-associated virus
ABC	Ammonium bicarbonate
ACN	Acetonitrile
AD	Alzheimer's disease
ADL	Acetyl-D-leucine
ADLL	Acetyl-DL-leucine
ADP	Adenosine diphosphate
AL	Acetyl-Leucine
ALL	Acetyl-L-leucine
AM013	Compound 13
AM014	Compound 14
AMP-DNM	N-(5-adamantane-1-yl-methoxy)- pentyl-1-deoxynojirimycin
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BCAA	Branched chain amino acids
BCKADHA	Branched chain keto acid dehydrogenase enzyme A subunit
BCAT1	Branched chain amino acid transferase 1
CCD22	D-Coiled-coil domain-containing protein 22
CerS6	Ceramide synthase enzyme 6
CERT	Ceramide transporter
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
DBA	Diamond-Blackfan anemia
DHB4	Peroxisomal multifunctional enzyme type 2
DL	D-leucine
DLL	DL-leucine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribo Nucleic Acid
DNMBP/Tuba	Dynamin Binding Protein
DTT	Dithiothreitol
ELP2	Isoform 2 of elongator complex protein
ER	Endoplasmic reticulum
Et-Leu	Ethyl leucinate
FA	Formic acid
FFA	Free Fatty Acid
Gba	Glucocerebrosidase
GDH	Glutamate dehydrogenase
GlcCerT	Ceramide glucosyl-transferase

GLUT	Glucose transporter
GM3S	GM3 synthase
GSLs	Glycosphingolipids
HDL	High-density lipoproteins
HEXA	Beta hexosaminidase a
HFD	High fat diet
HOMA-IR	Homeostatic model assessment insulin resistance
HSF1	Heat Shock Factor 1
HSP70	Heat shock protein 70
IAA	Iodoacetamide
IR	Insulin receptor
IRS	Insulin receptor substrates
ITPR3	Inositol 1,4,5-trisphosphate receptor type 3
Lamp2	Lysosome-associated membrane protein 2
LC	Liquid chromatography
LC3	Vacuole-associated phospho-lipidated protein
LDH	Lactate dehydrogenase
LDL	Low-density lipoproteins
LL	L-leucine
LRP1/ CD91	Lipoprotein receptor-related protein 1
LSD	Lysosomal Storage Diseases
MBP	Myelin basic protein
MPR	Mannose-6-phosphate receptor
MS	Mass Spectroscopy
mTOR	Mammalian target of rapamycin
NAAD	Nicotinic acid adenine dinucleotide
NAD	Nicotinamide adenine dinucleotide
NMDA	N- methyl-D-aspartate
NME7	Nucleoside diphosphate kinase 7
NPC	Niemann-Pick Type C
NPC1	Niemann-Pick Type C 1
NPC1	Niemann-Pick Type C 2
OXPHOS	Oxidative phosphorylation
P62/SQSTM1	Sequestosome-1
p85/PI3K	p85 subunit of phosphatidylinositol 3-kinase
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinases
PDP	Pyruvate dehydrogenase phosphatases
PGAM1	Phosphoglycerate mutase 1
PGC-1a	Proliferator-activated receptor-gamma coactivator 1-alpha

PI3-K	Phosphatidyl inositol 3-kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SERCA	Sarcoplasmic / endoplasmic reticulum calcium ATPase
SM	Sphingomyelin
SOD	Superoxide dismutase
Sph	Sphingosine
SphK	Sphingosine kinase
SREBP	Sterol regulatory element-binding protein
SSD	Sterol Sensing Domain
TCA	Tricarboxylic acid cycle
TG	Triacylglycerols/triglycerides
TNF	Tumor necrosis factor
TPC	Two-pore channels
VLDL	Very low-density lipoproteins

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Chapter 1: Introduction

1.1. Lysosomal Storage Diseases

Lysosomes are membrane-bound organelles containing acid hydrolases. They work as a “recycling centre” of the cell and degrade macromolecules derived from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways [1]. Lysosomes are also needed for cell growth, nutrient sensing, amino acid and ion homeostasis and calcium signalling [1]. Lysosomes also form contact sites with other organelles, namely mitochondria and the endoplasmic reticulum[2], [3].

Inherited effects in genes that encode proteins required for lysosomal function cause a group of rare diseases termed lysosomal storage diseases (LSD). These disorders are caused by mutations in genes encoding lysosomal enzymes, integral membrane proteins, transporters and enzyme modifiers or activators[2]. They are typically neurodegenerative and are primarily autosomal recessive conditions. Macromolecule substrates build up in these diseases (“storage”) and leads to secondary defects in cell function and ultimately cell death[2]. LDSs

affect 1: 5,000 live births and comprise 50 lysosomal enzyme deficiencies, 12 disorders of lysosome-related organelles and 14 disorders involving the production of lipofuscin [2].

1.2. Niemann-Pick Disease Type C Disease

1.2.1 Overview

Niemann-Pick disease type C (NPC) is a devastating rare genetic neurodegenerative lysosomal storage disease, which affects both visceral organs (e.g. liver) and the central nervous system (CNS) and results in premature death[4].

The incidence of the disease is minimally estimated to be 1/100,000 live births [4]. Although it causes abnormalities in liver, spleen and sometimes lung, progressive neurodegeneration is the major clinical hallmark of the disease. Early neurological symptoms include a delay in developmental motor function and clumsiness, followed by ataxia and progressive dementia [4]. Life expectancy of NPC disease patients can vary from a few days to over 60 years; however, the vast majority of patients die between 10 and 25 years from complications related to neurologic degeneration and lung failure [4].

NPC is caused by mutations either in the *NPC1* (approximately 95% cases) or *NPC2* genes, which are both lysosomal proteins, resulting in identical clinical phenotypes[5]. Although the exact functions of the NPC1 and NPC2 proteins remain unknown, studies have shown that mutations in both proteins interrupt intracellular cholesterol and sphingolipid trafficking. While the NPC1 gene maps

to chromosome 18q11-12, and encodes a 1278 amino acid protein, *NPC2* gene is located on chromosome 14q24.3[6], 151 amino acid glycoprotein [7].

NPC1 protein is 13-transmembrane protein localized to the limiting membrane of late endocytic organelles that are positive for Rab7 and lysosome-associated membrane protein (Lamp)-2 but negative for cation-independent mannose-6-phosphate receptor (MPR) [8], [9]. It contains three luminal loops, small cytoplasmic loops, and a C-terminal cytoplasmic tail [10]. The structure of *NPC1* includes a sterol sensing domain (SSD), which is large enough to accommodate a cholesterol molecule [10].

The *NPC2* protein is a small, soluble 151 amino-acid glycoprotein found in the lysosomal lumen that contains a mannose-6-phosphate receptor-binding domain [7]. It has been shown that *NPC2* can also bind free cholesterol with a 1:1 stoichiometry[11], is able to specifically and efficiently transport cholesterol from inner lysosomal vesicles, and this transport can be stimulated by low pH or low sphingomyelin [12].

The cellular pathophysiology of NPC disease includes normal uptake of LDL cholesterol and other lipids via endosomes and delivery to late endosomes and lysosomes. However, lipids become trapped in the lysosomes. Therefore, unesterified cholesterol, free fatty acids and multiple sphingolipids accumulate in late endosomes/lysosomes [13].

Although lipid storage occurs in potentially every cell in NPC disease, the types of lipids that accumulate differ between visceral and neural tissues. Cholesterol is the main storage product in fibroblasts, whereas glycosphingolipids (GSLs), especially GlcCer, LacCer, GM3, GM2 and GM1, are stored in the brain. These GSLs have important roles in various physiological

mechanisms, including neuron myelination, growth factor, signal transduction, cell adhesion, membrane trafficking, molecular sorting, neural electrical activity/ion channel activity and synaptic function[14]. Alterations in GSL levels/intracellular distribution may therefore disturb these functions.

When NPC1 is pharmacologically inhibited the first lipid to be stored is sphingosine. Sphingosine accumulation causes inhibition of Protein Kinase C (PKC), and this may be a contributing factor of the reduced activity in acid sphingomyelinase [15]. Sphingosine storage interferes with lysosomal calcium signalling by lowering luminal acidic store calcium levels. Calcium release from lysosomes via two pore channels (TPCs) is regulated by the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP). The calcium defect in NPC cells disrupts calcium dependent processes; such as vesicular fusion. As a result, impaired endocytic transport, and cholesterol and glycosphingolipid storage occur [16], [17] **(Figure 1.1)**. Secondary effects of NPC protein loss of function includes mitochondrial dysfunction; increase in mitochondrial cholesterol content, altered composition of the respiratory chain complex, substantial reduction in cellular ATP levels [18], increase in oxidative stress (with increases in reactive oxygen species (ROS)) and activated pro-apoptotic cascades[19]. CNS function strongly depends on efficient mitochondrial function, because the brain has a high-energy demand. Defects in mitochondrial dynamics, generation of ROS, protein aggregate-associated dysfunction and environmental factors can all alter energy metabolism and in many cases are associated with neuroinflammation and neurodegeneration [20], [21] **(Figure 1.1)**.

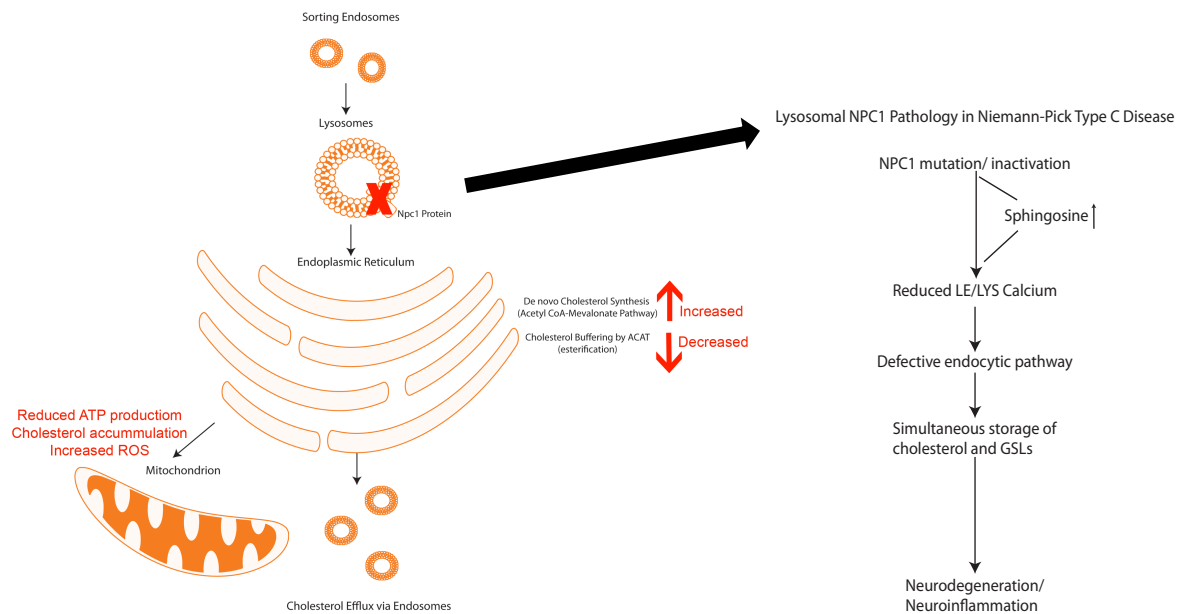


Figure 1.1. Schematic summarizing some key aspects of the cellular pathology in Niemann-Pick disease type C1, modified from [16].

The loss of function of NPC1 and/or NPC2 affects all organs in the body and causes systemic, neurologic and psychiatric abnormalities. Cholesterol accumulation affects the liver (foetal ascites or severe neonatal liver disease and/or lung failure), causes infantile hypotonia and developmental delay, vertical supranuclear gaze palsy, enlargement of the liver or spleen (beginning in childhood), progressive ataxia, dysarthria, dystonia, seizures and cataplexy, depression, dementia, or schizophrenia (beginning in adolescence or adulthood)[4], [5]. Death from inhalation pneumonia usually occurs in the late second or third decade[22].

Magnetic resonance imaging of the brain is usually normal until the late stages of the illness, which manifest atrophy of the superior and anterior cerebellar vermis, diminishing of the corpus callosum, and minor cerebral atrophy, as well as minor demyelination in the peritrial white matter. Quantitative magnetic resonance imaging studies in adults with NPC disease also

displayed grey and white matter anomalies, resulting in a reduction in total cerebellar volume, correlating to ataxia and ocular motor function deterioration [23] (**Summary Figure 1.2**).

NPC1 patient metabolic profiling showed abnormal plasma profiles, with significantly reduced levels of LDL-C (low density lipoprotein-cholesterol), HDL-C (high density lipoprotein-cholesterol) and increased total triacylglycerols (triglycerides-TGs) [24].

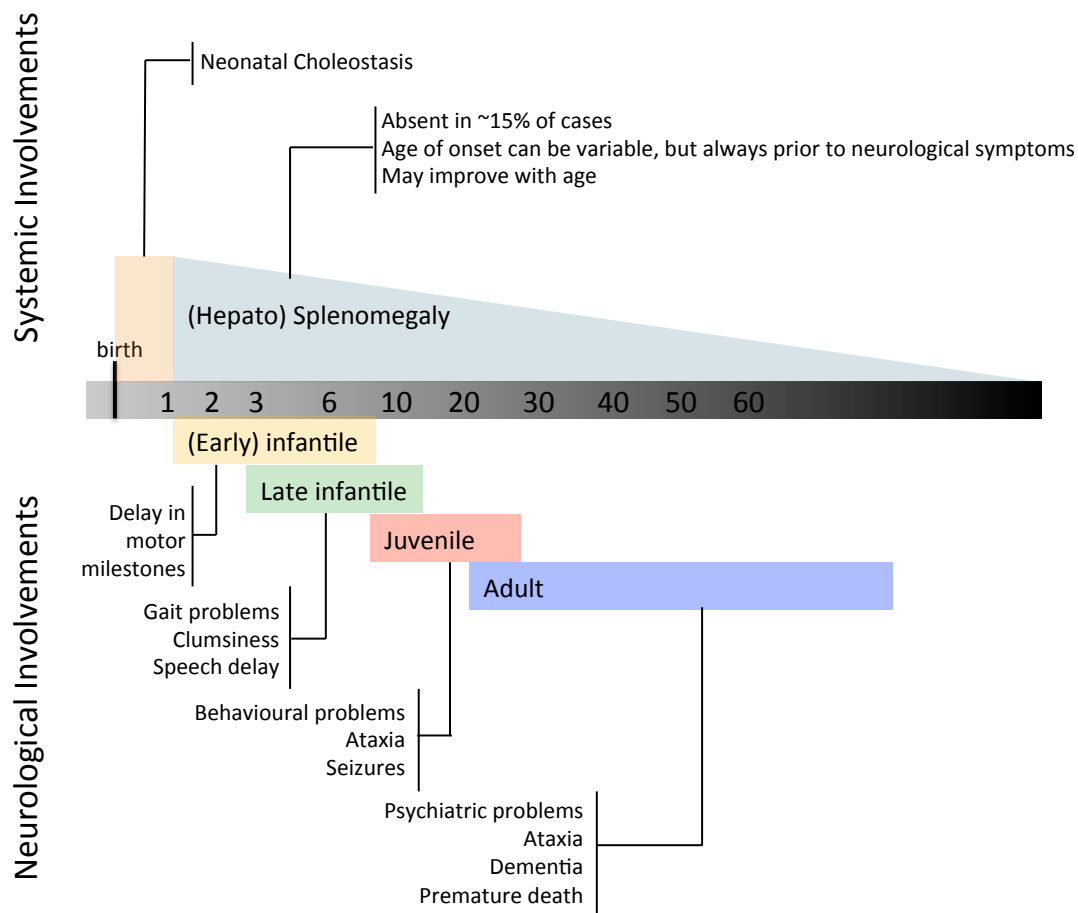


Figure 1.2. Summary of systemic and neurological disease onset and progression in Niemann-Pick disease type C. Figure obtained from Alexandria Colaco, modified from reference[4].

1.2.2 Animal models of NPC1 disease

Loss-of-function mutations in NPC1 orthologs *in vivo* have been described for *Drosophila*[25], yeast[26], zebrafish [27], dogs[28], cats[29], and mice. To date, five NPC1 mouse models have been identified with either spontaneous or engineered mutations and have been used to investigate the pathophysiology of this complex disorder. The mouse models are denoted as *Npc1*^{spm}[30], *Npc1*^{nih}[31], *Npc1*^{nmf164}[32], *Npc1*^{pf/pf} [33]*Npc1*^{tm^(I1061T)Dso}[34] and *NPC*^{imagine} [35]. Mice affected with the NPC1 mutation or deletion display similar phenotypes to humans, including cholesterol accumulation in cells, shortened lifespan, hepatomegaly, ataxia, Purkinje cell loss, neuroinflammation, and other signs of neurologic impairment. These models have led to a better understanding of NPC1 protein function. In this thesis, we used the BALBc/*Npc1*^{nih} mouse (BALB/cNctr-*Npc1*^{m1N}/J) and its wild type *Npc1*^{+/+} counterpart. This model is very well characterised as it was the first to be described in the 1980s, and displays neurological, visceral and cellular abnormalities similar to patients [31], [36]–[38] (**Figure 1.3**).

Metabolically, NPC1 protein loss of function results in poor growth and progressive weight loss in mice [39]. NPC mice display high plasma glucose levels, high total cholesterol levels in visceral organs, and larger and more heterogenous HDL particles when they are fed on a standard diet, these phenotypes are more pronounced when the diet contained high-cholesterol; conversely, TG levels are reduced in *Npc1*^{-/-} mouse serum [24], [40]. *Npc1*^{-/-} liver manifests with increased secretion of VLDL and a increased cholesterol ester/TG ratio compared with *Npc1*^{+/+} [41].

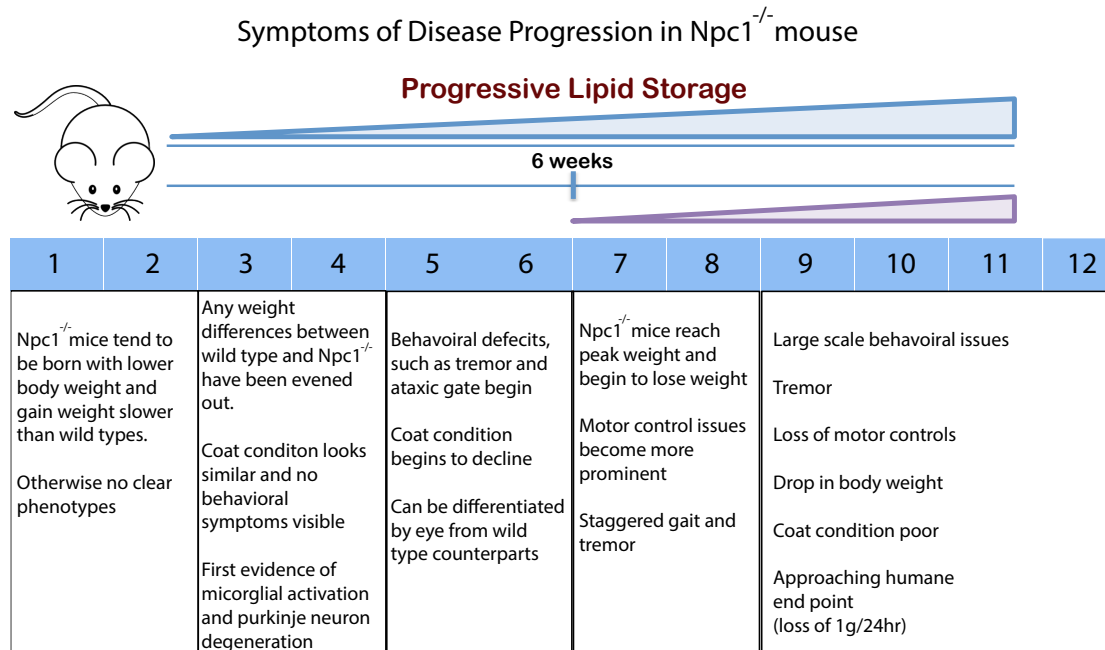


Figure 1.3. Symptoms of disease progression in the $Npc1^{nih}$ (BALB/cNctr- $Npc1^{m1N}$ /J) mice modified from [42].

1.2.3 Niemann-Pick Disease Type C as a metabolic syndrome

NPC1 and NPC2 proteins are crucial checkpoints of cell lipid metabolism. Therefore, NPC manifests a very wide spectrum of metabolic syndromes due to not only the dysfunction of these proteins, but also due to the resulting lipid storage.

1.2.3.1. NPC1 gene/protein

LDL lipoproteins are internalised with clathrin-coated vesicles, that later fuse with acidified early endosomes. The transport of LDL-derived cholesterol and fatty acids from late endosomes/ lysosomes other cellular compartments is mediated by the NPC1 protein[43]. Hepatocytes are central for lipid metabolism and take up cholesterol and TGs from the circulation via endocytosis of low-density lipoproteins (LDLs) and secrete cholesterol and TG in very-low density lipoproteins (VLDLs)[44]. When lipid levels reach high levels, lipid droplets

provide a cytoplasmic depot for TG and fatty acid esters derived from cholesteryl esters. It was shown in *Npc1*^{-/-} mice that lost NPC1 function reduces hepatocyte TG content and its secretion by increasing the metabolic flux of carbons into cholesterol biosynthesis; and also common genetic variation of NPC1 contributes to serum TG levels in humans [40]. Lipid metabolism is closely linked to glucose and general energy metabolism. Indeed, it is associated with two of the most common metabolic syndromes: obesity and diabetes. Insulin receptor (IR) functions via lipid rafts (cholesterol- and sphingolipid-rich membrane micro domains)[45] and its ligand insulin plays a central anabolic role in controlling both glucose and lipid metabolism [46].

Insulin binds to insulin receptor on the cell surface of cells (especially hepatic cells) and leads to autophosphorylation of the cytoplasmic domains (β -subunits) of the receptor[47]. Autophosphorylated insulin receptor activates insulin receptor substrates (IRS) (IRS-1, IRS-2, Src homology collagen (Shc), and an adaptor protein with a pleckstrin homology (PH) and Src homology (SH) 2) by intrinsic tyrosine phosphorylation. These phosphorylated docking proteins bind and activate several downstream components of the insulin signaling pathways. Activated IRS-1 associates with phosphatidyl inositol 3-kinase (PI3-K), which then activates Akt. IRS-2 is more linked to lipogenesis with its regulatory effect on lipogenic enzyme sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase; which emphasize the role of insulin's dual effect on shutting off the glucose synthesis and activating synthesis/catabolism of lipids [48]. After insulin triggers the downstream cascades, glucose transporter 4 (GLUT4), senses insulin signalling and initiates glucose transport into the cell [49] **(Figure 1.4)**.

Vainio *et al.* showed that in *Npc1*^{-/-} liver plasma membrane preparations contained high IR, however IR receptor activation was compromised, and cholesterol removal (with β -cyclodextrin) inhibited IR activation in control membranes but restored IR activation in NPC membranes [50]. A genome-wide association study done on 1,380 Europeans with early-onset and morbid adult obesity and 1,416 age-matched normal-weight controls revealed that a coding missense variant of *NPC1* gene is associated with early-onset and morbid adult obesity[51]. It was confirmed that the *NPC1* gene loss of function in the heterozygous state promotes weight gain by feeding *Npc1*^{nih} heterozygous mouse with a high fat diet (45% kcal fat)[52]. These animals displayed increased liver and inguinal adipose weights compared to *Npc1*^{+/+} mice [52]. This study was further confirmed in human *NPC1*^{+/-} carriers in 2017[52]. A very interesting follow-up study was performed to investigate the *Npc1* gene in relation to weight gain and metabolic features associated with insulin resistance. This involved interbreeding BALB/cJ Npc11/2 mice with wild-type C57BL/6J mice (the mouse strain commonly used to study aspects of diet-induced obesity and insulin resistance). This hybrid mouse model challenged with high fat diet manifested higher susceptibility to weight gain and insulin resistance, impaired fasting glucose, glucose intolerance, hyperinsulinemia, hyperleptinemia and dyslipidemia marked by an increased concentration of cholesterol and TG and LDL. This study highlighted that the *NPC1* gene–diet interaction can promote both weight gain and metabolic features associated with insulin resistance, putting the *NPC1* gene as a key player in maintaining energy and metabolic homeostasis[52].

Another metabolic study done in *Npc1^{nih}* heterozygotes mouse model revealed how the *NPC1* gene interacts with high fat diet (HFD) and effects the general energy metabolism pathways. Liver of HFD-fed *Npc1^{+/-}* mice displayed significantly higher levels of SREBP-1 and increased expression of SREBP-1 target genes that regulate glycolysis and lipogenesis, with an accumulation of triacylglycerol and cholesterol **(Figure 1.4)**. Consistently, cellular energy metabolism studies showed that *Npc1^{+/-}* fibroblasts had significantly increased glycolysis and lipogenesis (via flux of acetyl-CoA through the lipogenesis pathway to promote storage of neutral lipids), in addition to significantly decreased substrates of oxidative metabolism (glucose and endogenous fatty acids) with an accumulation of TG and cholesterol[54]. A study performed on adipocytes, which are an important cell population to study obesity and diabetes, illustrated that NPC1 activity is required for full Akt activation and NPC1 inhibition impaired GLUT4 trafficking [55].

Fat and glucose metabolism abnormalities display a very critical balance for the health of the central neural system and neuronal survival. At 9 weeks of age (symptomatic disease) the homozygotes *Npc1^{nih}* mouse brain presented with a dysfunction in insulin signalling, with reduced expression of insulin receptor substrate 2 (IRS2), and almost undetectable levels of insulin receptor substrate 1 (IRS1) expression. This abnormal insulin signalling was associated with loss of activation of regulatory p85 subunit of phosphatidylinositol 3-kinase (p85/PI3K) and highly reduced Akt phosphorylation **(Figure 1.4)** [56].

1.2.3.2. NPC2 gene/protein

About 5% of NPC cases are due to mutations in *NPC2*, a small soluble mannose-6-phosphorylated protein found in lysosomes and secretory fluids such as epididymal fluid and plasma[7]. It appears that the NPC2 protein provides cholesterol sensing/sorting and trafficking by binding and releasing cholesterol in a rapid way, working in concert with NPC1 [57]. For example NPC2 is crucial for keeping inner lysosomal membranes low in cholesterol, a prerequisite for efficient sphingolipid catabolism [58]. However, it is known that NPC2 can interact with other proteins and pathways not just NPC1; for instance, NPC2 is essential for cholesterol trafficking to mitochondria and therefore mitochondrial membrane structure and function [59]. Kennedy *et al.* showed that endogenous NPC2 depletion resulted in cholesterol accumulation in mitochondria and lactate accumulation, and this trend was not observed when mutant NPC2, which is unable to support endosomal cholesterol mobilization to mitochondria, was introduced to the cells [60]. Cholesterol accumulation in mitochondria led to altered energy metabolism and elevation of antioxidant response factor Nrf2 mRNA levels [60]. Interestingly, it was shown in a Korean cohort that the *NPC2* gene also has a strong obesity-related association [61]. Another study demonstrated that anti-obesity associated protein NEGR1 (its up regulation rescues obesity phenotype) might have an impact on human weight through its effect on cholesterol metabolism by its interaction with NPC2, acting as a membrane binding partner of NPC2 [62]. Furthermore, Guo *et al.* showed that NPC2 is an important regulator of mitophagy and autophagy (possibly via mTORC2 and Akt pathway-**Figure 1.4**), and can localise on mitochondria[63]. In addition, NPC2 knockdown adipocytes manifested decreased basal and maximal

respiration as well as reduced proton leak, increased basal levels of glucose uptake suggesting NPC2 could be a novel therapeutic target for adipocyte dysfunction in obesity and diabetes [63].

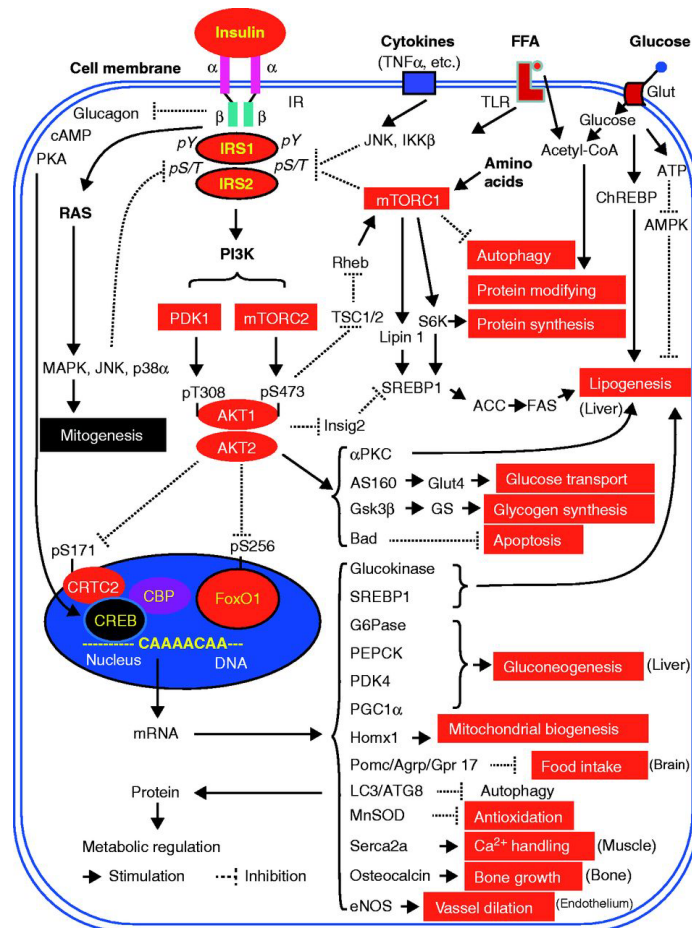


Figure 1.4. Cellular insulin metabolism figures modified from [64] .

1.2.3.3. Sphingolipids

All sphingolipids are stored in NPC disease resulting in impaired sphingolipid metabolism and signalling [65]. Sphingosine and its de novo precursor sphinganine (both elevated in NPC) impair insulin signalling[66]. Hamsters fed with either high fructose or high fructose and fat diets showed increased hepatic sphinganine and VLDL-TG levels and these effects were reduced with myriocin treatment (an inhibitor of *de novo* sphingolipid

biosynthesis), restoring insulin sensitivity and reducing hepatic SREBP-1c mRNA. However, myriocin treatment did not ameliorate the activation of hepatic inflammatory pathways [67]. Sphingosine kinase (SphK) is an important signalling enzyme that catalyses the phosphorylation of sphingosine (Sph) to form sphingosine-1-phosphate (S1P), which are involved in the progression of cancer, and the development of other inflammatory and metabolic diseases [68]. SphK and S1P are critical molecules involved in the regulation of cellular metabolic processes; such as cell proliferation, survival, apoptosis, adhesion and migration[68]. Previous studies showed that SphK activation improves hepatic insulin signalling in obesity and diabetes, with detected low SphK activity in the livers of mice fed a HFD [68]. SphK1 overexpression in the liver reduced hepatic TG synthesis and the total TG content in the HFD-fed mice; and SphK2 overexpression has been shown to elevate hepatic S1P expression and improve glucose/lipid metabolism in diabetic mice [68]. Finally, SphK/S1P-signalling activation ameliorates hepatic insulin resistance induced by endoplasmic reticulum stress [68].

Ceramide (the simplest sphingolipid) directly inhibits phosphorylation of the insulin-signalling mediator Akt/Protein Kinase B. More complex GSLs, block phosphorylation of the insulin receptor and down-stream signalling [69].

Ceramide is of great interest in diabetes and obesity research. During obesity, ectopic fatty that over-accumulate in non-adipose tissues (critical mediator in obesity-induced insulin resistance) are *de novo* synthesised to sphingolipid derivatives, namely ceramides [70]. Ceramides are structurally diverse in terms of chain lengths and are synthesised by a series of chain length selective ceramide synthases (CerS) [71], [72]. It was shown that certain

ceramide species and ceramide synthases are involved in insulin resistance and obesity. C16:0 mRNA expression increased in obese humans with an increase in ceramide synthase enzyme 6 (CerS6), and significantly correlated with the insulin resistance. CerS6 deficient mice showed a protection from high-fat-diet-induced obesity and glucose intolerance, increased lipid utilisation in brown adipose tissue and in liver [73]. HFD-fed mice displayed increased C18:0 ceramide content, associated with increased expression of CerS1. CerS1 knocked out in skeletal muscle exhibited improved insulin stimulated suppression of hepatic glucose production improving systemic glucose regulation. In contrast, muscle-specific deficiency of C16:0 ceramide-producing CerS5 and CerS6 failed to protect mice from obesity-induced insulin resistance. These studies reveal that ceramide and CerS have a direct function on the development of obesity-associated insulin resistance, in a tissue specific manner [74].

Ceramides synthesized in the endoplasmic reticulum (ER) are transported to the Golgi apparatus to be metabolized into complex sphingolipids. Ceramide transport occurs in two ways: vesicular (allows transport of ceramides from the ER to the cis Golgi network-no ATP required) and non-vesicular transport [73] [74]. Ceramides transported in vesicles are metabolized into glucosylceramides (GlcCer). Non-vesicular ceramide is transported from the ER to the trans Golgi network by the ceramide transporter (CERT) that functions in an ATP-dependent manner. This pool of non vesicular ceramide is metabolized into sphingomyelin (SM) [76]. Glucosylceramide synthase catalyzes the synthesis of GlcCer, the core structure of most GSLs [77], which are stored in NPC cells and organs including the brain. In cultured adipocytes, with tumor necrosis factor (TNF) α -induced insulin resistance, the depletion of GSLs (by an inhibitor of

glucosylceramide synthase, D-PDMP) resulted in nearly complete recovery from the resistance of insulin receptor signalling [78]. GSLs have been found to negatively correlate with insulin response in also leukemic cell lines [79].

When the GSL ganglioside GM3 is added to cultured adipocytes, it suppresses phosphorylation of IR and its down-stream substrate IRS-1, resulting in reduced glucose uptake [78]. *In vivo* studies in rodents proved the lack of GM3 ganglioside enhanced phosphorylation of the skeletal muscle IR and protected mice from HFD induced insulin resistance [69]. Additionally, insulin sensitivity and glucose tolerance were improved in mice with increased expression of the GM3-degrading sialidase Neu3 in the liver [69]. Sato *et. al.* reported that serum GM3 concentrations were higher (1.4- to 1.6-fold) in hyperlipidemic individuals and type II diabetics with and without hyperlipidemia compared to normal subjects [80]. Pharmacologically diminishing GM3 levels with the iminosugar drug N-(5-adamantane-1-yl-methoxy)- pentyl-1-deoxynojirimycin (AMP-DNM) strikingly improved glycaemic control in rodent models and restored insulin signalling in isolated *ex vivo* insulin-stimulated adipocytes. As a consequence of this improved function of fat tissue, less inflammation that was characterized by reduced numbers of adipose tissue macrophages (crown-like structures) was observed [81]. Embryonic fibroblasts obtained from GM3 synthase (GM3S)-deficient mice have increased insulin signalling compared to wild-type embryonic fibroblasts, which in turn led to enhanced adipogenesis. In addition, GM3S-deficient mice fed high-fat diet became obese but were resistant to the development of insulin resistance and chronic low-grade inflammatory states [82]. Serum GM3 species levels quantified in 125 Japanese residents identified GM3 (d18:1-h24:1) to be the best candidate for metabolic screening, proving to

be significantly correlated with atherosclerotic disease in humans, and homeostatic model assessment insulin resistance (HOMA-IR)[83].

1.2.3.4. Cholesterol

In early 1970s, it was discovered that excess body weight correlated with the daily production rate of cholesterol and slow cholesterol turnover in adipose tissue, but not necessarily in plasma[84], [85]. In the cell membrane, cholesterol is mainly found in a detergent-insoluble membrane sub-domain called lipid rafts, which are known to be associated with insulin signalling [45]. Type 2 diabetic patients' cholesterol metabolism differs from nondiabetic patients; with high cholesterol biosynthesis [86] and it was reduced by insulin[87]. Serum plant sterol levels (indicators of cholesterol absorption efficiency) were found to be low in type 2 diabetes [88]. Another study on obese and obese-diabetic patients showed no difference in serum total cholesterol but LDL and HDL cholesterol were significantly lower and VLDL cholesterol and serum total and VLDL, TG were higher in the diabetic-obese group. Cholesterol absorption efficiency dropped in diabetic subjects, and cholesterol biosynthesis, neutral sterol and bile acid excretion and cholesterol turnover tended to be higher [89]. Blood glucose was positively correlated with cholesterol biosynthesis in the diabetic group, suggesting that the higher the blood glucose level, the higher the rate of cholesterol biosynthesis. This highlights that diabetes (glucose metabolism) modulates cholesterol metabolism more than obesity alone [89]. However, deficiency of cholesterol also affects cellular metabolism. Reduction of cellular cholesterol with β -cyclodextrin altered insulin-stimulated glucose uptake, and

this effect was restored subsequent to cholesterol replenishment [90] as IR has been detected in membrane domains enriched in cholesterol and GSLs[69].

1.2.3.5. Nutrient Metabolism in NPC1 disease

The cells of the body strive to achieve balance between nutrient utilisation and metabolism, depending on nutrient availability. Barbara Karten and her colleagues, showed the accumulation of cholesterol in different organelles (lysosome and mitochondria) in NPC1 deficiency leads to alterations in mitochondrial function [60]. They showed that shifts in mitochondrial function affect glucose/energy and neurotransmitter metabolism, and leads to serious harms in central nervous system. For energy production, glucose should be converted to pyruvate, and pyruvate dehydrogenase (PDH) transforms pyruvate (obtained from glycolysis) to acetyl-coA. Acetyl CoA is then used in Tricarboxylic acid cycle (TCA), and yields cofactors for oxidative phosphorylation (OXPHOS) (produces 38 ATP) (**Figure 1.5**) [91]. If oxygen is not available for ATP production by OXPHOS, pyruvate is converted to lactate by lactate dehydrogenase (LDH), which yields 3 ATP [92]. Metabolic profiling of *Npc1*^{-/-} mice revealed markedly increased lactate production and decreased PDH activation (due to the increased pyruvate dehydrogenase kinase 1 and 2), and low acetate/acetyl-CoA levels in *Npc1*^{-/-} cerebellum and cerebral cortex at all ages, which indicate anaerobic glucose metabolism and respiration. They also showed an upregulation of glycolytic gene expression at the early symptomatic stage [93]. As the brain is highly sensitive to a decrease in PDH activity because of its strong reliance on the oxidative metabolism of glucose, increase in lactate and lactate dehydrogenase activation has been linked to several CNS diseases,

such as Alzheimer's disease and aging phenotype [92]. In addition, PDH deficiencies are known to manifest neurological symptoms such as ataxia and seizures, which also seen in NPC1 disease[94].

When glucose oxidation and utilization is insufficient, free fatty acids (FFA) could be converted to acetyl-CoA by β -oxidation and then further processed to ketone bodies, which act as an energy source. The other one-third of FFAs is then used by the TCA cycle to generate ATP to meet energy demands[95]. In NPC disease, peroxisomal β -oxidation of fatty acids and catalase activity in the brain and liver was significantly reduced[96]. In addition, amino acids and glutamine/glutamate neurotransmitters become valuable energy substrates when glucose is limited in the brain. Kennedy *et al.* showed increased expression of glutamine synthetase and branched chain amino acid transferase (BCAT1) in NPC1 mouse cerebellum, which could ultimately allow astrocytes to increase *de novo* glutamine synthesis to feed neurons with sufficient glutamine to both maintain the levels of neurotransmitter glutamate and oxidize glutamate in the Krebs' (TCA) cycle (via BCAT1)[97] **(Figure 1.5)**.

The insulin signalling, which is responsible for an uptake of glucose is closely related and regulated by glucose metabolism. It has been recently observed that insulin attenuated the phosphorylation (activation) in the PDH E1a subunit in normal rat hepatocyte [98].

The activity of PDH is regulated by acetyl-CoA and NADH, inhibitor pyruvate dehydrogenase kinases (PDK 1-2-3-4) and activator pyruvate dehydrogenase phosphatases (PDP 1-2)[99] **(Figure 1.5)**. Diet-induced obese mice treated with a liver-specific pan-PDK inhibitor 2-[(2,4-dihydroxyphenyl)]

sulfonyl]isoindoline-4,6-diol presented significantly improved glucose, insulin and pyruvate tolerance with lower plasma insulin levels and increased insulin signalling, and decreased lipogenic enzymes in liver. PDK inhibitor treated obese mice as well as untreated obese mice displayed predominant fatty acid utilisation over glucose oxidation. However, obese mice with increased PDH do not raise the plasma total ketone body level harmful ketoacidosis[100].

PDK4 is highly inducible by starvation and it is also elevated under insulin resistance and fatty acid elevation [101], [102]. The *Pdk4* gene expression can be suppressed by insulin under normal physiological conditions [103]. When *Pdk4* knockout mice challenged by a high-fat diet, they exhibited lower blood glucose levels and better glucose tolerance relative to the control wild-type mice[104]. On the other hand, *Pdk2*-null mice only manifested a moderate reduction in blood glucose under non-fasted conditions [17]. In another study, *Pdk2*-null mice were crossed with a mouse model lacking IRS1 and IRS2, and the hybrid model showed better insulin tolerance as compared to the *Pdk4* gene inactivation on the *Irs1/2* knockout genetic background [101]. Wild-type mice fed a HFD exhibited hepatic steatosis, insulin resistance, and increased levels of pyruvate, but reduced ketogenesis and PDH activity due to PDK2 induction. Hepatic PDH activation by PDK2 inhibition attenuated hepatic steatosis, improved hepatic insulin sensitivity, reduced hepatic glucose production, increased capacity for β oxidation and ketogenesis, and decreased the capacity for lipogenesis. These data shows PDK2 and PDK4 are tightly associated with obesity and diabetes.

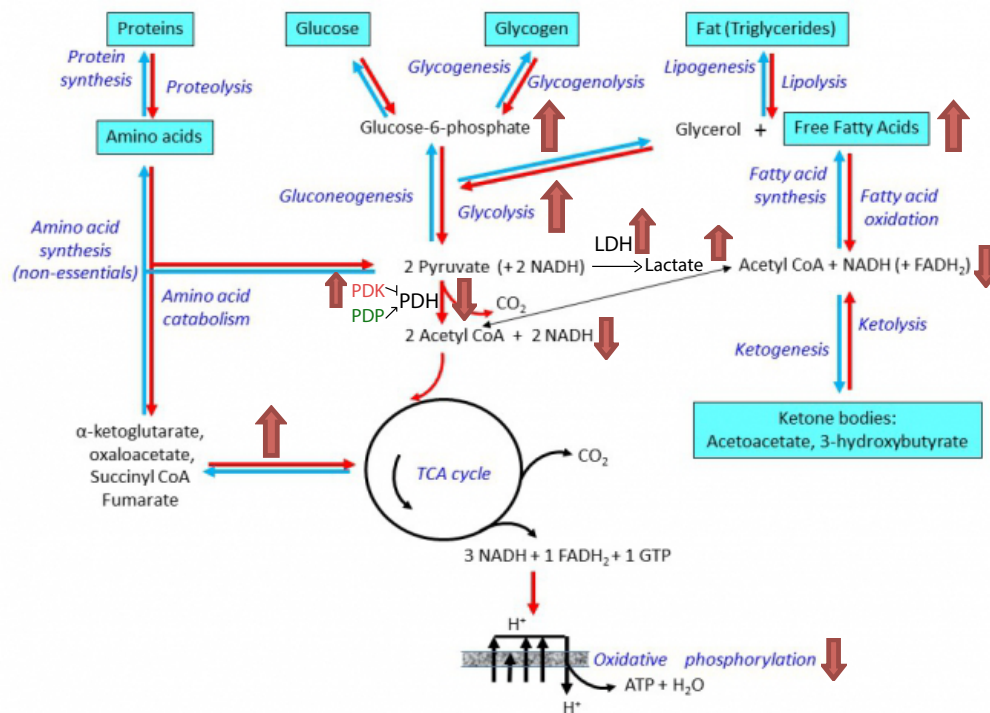


Figure 1.5. Cellular nutrient metabolism, with modifications from [67]. Red arrows indicate the NPC1 pathologies.

1.3. Relevant Neurodegenerative Metabolic Diseases

Aberrant cholesterol and lipid metabolism has been implicated in several neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) [105]. Another metabolic connection between NPC and other neurodegenerative diseases is altered glucose metabolism and glucose utilisation, which directly impacts neuronal survival, regulation of neuronal proliferation, apoptosis, synaptic transmission, neuronal degeneration and learning [60]. Defective glucose metabolism can trigger oxidative phosphorylation (OXPHOS) and mitochondrial nutrient metabolism (i.e. fat utilisation), which coincide with protein aggregation in the majority of common neurodegenerative diseases[106].

Patients suffering from AD and PD exhibit reduced expression of IR in the brain, resulting in insulin signalling impairment [107]. Brain-neuron insulin receptor knockout mice exhibited complete loss of insulin-mediated activation of PIP3K, neuronal apoptosis and markedly reduced phosphorylation of Akt. Interestingly, this led to substantially increased phosphorylation of the microtubule-associated protein Tau, a hallmark of neurodegenerative diseases including AD and NPC [108].

Alzheimer's disease is the most common cause of dementia (60–70% of cases) and shares many pathological features with NPC disease; including A β and tau tangles, mitochondrial dysfunction, oxidative stress/damage, proinflammatory responses and defective energy metabolism [109].

In AD, storage material amyloid precursor protein (APP) deposits are commonly observed in pancreatic islets of diabetic patients. On the other hand, individuals suffering from type 2 diabetes show an increased prevalence of dementia, which confirms the finding that the elevation in serum insulin levels (as observed in prediabetes and early type 2 diabetes) positively correlate with impaired cognitive function or amyloid protein levels [110]. In recent years, cholesterol was found to modulate synthesis of b-amyloid, and 3-hydroxy-3-methylglutaryl coenzyme A synthase inhibitors (which affects cholesterol biosynthesis) may offer a treatment option for AD[106].

Several transgenic mouse models overexpressing APP developed diabetes mellitus subsequent to amyloid deposits. Targeted reduction of APP leads to enhanced insulin secretion and improved glucose tolerance. Interestingly, disruption of insulin-degrading enzyme causes increased amyloid plaque formation, glucose intolerance along with hyperinsulinemia [106]. It has been

also shown that PDH levels and its activity are reduced in AD patients' brain[111].

Parkinson's disease results from the complex interaction between genetic and environmental factors and also manifests oxidative stress, mitochondrial dysfunction, neuroinflammation and excitotoxicity in the CNS. The production of protein aggregates (alpha-synuclein) can disrupt the function of mitochondria in PD and result in low ATP generation and high ROS levels[112].

Impaired glucose tolerance (up to 80% of the cohort[106]) and reduced PDH activity is also observed in PD[113]. While little is known about the pathogenesis of prediabetes in PD, therapy with levodopa found to exacerbate glucose intolerance [106].

Another pathological feature of NPC is ataxia as a result of Purkinje cell degeneration. Very interestingly, some ataxias have been found to have glucose metabolism abnormalities. Ataxia-telangiectasia [OMIM 208900] shows extreme insulin resistance [106]. Cerebellar ataxia, deafness, and narcolepsy (CADN) [OMIM 604121], Friedreich ataxia [OMIM 229300], Herrmann syndrome [OMIM 172500] (manifests with nephropathy, epilepsy, ataxia and deafness), spinocerebellar ataxia 3 [OMIM 109150] and 6 [OMIM 183086] show a high prevalence of diabetes mellitus[106]. These disorders are linked directly or indirectly to mitochondrial energy transfer or antioxidant system abnormalities[106]. Insulin secretion and glucose utilisation is dependent on energy needs, sugar, fat or amino acid availability, OXPHOS, glycolytic flux and mitochondrial capacity. Therefore it is very likely that any neurodegenerative disease that affects the energy metabolism would have a consequence on glucose metabolism, which neurons are highly dependent on.

1.4. Therapy

Pharmacological approaches for treating NPC are currently limited and are either symptomatic treatment, substrate reduction therapy to diminish lipid storage and promote cellular trafficking, or targeting secondary pathologic processes such as inflammation and oxidative stress[114].

Currently, the therapeutic goal is to understand pathogenesis in NPC to an extent that a combination of small molecule drugs can be devised, which slow disease progression and improve quality of life for patients. The first and currently the only drug approved for NPC was miglustat (world-wide approval except USA), which is small molecule inhibitor of GSL biosynthesis [114]. Miglustat is an N-acylated glucose mimetic imino sugar drug that reversibly inhibits the enzyme ceramide glucosyl-transferase (GlcCerT) [IC_{50} 20-40 μ M] (this enzyme catalyses the first step of GSL biosynthesis) [115]. Miglustat differentially inhibits the non-lysosomal β -glucosidase 2 (GBA2) [IC_{50} 0.31 μ M] and glucocerebrosidase (GBA) [IC_{50} 520 μ M][116]. Miglustat is already in use for the treatment of type 1 Gaucher disease since its approval in Europe (2002) and in the USA (2003). It was later given orphan drug designation for NPC in 2006 and approved by the EMA for the treatment of NPC in 2009. Miglustat treatment induces weight loss due to central appetite suppression [117] and in patients the major side effect is osmotic diarrhoea due to intestinal disaccharidase inhibition (sucrase/isomaltase)[118]. The galactose analogue of miglustat, lucerastat (currently in clinical trials for Fabry disease) [*N*-butyl-deoxygalactonojirimycin (NB-DG)], is also a ceramide glucosyl-transferase inhibitor, inhibiting GBA2 activity, but neither GBA, nor disaccharidases in the gastrointestinal tract [119].

Studies on the GM1 gangliosidosis mice showed that Lucerastat was better tolerated than miglustat and does not induce weight loss, while miglustat shows greater functional improvement in mice, possibly due to additional anti-inflammatory properties[120].

A second NPC disease modifying drug, 2-hydroxypropyl- β - cyclodextrin (currently in clinical trials in USA, intrathecal and IV trials) is proposed to act as a cholesterol-sequestering compound and bypasses NPC1 and NPC2 proteins to transport lipid. This treatment not only reduces levels of cholesterol accumulation, but also glycosphingolipids and free sphingoid bases [121]. A recent trial of intrathecal administration of the drug failed to demonstrate clinical efficacy[122].

Sarcoplasmic / endoplasmic reticulum calcium ATPase (SERCA) antagonist curcumin was tested in NPC models and observed to increase cytosolic calcium levels, therefore compensating for the reduced lysosomal calcium release in NPC disease [13], [17]. Another interesting treatment approach that has completed a Phase 3 trial (has most recently been designated Fast Track status by the FDA) is Arimocromol, an inducer of heat shock protein 70; which stabilises the interaction of Heat Shock Factor 1 [HSF1] with Heat Shock Elements leading to elevated production of HSP70. Arimocromol was found to reduce ataxia and extend the lifespan of the treated mice demonstrating the drug's neuroprotective ability [123].

A third compound valuated in clinical trials is the HDAC inhibitor vorinostat, an FDA-approved drug [124], was found to elevate mutant NPC1 levels in patient fibroblasts , reduce lysosomal cholesterol levels as well as

reduce sphingolipid cellular accumulation [125]. This drug was able to improve liver pathologies (with lipid level reduction)[126], however no lifespan increase obtained with this treatment on *Npc1^{nmf164}* mouse model[127], most probably due to its inability to cross the blood-brain barrier to have a significant drug accumulation in CNS tissues [127]. It failed to show efficacy in a clinical trial (Clinical trial no: NCT02124083).

In the last few years, gene therapy to deliver a wild type *NPC1* gene to NPC mouse models have been evaluated and look very promising. In 2017, the human *NPC1* gene was delivered for the first time, via adeno-associated virus serotype 9 (AAV9) vectors under the transcriptional control of the neuronal-specific (CamKII) or a ubiquitous (EF1a) promoter with retro-orbitally single injection to NPC mice, and showed greatly increased life span, improved motor function, neuroprotection and cholesterol storage reduction[128]. Another study, also using AAV9 gene delivery system confirmed the beneficial effect with single intracardiac injection, showing a significant robust NPC1 expression in various tissues, including brain, heart, and lung, along with increase in Purkinje cell survival, locomotor activity, coordination lifespan of mice[129]. Finally, in 2018, Hughes *et. al.* further proved the utility of gene therapy by showing intracerebroventricular delivery of the human *NPC1* gene using the synapsin I promotor and administered to neonatal mice was able to substantially extend lifespan, improve quality of life, prevent neurodegeneration, reduce biochemical pathology and normalize motor function[130].

1.5. Aims of the Following Chapters

In recent years, the modified amino acid Acetyl-Leucine has been evaluated as a symptomatic therapy for NPC [131], [132]. This leucine amino acid derivative has an excellent safety profile and is used as an antivertigo agent in France [133]. It significantly improved ataxia (possibly via its regulatory effect on membrane polarization[134]) tremor, dysmetria, gait in 12 NPC patients [131].

The branched chain amino acid leucine can cross the blood-brain barrier faster than other amino acids. It is one of ten essential amino acids that the human body is unable to synthesize and must therefore obtain from the diet, primarily in the form of protein[135]. Dietary amino acids (especially leucine) are able to modulate mitochondrial function, nutrient utilization and regulate abnormal glucose metabolism, and they are being trialed for obesity, diabetes and for aging diseases [136] as l-leucine is a known insulin secretagogue [137], [138]

Nozomi *et al.* showed that branched chain amino acids (including leucine) act as a neuroprotective agent for cerebellar neurons by giving Aminoleban (amino acid mixture) to patients who have spinocerebellar degeneration and measuring ataxia in their study[139].

Therefore we hypothesized that Acetyl-leucine could have benefit beyond symptomatic relief of ataxia and change cellular metabolism in NPC and other LSDs.

In **Chapter 2**, we

- Explore the effect of modified leucine amino acid Acetyl-DL-Leucine and its individual enantiomers in a *Npc1*^{-/-} mouse model
- Assess the effect of Acetyl-DL-Leucine in combination with miglustat
- Investigate acetyl-leucine isomers' (ALs) mechanism of action in the cerebellum; with a focus on nutrient sensing, glucose/energy metabolism, the antioxidant system and glutamate metabolism

In **Chapter 3**, we

- Assess Acetyl-DL-Leucine's effect on another metabolic neurodegenerative disorder, Sandhoff disease, and its impact on animal when combined with miglustat
- Characterise Sandhoff mouse cerebellar metabolism with emphasis on nutrient sensing, glucose/energy metabolism, the antioxidant system and glutamate metabolism
- Understand the effect of ADLL treatment on Sandhoff mice cerebellum on the same metabolic pathways

In **Chapter 4**, we

- Assess leucine amino acids ' (DL-Leucine, L-Leucine and D-Leucine and an ester form (L-Leucine ethyl ester) efficacy in NPC1 mouse model measuring behaviour and biochemical pathologies
- Test the effect of leucine treatment in an *in vitro* model (NPC1 deficient CHO cells)

Finally, in **Chapter 5**, we

- Assessed the effects of ALs in NPC1 CHO cells in terms of lysosomal and mitochondrial parameters.
- Explore Acetyl-L-Leucine's protein interactors using affinity approaches coupled with discovery proteomics in *Npc1*^{-/-} murine fibroblasts.

Chapter 2: Evaluation of Acetyl-Leucine treatment in NPC1 Mouse

2.1.Introduction

Acetyl-DL-leucine (ADLL) is a derivative of the branched chain amino acid leucine. It has been used since 1957 as an across the counter medication (Tanganil®) in France and former French colonies, for the treatment of acute vertigo and vertiginous symptoms. It is orally available with an excellent safety profile; however, its mechanism of action remains unclear[133]. Studies utilising a lesioned guinea pig model of acute vertigo demonstrated the ability of ADLL to restore the membrane potential of abnormally polarised neurons of medial vestibular nucleus[140]. In the same year, Bernard *et al* delivered ADLL via a parenteral route in macaques and found rapid distribution to the brain [141]. High uptake into cerebral tissues and cortical regions as well as nuclei, some of which are involved in reflex circuits required for maintaining balance, was

observed [141]. A clinical study of patients suffering from acoustic neurinoma or unilateral and intractable Ménière's disease (post-acute stage of recovery) who had undergone neurotomy and labyrinthectomy confirmed the benefit of ADLL relative to placebo[142].

ADLL has also been evaluated clinically in patients with monogenic forms of cerebellar ataxias, in which symptomatic improvement, including reduced gait variability[143], was observed[144]. A placebo-controlled trial involving a larger cohort of cerebellar ataxia patients is currently in progress[145]. However, a case series that tested an injectable form of the drug did not report significant correction, suggesting pharmacokinetic parameters may be important for achieving efficacy. More recently, significant improvements in ataxia and dysmetria were documented in 12 patients with Niemann-Pick disease type C (NPC) given ADLL orally[131]. Interestingly, improvements in cognition were anecdotally reported by the parents of some of the patients who responded to treatment, suggestive of functional benefit beyond the cerebellar system [131]. Finally, a case report in an elderly subject reported improved balance [146], suggesting ADLL may have the potential for therapeutic use in a broader range of diseases/conditions, that involve including both ataxia and defective balance. In all of the studies described ADLL was provided as a racemic mixture of acetyl D and L leucine isomers (ADL and ALL). A pre-clinical investigation implicated acetyl-L-leucine as the active isomer responsible for postural compensation after unilateral vestibular damage [147].

In this current chapter, we have investigated ADLL and its enantiomers' (hereafter referred to as AL). Firstly we explored whether the beneficial effects of ALs on ataxia in NPC patients, could be replicated in the *Npc1* deficient

mouse[133]. Then we initiated AL treatments in the pre-symptomatic phase of the disease and characterised the drugs' effect on behavioural parameters, biochemical pathologies and neuropathologies. Later, we explored the potential mechanistic targets of these three treatments. Currently, the only approved clinical treatment for NPC (in Europe and worldwide, but not USA) is the substrate reduction therapy drug, miglustat, which slows disease progression by virtue of its inhibition of glycosphingolipid (GSL) biosynthesis [115]. We therefore combined the lead compound ADLL with miglustat to assess the potential synergistic effect.

2.2. Materials and Methods

2.2.1. Mice

BALBc/NPCⁿⁱ [148] mice (*Npc1*^{-/-}) and (*Npc1*^{+/+}) were used. Mice were bred as heterozygotes to generate affected mice and control genotypes, and were housed under non-sterile conditions, with food and water available *ad libitum*. All experiments were conducted using protocols approved by the UK Home Office Animal Scientific Procedures Act, 1986.

2.2.2. Drug treatments

ADLL and enantiomers (ADLL: Molekula #73891210, ALL: Sigma Aldrich #441511, ADL: Sigma #A0876) were administered as dry admix in powdered mouse diet (PicoLab Rodent Diet 20/5053, LabDiet) at a dose of 0.1g/kg/day. Treatment groups for *in vivo* study consisted of at least 4 mice per group.

2.2.3. Mouse behavioural analysis

Weight and activity of each mouse were recorded weekly until reaching the humane endpoint (defined as a loss of 1g body weight within 24h). Gait analysis was performed using CatWalk 10.5 system (Noldus) according to the manufacturer's instructions and five runs were recorded per animal at each time point. The camera was set to 40cm below the walkway, the walkway was approximately 4cm wide, and detection settings set to camera gain 14.05 and green intensity 0.12. Motor function was measured using an accelerating NG Rota Rod for mice (Ugo Basile), starting from 1rpm to 10 rpm, accelerating every 30 seconds by one rpm.

2.3.4. Tissue processing and analysis

2.3.4.1. Biochemical and immunohistochemical analyses

Mice were saline perfused all cases. Tissues for biochemical analyses were perfused were then snap-frozen in cold isopentane. For immunofluorescent staining, tissues were perfused with 4% PFA followed by PBS and kept in 4% PFA solution for 24h and stored in PBS containing 30% sucrose. Brains were cut parasagittally (20 micron sections) and kept in glycerol/ethylene glycol storage solution (freezing media) and stored in -20°C. Biochemical analyses were performed on water-homogenised tissues (mg/ml) and protein content was determined using a BCA protein assay (Thermo Fisher, #23227) according to the manufacturer's instructions.

2.3.4.2. Western blot analysis

Western blot analysis was carried out by homogenising half cerebellums (ranging from 25 to 35 mg) in sufficient RIPA buffer (Pierce RIPA Buffer, Thermo Fischer Scientific-89900) with protease/phosphatase inhibitor cocktail (Halt™ Protease and Phosphatase Inhibitor Cocktail-100X, Cat No: 78440) to gain 50mg/ml w/v. Samples then incubated for 30 minutes on ice. They were centrifuged at 13,000 g at 4°C and supernatants were retained for further BCA protein assay and western blotting.

2.3.4.3. ADP and ATP extraction

ADP and ATP were extracted with Phenol-TE from tissues according to published method[149]. Briefly, freshly prepared tissues were homogenized with 3.0 mL of ice-cold phenol-TE (Sigma-77607) and processed with three cycles of 30-s homogenization and 30-s cooling. One mL of homogenate was

transferred into tubes containing 200uL of chloroform and 150uL of de-ionized water. The homogenate was thoroughly shaken for 20s and centrifuged at $10,000 \times g$ for 5 min at 4°C (standardise all temps to this format). The supernatant was diluted 1000-fold with deionized water, the diluted extract was used for ADP/ATP analyses.

2.3.4.4. NAD and NADH extraction

NAD and NADH extraction was performed with 20mg fresh tissues, PBS washed on wet ice. Tissues were homogenised with a Dounce homogeniser with 400uL of NADH/NAD extraction buffer (Abcam NAD/NADH Assay kit-ab65348), followed by centrifugation for 5 minutes at 4°C at 13.000g. Supernatants were collected into a new tube and filtered through 10kD Spin column (Abcam, #ab93349) at 10,000 g at 4°C until the majority of the liquid had passed through (approximately 20 minutes).

2.2.5.Sphingosine and Sphinganine Measurements

Sphingoid bases were extracted from 100uL tissue homogenates in 500uL of chloroform:methanol (1:2) followed by sonication for 10 minutes at room temperature. Subsequently, 1M 500-uL sodium chloride, 500 uL chloroform, and 3M 100uL sodium hydroxide were added to the samples, and vortexed every five minutes for 15 minutes at room temperature. Homogenates were then centrifuged at 13,000 g for 10 minutes and the lower organic phase retained. Sphingoid bases were purified from the samples by pre-equilibrating SPA-NH₂ columns with 2x1mL chloroform followed by sample elution with 3x300uL acetone. The samples were dried down under nitrogen. Lipids were resuspended in 50uL pre-warmed ethanol (37°C), and 50 uL OPA labelling solution (1mg

OPA/20 uL Ethanol/1uL 2-mercaptoethanol; dilution 1:2000 in 3% boric acid pH 10.5) was added. Samples were kept at room temperature in the dark for 20 minutes, vortexing every 10 minutes. Samples were buffered with 100uL methanol: 5mM Tris pH 7 (9:1) and centrifuged at 5,000 g for 2 minutes. Supernatants (150uL) were loaded onto a normal phase HPLC. Separation was carried out at 320nm emission, 455nm excitation. Solvent A was 100% methanol, C was 100% acetonitrile and D was 80% mQ water 20% acetonitrile mixture.

2.2.6. Glycosphingolipid measurements

Glycosphingolipids were extracted from tissue homogenates in chloroform/methanol (C:M) (1:2 v/v) overnight at 4°C. The mixture was centrifuged (3000rpm/10min) and 1mL chloroform and 1mL PBS were added to the supernatant and centrifuged (3000rpm/10min). The lower phase was dried under N₂, resuspended in 50uL C:M 1:3 v/v and combined with the upper phase. Subsequently, GSLs were recovered using C18 Isolute columns (100mg) (Biotage) pre-equilibrated with 4x1mL MeOH and 2x1mL H₂O. Samples were washed 3x1mL H₂O and eluted with 1mL C:M 98:2, 2x1mL C:M 1:3, 1mL MeOH. The column eluate was dried under N₂, resuspended in 100uL C:M 2:1, dried again under N₂ and resuspended in ceramide glycanase (CGase) buffer (50mM sodium acetate pH 5.5, 1mg/mL sodium taurodeoxycholate). 50mU CGase was added, and samples incubated at 37°C overnight. Released oligosaccharides were anthranilic acid (2-AA) labelled and purified by mixing with 1mL Acetonitrile:H₂O 97:3 and added to Discovery DPA-6S columns (SUPELCO, # 52625-U) pre-equilibrated with 1mL acetonitrile, 2x1mL H₂O, and 2x1mL acetonitrile. Columns were washed with 2x1mL Acetonitrile:H₂O 95:5 v/v, and

eluted in 2x 0.75mL H₂O. Samples were loaded 30:70 H₂O: MeCN (v/v) for normal phase HPLC. Solvent A was acetonitrile, solvent B was mQ water, and solvent C was 100mM NH₄OH (pH 3.85) in mQ H₂O. Separation was carried out at 30°C using Waters Alliance 2695 separation module, excitation 360, emission 425 using Waters 2475 Fluorescence detector.

2.2.7.Cholesterol measurements

Cholesterol was measured with the Amplex Red kit (Molecular Probes) according to the manufacturer's instructions. Briefly, cell and tissue homogenates in 100uL mQ water were Folch extracted [150] and dried down under nitrogen. The pellets containing cholesterol were resuspended in 1X Reaction Buffer, and 50 uL was loaded for an each sample in an F bottom 96-well plate. The reaction was initiated by adding Working Solution per sample (25% Amplex® Red, 2U/mL HRP stock solution, 2 U/mL cholesterol oxidase stock solution, 0.2 U/mL cholesterol esterase stock solution in 1X Reaction Buffer). Samples were incubated at 37°C for 30 minutes and the fluorescence was measured in a microplate reader (Optima, BMG Labtech) using excitation in the range of 530–560 nm and emission detection at ~590 nm.

2.2.8.Fluorescent Staining

Brain sections in “freezing media” were rinsed 3 times with PBS and blocked with 2% goat serum in 0.3% PBS Triton at room temperature. Sections were stained with primary antibodies; monoclonal rabbit Calbindin (1:2000) (Swant, #CB38a) and monoclonal rat CD68 (1:500) (Biorad, #MCA1957), in 2% goat serum in 0.3% PBS for 16 hours at 4°C, followed by washing with 0.3% PBS Triton, then 3 times with PBS. Secondary antibody stainings Alexa Fluor 647

Goat anti rabbit (abcam, #150080) and Alexa Fluor 488 Goat anti rat (abcam, #150157) were conducted at room temperature for 2 hours. Samples were washed once with 0.3% PBS Triton, 3 times with PBS and manually mounted on slides. Slides left on the bench overnight to dry the sections followed by application of ProLong Gold (Invitrogen, #P36930).

2.2.9. Western Blotting

Cerebellum homogenates (50mg/ml) were stored with 1% Igepal CA, 0.5% sodium deoxycholate, 0.1% SDS, and 1% protease-phosphatase inhibitor cocktail. Samples were resolved using 4-12% SDS Tris-Glycine (Invitrogen, #WXP01026BOX) gel electrophoresis and transferred to low fluorescent PVDF membrane (Biorad Immunblot Low Fluorescence PVDF paper-#1620262) using a semidry transfer apparatus (Trans-Blot Turbo Transfer System (Biorad #1704150)). The antibodies used are summarised in **Table 2.1**.

Abv/acronym	Antibody type	Host	Source	Catalogue code	Dilution
LC3B	Polyclonal	Rabbit	abcam	ab51520	1:2000
P62	Mab	Mouse	abcam	ab56416	1:2000
MBP	Mab	Mouse	Biolegend	836504	1:2000
GDH	Mab	Rabbit	CellSignalling	D9F7P	1:1000
pMTOR (ser 2448)	Polyclonal	Rabbit	CellSignalling	2971	1:500
mTOR	Polyclonal	Rabbit	CellSignalling	2972	1:500
PDH Complex	Polyclonal	Mouse	abcam	ab110416	1:1000
pPDH (s293)	Polyclonal	Rabbit	abcam	ab92696	1:500
SOD1	Polyclonal	Rabbit	abcam	ab13498	1:1500
SOD2	Polyclonal	Rabbit	abcam	ab56416	1:1500
PDP1	Polyclonal	Rabbit	abcam	ab228578	1:1000
PDK1	Mab	Mouse	abcam	ab110025	1:1000
PDK2	Mab	Rabbit	abcam	ab68164	1:1000
PDK4	Mab	Rabbit	abcam	ab214938	1:1000
BCKADH-A	Polyclonal	Rabbit	abcam	ab90691	1:1000
pBCKADH-A	Polyclonal	Rabbit	abcam	ab200577	1:1000
LDHB	Mab	Mouse	abcam	ab85319	1:1500
PGC-1 alpha	Mab	Mouse	Merck Millipore	ST1202	1:1500

Anti-Mouse 800CW IgG (H + L)	Secondary Ab	Goat	Licor-IRDye	925-32210	1:10,000
Anti-Rabbit 680RD IgG (H + L)	Secondary Ab	Goat	Licor-IRDye	925-68071	1:10,000
HRP conjugated beta actin	Mab	Mouse	Invitrogen	MA5-15739- HRP	1:15,000
Pierce ECL Substrate Kit	-	-	Thermo Fisher	32106	-

Table 2.1. Summary of the antibodies used for western blotting.

2.2.10. Image Acquisition and Quantification

Brain section imaging was performed with a Leica-SP8 confocal microscope in tile imaging mode. Western blot data acquisition was conducted with Licor Odyssey Infrared Imaging system model no 9120. Mean fluorescence values, cerebellum diameters and areas, cell quantifications were calculated with Fuji Version 1.0 software.

2.2.11. ADP/ATP measurements

ADP/ATP measurement kit was purchased from Sigma Aldrich (MAK135) and conducted according to the manufacturer's instructions. 10ul of Phenol-TE extracted samples were mixed with 90 uL ATP reagent in F bottom black 96 well plates, briefly mixed and luminescence values were immediately measured using plate reader (background). Mix was incubated for 1 minute and luminescence was measured for ATP (RLU-A). The plate was incubated for an additional 10 minutes and read for luminescence (RLU-B). Then the ADP reagent was added and samples were briefly mixed and incubated for 1 minute, and luminescence read (RLU-C). The ADP/ATP ratio was calculated with following equation: $(RLU-C - RLU-B) / RLU-A$.

2.2.12.NAD, NADH measurements

NAD, NADH and total NAD (NADt) ($\text{NAD} + \text{NADH} = \text{NADt}$) were measured with the NAD/NADH assay kit (Abcam-#ab65348). Fresh samples were processed for ADP ATP extraction. Each sample was divided into 2 groups: 1) Normal samples 2) Decomposed samples. Decomposition of each sample was achieved by heating samples at 60°C for 30 minutes on a heating block. 30uL extracts were loaded into F bottom clear 96 well plates, each sample comprising 3 wells: 1) Background 2) NADt 3) Decomposed samples (contains only NADH). Background reaction mix (60uL) was loaded into “background wells”, and Reaction Mix was loaded into “NADt” and “Decomposed sample” wells. Plate was incubated at room temperature for 5 minutes, and 6 uL NADH developer solution was added to the “Decomposed sample” wells. Absorbance readings were taken at 30 mins-1h-1.5h-2h-3h at 450 nm.

2.2.13.Statistical Analyses

Differences between groups were identified either by 1-way or 2-way analyses of variances (ANOVA); comparisons of groups were made after 2-way ANOVAs with Fisher’s LSD test. Statistical tests were performed using GraphPad Prism 6 software (La Jolla, CA).

2.3.Results

2.3.1. AL treatments initiated during the symptomatic phase of disease correct ataxia in *Npc1*^{-/-} mice.

The BALBc/*Npc1*^{nih} [148] mouse strain (hereafter referred to as *Npc1*^{-/-}) is devoid of functional Npc1 protein and authentically replicates the main pathologies of the clinical disorder[151]. Mice have a 10-12-week life span, with onset of pathological symptoms, that include progressive ataxia and motor deterioration, tremor and weight loss beginning at 6-7 weeks of age [151]. ALs were administered to *Npc1*^{-/-} mice in the diet to achieve approximately the same dose (0.1/kg/day in mice) as used previously in clinical studies [131]. Untreated 9-week-old *Npc1*^{-/-} mice exhibited ataxia that presents as a pronounced sigmoidal gait relative to wild-type mice, whereas 9-week-old *Npc1*^{-/-} mice treated with ALs from 8-weeks of age displayed significantly reduced ataxia as indicated by improvement in gait indistinguishable from those of wild-type mice ($p<0.0001$ for all treatments compared to *Npc1*^{-/-} untreated group) (**Figure 2.1a, b**).

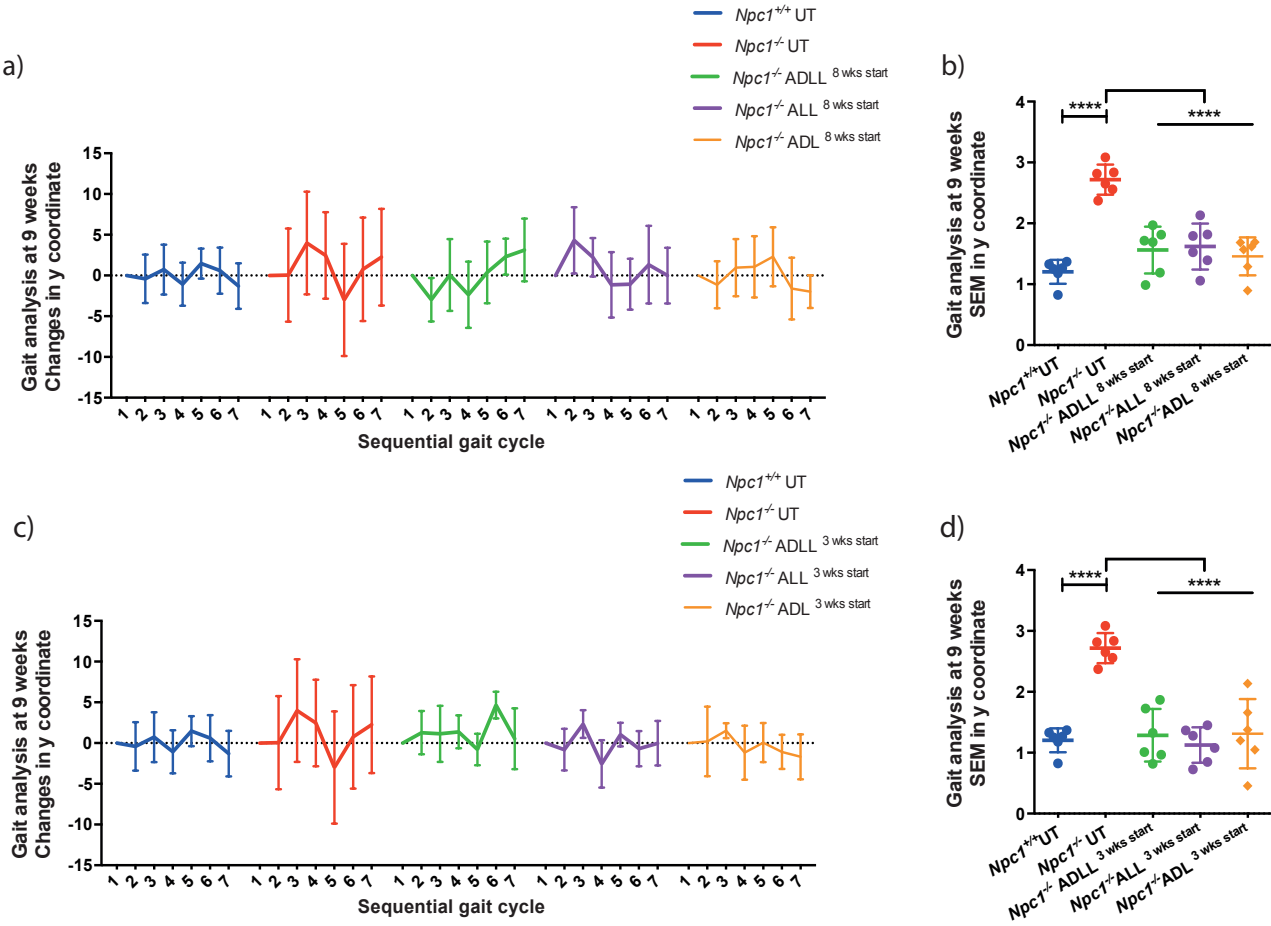
2.3.2. AL treatments initiated during the pre-symptomatic phase of disease delay disease progression.

We investigated whether any additional benefit was conferred by AL isomers if animals were treated before symptom onset. *Npc1*^{-/-} mice were therefore treated pre-symptomatically from weaning (3 weeks of age) and assessed at 9 weeks of age (6 weeks of treatment). All three AL isomers tested significantly reduced the magnitude of ataxia (**Figure 2.1 c and d**) ($p<0.0001$ for all treatments) with the magnitude comparable to the effects observed with one week of treatment (**Figure 2.1 a and b**).

Automated gait analysis revealed that untreated *Npc1*^{-/-} mice had increased hind paw duty cycle ($p=0.0031$)[123] (percentage of hind paws in contact with the platform relative to the elapsed time between two consecutive contacts of the same paw) and also reduced hind paw stand mean (average duration of paw in contact with the platform) ($p=0.0026$) relative to wild-type mice (**Figure 2.1 e, f**) [123]. ADLL and ALL treatment of *Npc1*^{-/-} mice resulted in a significantly reduced (improved) duty cycle of hind paws (ADLL: $p=0.0005$, ALL: $p<0.0001$) (**Figure 2.1 e**) and hind paw stand mean (ADLL: $p=0.0179$, ALL: $p=0.0014$) relative to untreated mice (**Figure 2.1 f**). Rotarod performance (forced task for evaluating motor strength, function and coordination[152]) was significantly impaired in the untreated *Npc1*^{-/-} mice relative to wild-type littermates ($p<0.0001$). Treatment with ADLL ($p=0.0088$) and ALL ($p=0.0070$) was associated with a significant improvement in rotarod performance by the *Npc1*^{-/-} mice (**Figure 2.1 g**). ADL treatment had no significant benefit on *Npc1*^{-/-} mouse motor function, as assessed using Catwalk ($p<0.95$) or rotarod ($p=0.2218$) (**Figure 2.1 e-g**), suggesting the D enantiomer has no benefit on behavioral parameters.

In comparison to untreated *Npc1*^{-/-} mice, the life span of animals was increased by 8 days (median, 9.1%) with ALL treatment ($p=0.0334$), 4 days with ADLL ($p=0.0305$) (median, 4.5%) and was not changed with ADL ($p=0.6908$) (**Figure 2.1 h**).

Symptomatic treatment with ALs



Pre-Symptomatic treatment with ALs

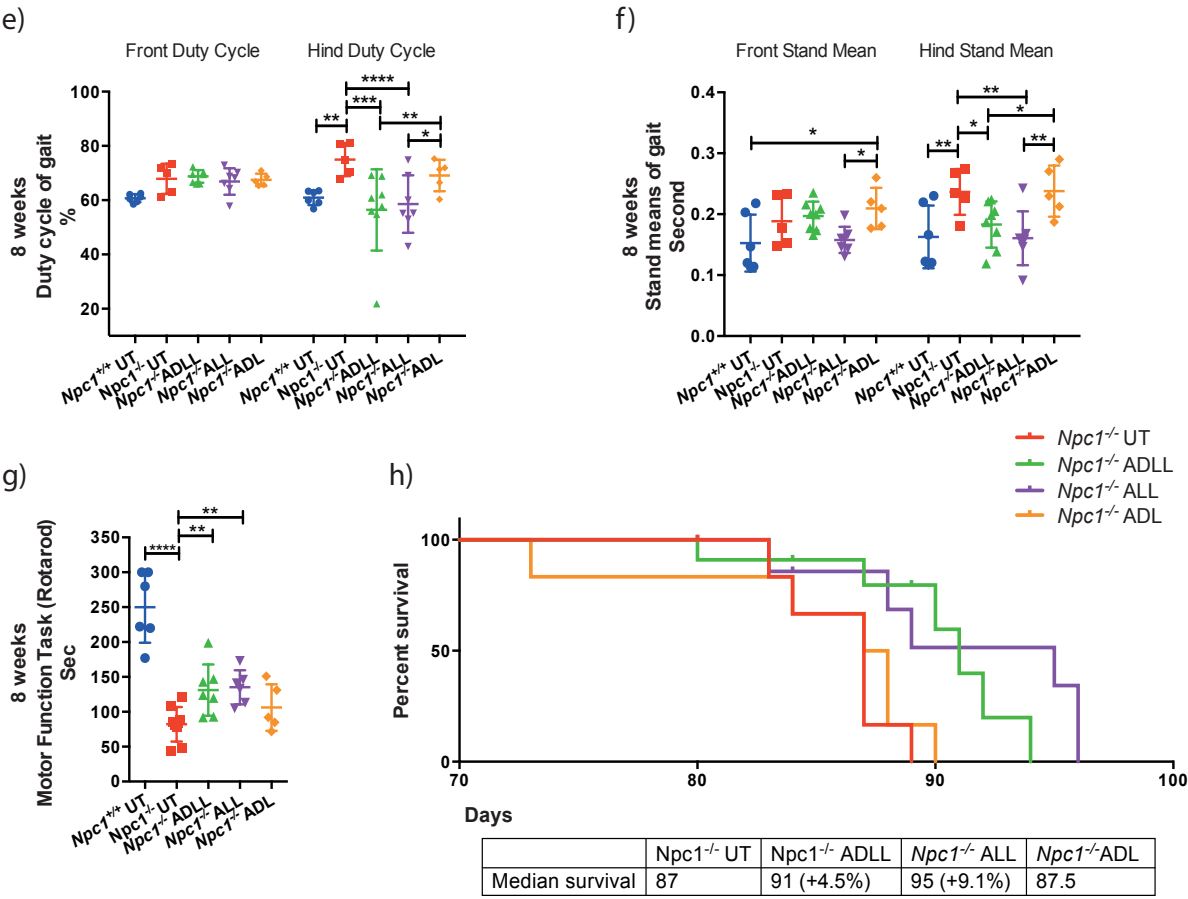


Figure 2.1. Acetyl-leucine isomers' impact on behavioural parameters in NPC1 mice. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), ADLL (*Npc1*^{-/-} ADLL), ALL (*Npc1*^{-/-} ALL), ADL (*Npc1*^{-/-} ADL) treatments min 5 animals, max 8 for each group. **a)** Late stage AL treatment Y coordinates displacement of each consecutive foot from a straight-line trajectory. (Mean $\pm$ SD, n=6) **b)** Late stage AL treatment SEMs of Y coordinate changes (Mean $\pm$ SD, n=6; $p < 0.0001$, One-way ANOVA). **c)** Early stage AL treatment Y coordinates displacement of each consecutive foot from a straight-line trajectory (Mean $\pm$ SD, n=6) **d)** Early stage AL treatment SEMs of Y coordinate changes (Mean $\pm$ SD, n=6; $p < 0.0001$, One-way ANOVA). **e)** Early stage AL treatments Duty Cycle. Front and hind duty cycle measurements, mean $\pm$ SD, $**p < 0.032$, $***p < 0.0005$, $****p < 0.0001$ (2-way ANOVA) **f)** Early stage AL treatment front and hind stand mean measurements, min 5 animals per group, Mean $\pm$ SD, $*p < 0.026$, $**p < 0.003$ (2-way ANOVA). **g)** Early stage AL treatment motor performance measurements, min 5 animals each group, Mean $\pm$ SD, $**p < 0.009$, $****p < 0.0001$ (One-way ANOVA). **h)** Life expectancy percentages, Mean $\pm$ SD, $*p < 0.046$, Median (Gehan-Breslow-Wilcoxon test) n=6.

2.3.3.Pre-symptomatic treatments with ADLL and individual enantiomers have differential effects on lipid storage in *Npc1*^{-/-}.

In light of the unexpected benefits ALs provided when started pre-symptomatically we conducted a series of experiments to evaluate the impact of AL treatment on biochemical storage and pathologies in NPC mice.

NPC1 disease is characterized by the accumulation of sphingoid bases (sphingosine and its *de novo* precursor, sphinganine) and secondary accumulation of other lipids, that include cholesterol and GSLs [16]. We therefore examined the impact of drugs administered to *Npc1*^{-/-} mice from 3 weeks of age on levels of sphingoid bases, GSLs and cholestreol in the liver, cerebellum and other brain regions at day 59 (8.3 weeks). As expected, at 8.3 weeks untreated *Npc1*^{-/-} mice display biochemical and histological pathologies indicative of disease[13] (**Figure 2.2, 2.3, 2.4, 2.5**). A cohort of mice was given miglustat as a positive control for reduction in GSLs (**Figure 2.2, 2.3, 2.4**). All acetyl-leucine isomers tested (ADLL, ALL, ADL) significantly reduced total GSL

levels in the livers of *Npc1*^{-/-} mice, in comparison to untreated animals (26.9% for ADLL ($p=0.03$) and ADL ($p=0.0253$) and 45.6% for ALL ($p=0.0003$) (**Figure 2.2 a**). Quantification of the major GSL species confirmed that levels of GM2Gc (the most abundant GSL in the liver) were decreased significantly (45.05%) with all compounds (ADLL: $p=0.0002$, ALL: $p<0.0001$, ADL: $p<0.0001$), while GM3Gc levels were only reduced significantly by ALL (54.8%) ($p=0.0314$) (**Figure 2.2 b**).

Sphingosine (ADLL 29.5%, $p=0.0233$; ALL 33.2%, $p=0.0148$; ADL 33.6% $p=0.0103$) and its *de novo* precursor sphinganine (ADLL 32.5% $p=0.0365$; ALL 37.4% $p=0.0189$; ADL 38.5% $p=0.0163$) were significantly lower, to an equivalent extent (**Figure 2.2 c, d**). Total free cholesterol in liver was significantly decreased after treatment with ADLL, ALL and ADL (ADLL 18.2% $p=0.0346$; ALL 23.4% $p=0.0091$; ADL 16.2 % $p=0.0210$) (**Figure 2.2 e**).

Liver

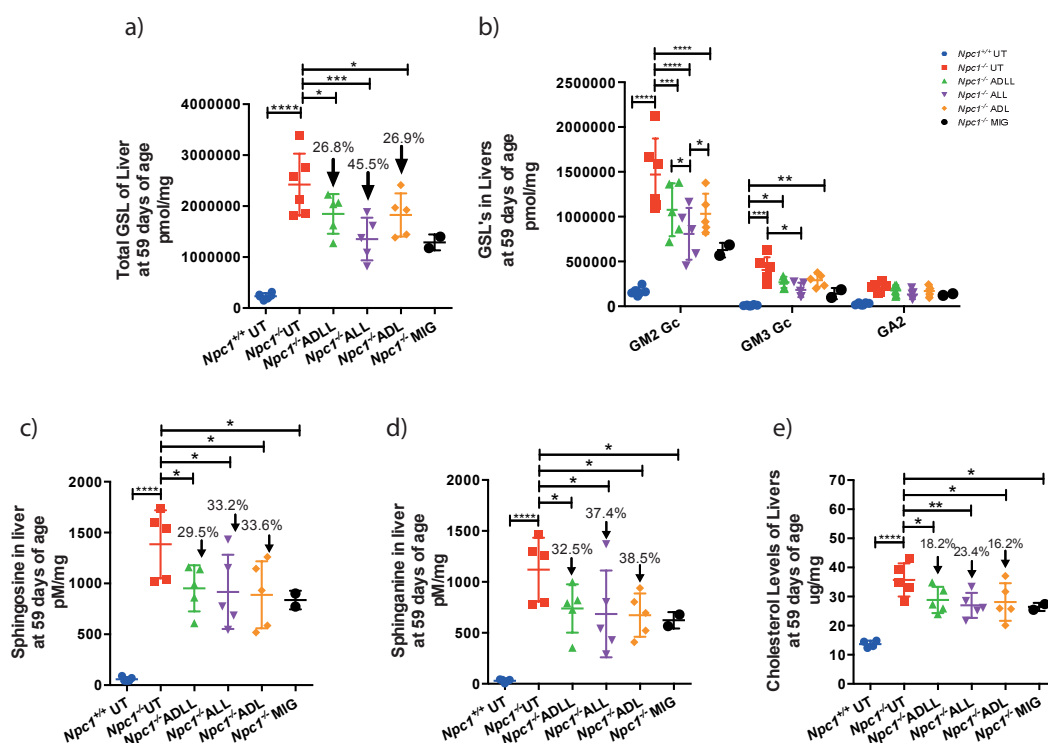


Figure 2.2. Acetyl-leucine compounds' efficacy on lipid storage in NPC liver at 59 days of age. For wild type untreated ($Npc1^{+/+}$ UT), NPC1 untreated ($Npc1^{-/-}$ UT), ADLL ($Npc1^{-/-}$ ADLL), ALL ($Npc1^{-/-}$ ALL), ADL ($Npc1^{-/-}$ ADL) treatments n=5 animals per group, Miglustat treatment ($Npc1^{-/-}$ MIG) n=2. **a)** Total glycosphingolipid (GSL) measurements in liver. GSLs were extracted from water-homogenised tissues with Svennerholm extraction, followed by recombinant EGCase enzyme digestion and anthralinic acid labelling. Mean $\pm$ SD * p <0.03, *** p =0.0003, **** p <0.0001 (One-way ANOVA). **b)** Glycosphingolipid (GSL) profiling in liver. Mean $\pm$ SD * p <0.038, *** p <0.0002, **** p <0.0001 (2-way ANOVA). **c)** Sphingosine measurements in livers. Livers were homogenised in water and sphingoid bases were extracted, purified with SPE NH2 column and eluted in acetone. Samples then labelled with OPA. Mean $\pm$ SD. * p <0.0393, **** p <0.0001 (One-way ANOVA). **d)** Sphinganine levels in livers. Sphinganine measured along with sphingosine in the same HPLC separation. Mean $\pm$ SD, * p <0.04, **** p <0.0001 (One-way ANOVA). **e)** Cholesterol levels in liver. Cholesterol was extracted from homogenates (in water) with Folch extraction and assayed with Amplex Red Cholesterol Assay Kit (Invitrogen). Mean $\pm$ SD, * p <0.034, ** p =0.0091, **** p <0.0001 (One-way ANOVA).

In order to evaluate the impact of the drugs in the CNS we analyzed the cerebellum, a part of the hindbrain that is particularly affected in NPC [13] and all of the other regions of the brain combined. Total GSLs in the brain {excluding cerebellum} were not significantly altered by AL treatments (**Figure 2.3 a**), whilst ALL significantly reduced GM1a (20.1% by mean; $p=0.0018$) and GM2 (19.6%; $p=0.0222$) (**Figure 2.3 b**). Interestingly, although sphingosine levels were not significantly affected (**Figure 2.3 c**), ADLL and ADL reduced sphinganine content by 13.5% ($p=0.0456$) and 18.2% ($p=0.0111$) respectively (**Figure 2.3 d**).

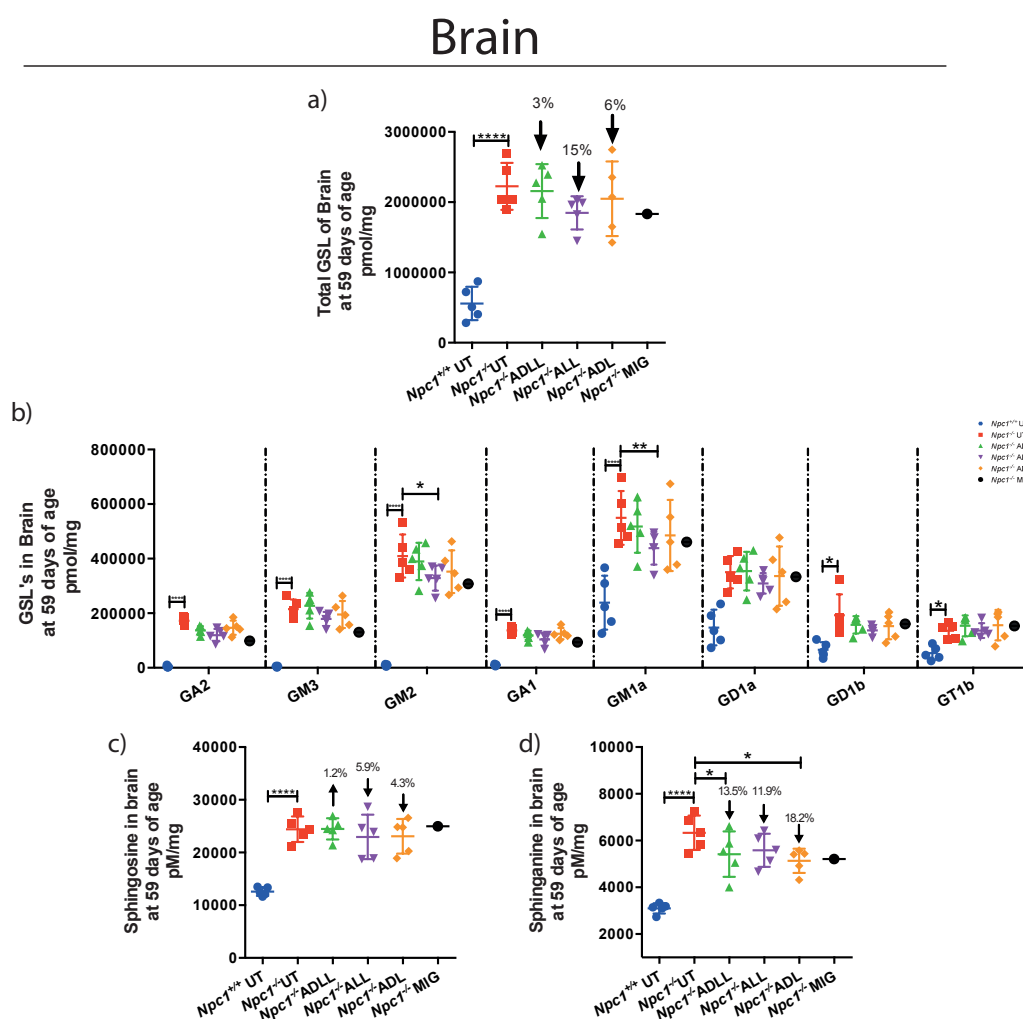


Figure 2.3. Acetyl-leucine compounds' efficacy on lipid storage in NPC brain at 59 days of age. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), ADLL

(*Npc1*^{-/-} ADLL), ALL (*Npc1*^{-/-} ALL), ADL (*Npc1*^{-/-} ADL) treatments n=5 animals per group, Miglustat treatment (*Npc1*^{-/-} MIG) n=1. **a)** Total GSL levels in brain, Mean $\pm$ SD (One-way ANOVA). **b)** GSL profiles in brain. Mean $\pm$ SD, * p <0.024, ** p <0.0019, *** p <0.0005, **** p <0.0001(2-way ANOVA). **c)** Sphingosine levels in brain. Mean $\pm$ SD, **** p <0.0001(One-way ANOVA). **d)** Sphinganine levels in in brain. Mean $\pm$ SD, * p <0.046, **** p <0.0001(One-way ANOVA).

Analysis of the cerebellum confirmed there was no significant alteration in total GSLs relative to untreated *Npc1*^{-/-} with any of the AL isomers that were tested (**Figure 2.4 a**). ADLL and ALL treatment significantly lowered levels of GA2 (ADLL: 21.3%, p =0.0102; ALL: %23.8 by mean, p =0.0042) whereas a significant reduction in GA1 was only observed with ALL treatment (23.5%, p =0.0254) (**Figure 2.4 b**). Comparable to the results for the whole brain, sphingosine was unchanged in the cerebellum (**Figure 2.4 c**), but sphinganine was significantly reduced following ADL treatment (42.3% by mean, p =0.0074) (**Figure 2.4 d**).

Cerebellum

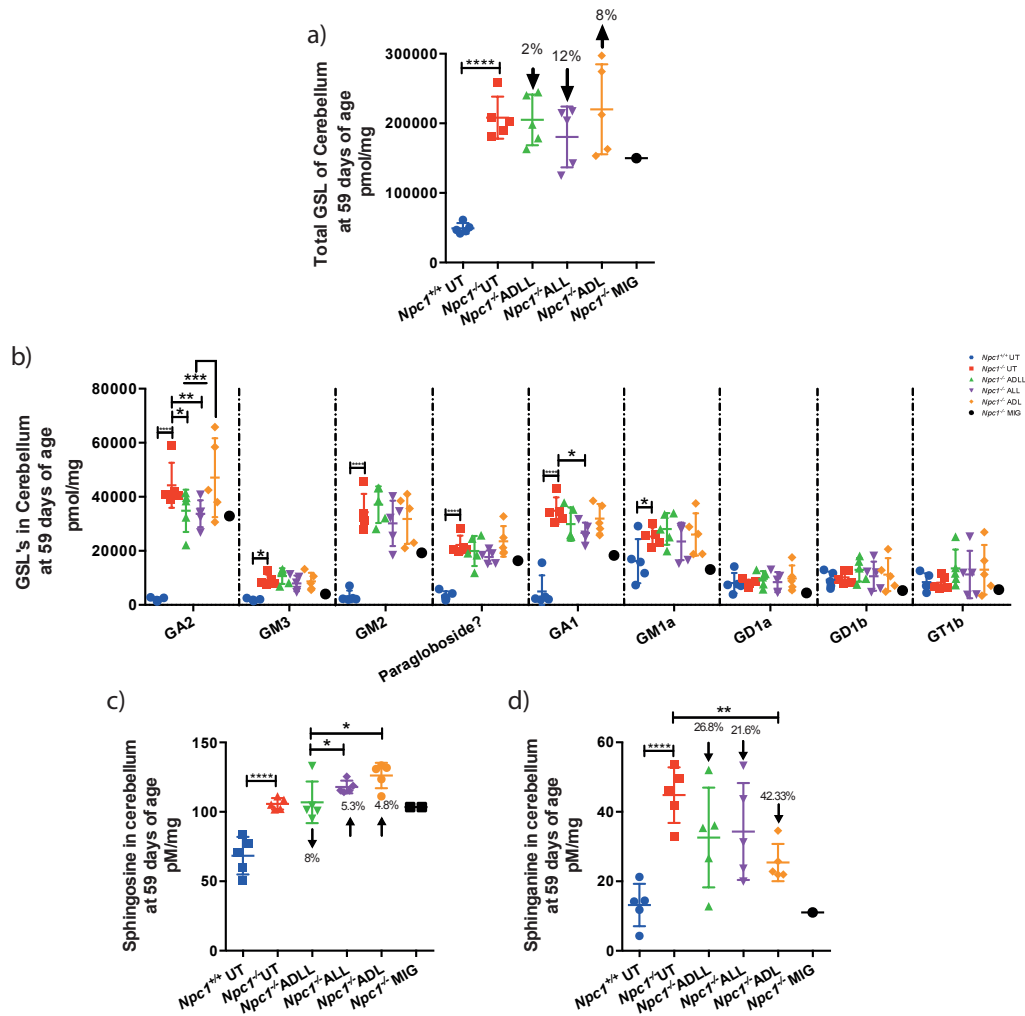


Figure 2.4. Acetyl-leucine compounds' efficacy on lipid storage in NPC cerebellum at 59 days of age. For wild type untreated ($Npc1^{+/+}$ UT), NPC1 untreated ($Npc1^{-/-}$ UT), ADLL ($Npc1^{-/-}$ ADLL), ALL ($Npc1^{-/-}$ ALL), ADL ($Npc1^{-/-}$ ADL) treatments n=5 animals per group, Miglustat treatment ($Npc1^{-/-}$ MIG) n=1. **a)** Total GSL levels in cerebellum, Mean $\pm$ SD (One-way ANOVA). **b)** GSL profiling in cerebellum. Mean $\pm$ SD, * p <0.026, ** p <0.005, *** p <0.0009, **** p <0.0001 (2-way ANOVA). **c)** Sphingosine levels in cerebellum. Mean $\pm$ SD, * p <0.046 **** p <0.0001 (One-way ANOVA). **d)** Sphinganine levels in in brain without cerebellum. Mean $\pm$ SD, ** p <0.075, **** p <0.0001 (One-way ANOVA).

2.3.4. Pre-symptomatic treatments attenuate neuropathology in $Npc1^{-/-}$ cerebellum.

$Npc1^{-/-}$ mice exhibit progressive neurodegeneration, with cerebellar Purkinje cell loss progressing from anterior to posterior lobes [153] (Figure 2.5 a). Microglial activation is also a feature of the CNS pathology (Figure 2.5 a).

ADLL treatment significantly increased Purkinje cell survival at 59 days of age relative to untreated); 133% more Purkinje cells in lobules I and II ($p=0.0027$), and 402% more Purkinje cells in lobule III ($p=0.0108$) relative to untreated littermates. **(Figure 2.5 a, b)**. In addition ALL treatment was observed to significantly reduce (20%, $p=0.0177$) the frequency of CD68 positive, activated microglia **(Figure 2.5 a, c)**. Loss of *Npc1* activity is recognized to result in hypomyelination [154] **(Figure 2.5 d, e)**. We therefore examined the impact of AL on myelin levels in the cerebellum, as reflected by the relative expression of myelin basic protein (MBP)[123]. None of the treatments had statistically significant effects on MBP (ADLL: $p=0.6414$, ALL: $p=0.2884$, ADL: $p=0.6518$ vs untreated *Npc1*^{-/-}) **(Figure 2.5 d, e)**.

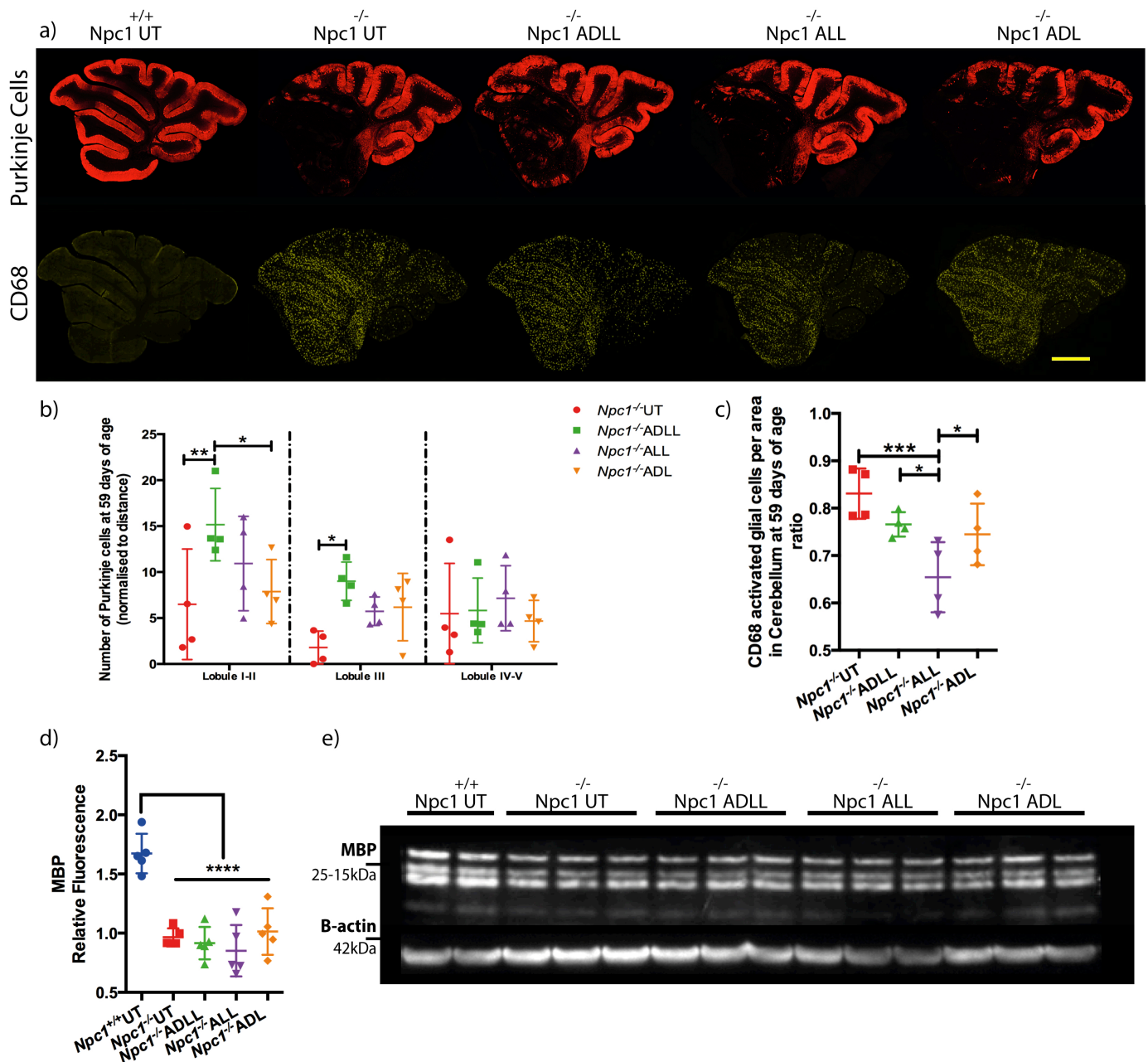


Figure 2.5. Effects of acetyl-leucine in NPC cerebellum at 59 days of age. For wild type untreated ($Npc1^{+/+}$ UT), NPC1 untreated ($Npc1^{-/-}$ UT), ADLL ($Npc1^{-/-}$ ADLL), ALL ($Npc1^{-/-}$ ALL), ADL ($Npc1^{-/-}$ ADL) treatments min 4 animals max 5 animals for each group. a) Fluorescence images showing changes between wild type and NPC and with treatments. Sections were stained with either calbindin-b (Purkinje cells, red) or CD68 (activated microglia, green). Scale bar, 800 μ m. b) Purkinje cell density in lobule I-II, III and IV-V at 59 days of age relative to NPC untreated. Mean $\pm$ SD, * $p < 0.011$, ** $p < 0.028$ (One-way ANOVA) c) CD68 activated glial cell density in cerebellum at 59 days of age. Mean $\pm$ SD, * $p < 0.046$, *** $p < 0.0009$ (One-way ANOVA). d) Fluorescence quantification of cerebellar MBP at 59 days of age, relative to NPC untreated group normalised to b-actin. Mean $\pm$ SD, **** $p < 0.0001$ (One-way ANOVA). e) Western blot illustrative image of MBP expression.

2.3.5. Potential targets of Leucine isomers in the cerebellum

No effect of ALs on autophagy and mTOR.

L-Leucine is a potent activator of the mammalian target of rapamycin (mTOR) [155] and inhibits autophagy[156], [157]. We therefore investigated possible changes in mTOR and its phosphorylation status after exposure to ALs. Western blotting indicated the amount of mTOR was not significantly changed and neither was the extent of phosphorylation (serine 2448[158]) by treatment with leucine isomers (**Figure 2.6 a-d**). Autophagic vacuoles have been demonstrated to accumulate in NPC disease, consistent with impaired lysosomal flux[159]. In order to assess whether leucine isomers impact upon autophagy we examined the relative amounts of the autophagic vacuole-associated phospho-lipidated protein LC3-II and its non-lipidated form, LC3-I [159]. As indicated by western blotting, none of the compounds significantly changed the levels of LC3-I and LC3-II proteins. In addition, we were unable to measure a significant change in levels of its substrate p62/SQSTM1[160]. (**Figure 2.6 e-g**). Furthermore, none of the leucine isomers changed autophagic function or flux (LC3 II/I) (**Figure 2.6 e**). The lack of autophagic correction post-treatment led us to investigate the effect of leucine derivatives on other pathways involving leucine.

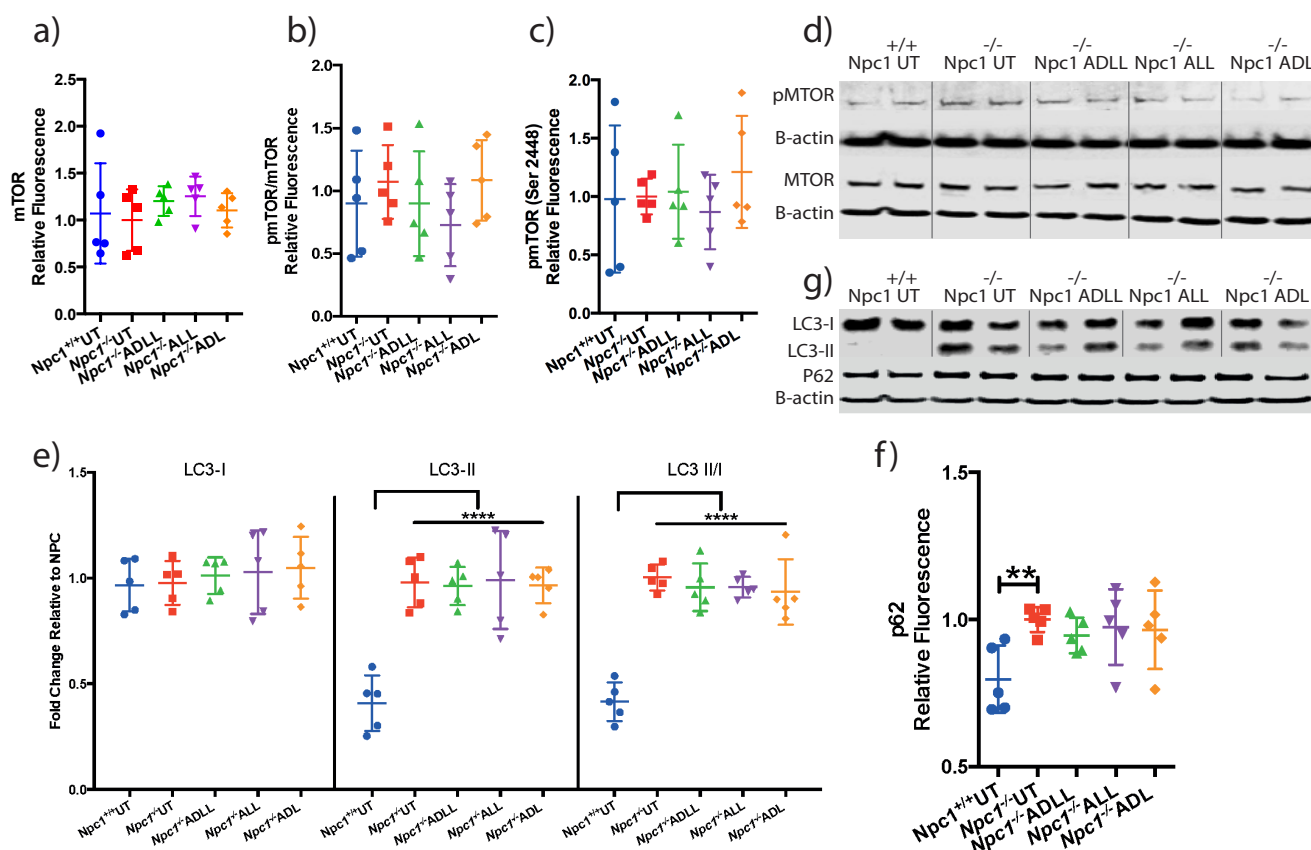


Figure 2.6. Acetyl-leucine isomers effects on autophagy in NPC cerebellum at 59 days of age. Wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), ADLL (*Npc1*^{-/-} ADLL), ALL (*Npc1*^{-/-} ALL), ADL (*Npc1*^{-/-} ADL) treatments, n=5 for each group. Proteins of interests were probed with fluorescent western blot, normalised to b-actin. Fluorescence values were obtained with ImageJ software and calculated relative to *Npc1*^{-/-} UT. a) mTOR expressions, Mean $\pm$ SD (One-way ANOVA). b) Phospho-mTOR expressions, Mean $\pm$ SD (One-way ANOVA). c) Ratio between phospho-mTOR and mTOR, Mean $\pm$ SD (One-way ANOVA). d) Western blots images of phospho-mTOR, mTOR and their b-actin loading controls. e) LC3-I, LC3-II expressions, and Ratio of LC3-II versus LC3-I. Mean $\pm$ SD **** p <0.0001 (2-way ANOVA). f) p62 levels, Mean $\pm$ SD, ** p <0.006 (One-way ANOVA). g) Western blot images of LC3s, p62 and loading control b-actin.

Effect on energy and antioxidative metabolism

In light of our data indicating the absence of significant effects of leucine derivatives on the autophagic-lysosomal system we explored alternative metabolic pathways that are influenced by the amino acid. For example, branched chain amino acids, especially leucine, are important as a protein source,

glutamine/glutamate balance and for glucose/energy metabolism when glucose metabolism is deficient/impaired[135][97]. Catabolism of leucine serves as a source of Acetyl CoA [161], and it also facilitates glutamate metabolism (via its direct effect on glutamate dehydrogenase that converts glutamate to alpha-ketoglutarate), which feeds into the TCA cycle[162].

In order to investigate potential disruption of branched chain amino acid catabolic activity in the cerebellum of *Npc1*^{-/-} mice we examined branched chain keto acid dehydrogenase enzyme A subunit (BCKADHA) (**Figure 2.7 a and c**), which functions in the last step of the pathway that yields Acetyl CoA[163]. Phosphorylation of BCKADHA, which represents the inactive form, was significantly reduced in *Npc1*^{-/-} cerebellum relative to wild type (37.6% by mean, $p=0.0254$) and enzyme activity (p/t) was significantly increased (36.6% by mean, $p=0.0269$) (**Figure 2.7 a, c**). None of the AL isomers tested had a significant impact on either total protein or extent of phosphorylation (**Figure 2.7 a,c**).

We next addressed whether ALs were able to interfere with glutamate homeostasis, which has been previously hypothesized [164]. We found by western blotting that there was no difference in glutamate dehydrogenase (GDH) protein abundance between untreated *Npc1*^{-/-} and *Npc1*^{+/+} and also following AL treatments (**Figure 2.7 b, c**).

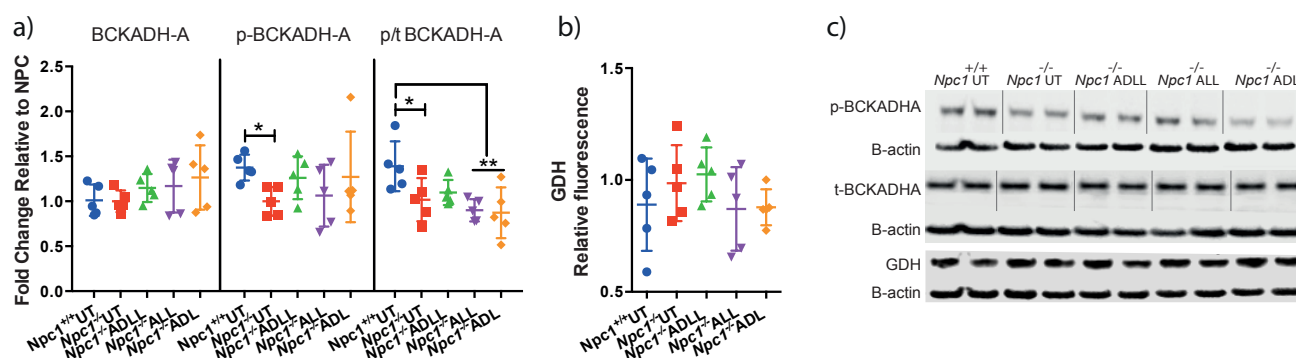


Figure 2.7. Effects of acetyl-leucine isomers on amino acid and glutamate catabolism in NPC cerebellum at 59 days of age. a) BCKADH, pBCKADH, p/t BCKADH expression, mean $\pm$ SD, * p <0.027, ** p <0.005 (2-way ANOVA). **b)** GDH expression, mean $\pm$ SD (One-way ANOVA). **c)** BCKADH, pPCKADH, and GDH western blot images with β -actin loading control.

The status of glucose metabolism can be assessed by determining adenosine diphosphate and adenosine triphosphate ratios (ADP/ATP), and the ratio of nicotinamide adenine dinucleotide and its reduced form (NAD/NADH). We measured ADP, ATP, NAD, NADH and total NAD (NADt = NAD+NADH) and determined ADP/ATP and NAD/NADH ratios (**Figure 2.8 a-c**). NAD and NADH were significantly decreased in *Npc1*^{-/-} relative to WT with statistical significance on NADH (mean 55.1%, p =0.0314) and sum of tNAD (mean 49.2%, p =0.0001) (**Figure 2.8 a**). Although there was no effect on the amount of coenzymes with acetyl-leucine treatments in comparison with NPC untreated (**Figure 2.8 a**), the NAD/NADH ratio was significantly decreased with ADLL treatment (mean 30.7%, p =0.0240) (**Figure 2.8 b**), which might be an indication of increased glycolysis. In accordance with this result, ADP/ATP ratios also favored ADP with ADLL (p =0.0101) (**Figure 2.8 c**).

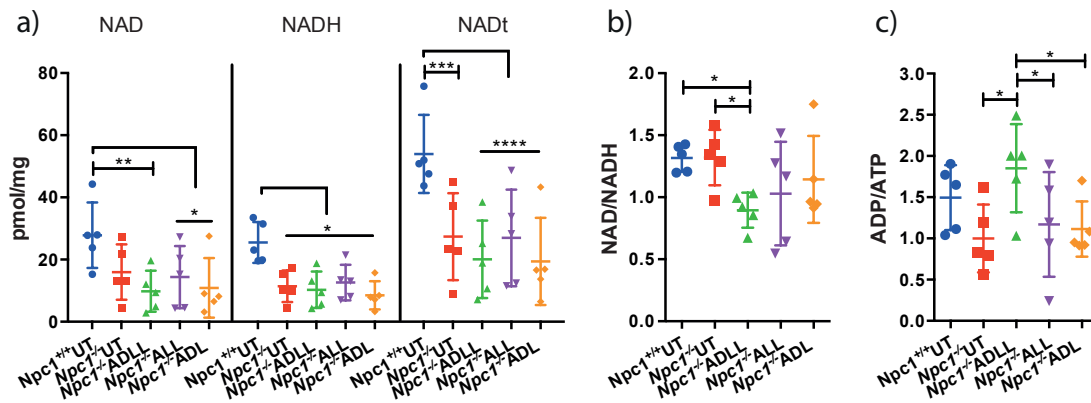


Figure 2.8. Effects of acetyl-leucine isomers on NAD NADH NADt coenzyme levels and ADP/ATP ratio in NPC cerebellum at 59 days of age. a) NAD, NADH, total NAD (NADt) measurements, mean $\pm$ SD, * $p < 0.05$, ** $p = 0.0065$, *** $p = 0.0001$, **** $p < 0.0001$ (2-way ANOVA). **b)** NADH/NAD ratios, mean $\pm$ SD, * $p < 0.025$ (One-way ANOVA). **c)** ADP/ATP ratios, Mean $\pm$ SD (One-way ANOVA).

ADLL increases PDH activity while ALL shifts glucose metabolism towards PDH from LDH

As a consequence of the potential increase in glycolysis, we investigated the effect of AL isomers on glucose utilization, by measuring the key enzymes pyruvate dehydrogenase (PDH) and lactate dehydrogenase (LDH). PDH is composed of 3 subunits: E1, E2, E3, with the active site located on the alpha subunit of E1, and enzyme activity by its phosphorylation that results in inactivation of the enzyme[165]. *Npc1*^{-/-} mice showed significantly decreased levels of PDH-E1 alpha (40.2%, $p = 0.0009$), PDH-E1 beta (61.2%, $p < 0.0001$), PDH-E3 binding protein (PDH-E3bp) (42.2%, $p = 0.0005$), while phosphorylation of PDH (41.4%, $p = 0.0092$), PDH enzyme inactivation (pPDH/PDH E1alpha) (55.5%, $p = 0.0006$) and LDH increased in the disease group (14.2%, $p = 0.0335$) (**Figure 2.9 a-f**). Accordingly, the glucose metabolism preference (PDHE1 alpha/LDH) favored LDH dependency in *Npc1*^{-/-} mice (74.2%, $p = 0.0060$), with no detectable difference proliferator-activated receptor-gamma coactivator 1-alpha

levels, a key regulator of oxidative energy metabolism (OXPHOS)[166] (**Figure 2.9 a-f**). Only E1 alpha subunit protein levels were significantly increased by ADLL (29.1%, $p=0.0146$), ALL (43.3%, $p=0.0004$) and ADL (34.17%, $p=0.0044$) in treated cerebellums (**Figure 2.9 a, f**). The protein levels of phosphorylated E1 alpha subunit were not significantly different following treatments compared to *Npc1*^{-/-} untreated group; however, the ratio of phosphorylated E1 alpha to total E1 alpha subunit was reduced with ADLL (%33, $p=0.0333$) (**Figure 2.9 b, f**). LDH levels were the same in untreated and treated *Npc1*^{-/-} mice (**Figure 2.9 c, f**). Nevertheless, the ratio of E1 alpha subunit to LDH was significantly shifted with only ALL in favor of PDH (49.1%, $p=0.0494$) (**Figure 2.9 d**). Lastly, we measured the protein expression levels of PGC-1 alpha to see whether this metabolic activation and shift towards PDH dependency had an impact on oxidative phosphorylation. There was no difference in protein levels between WT and NPC cerebellum, as well as with the treatments (**Figure 2.9 e, f**).

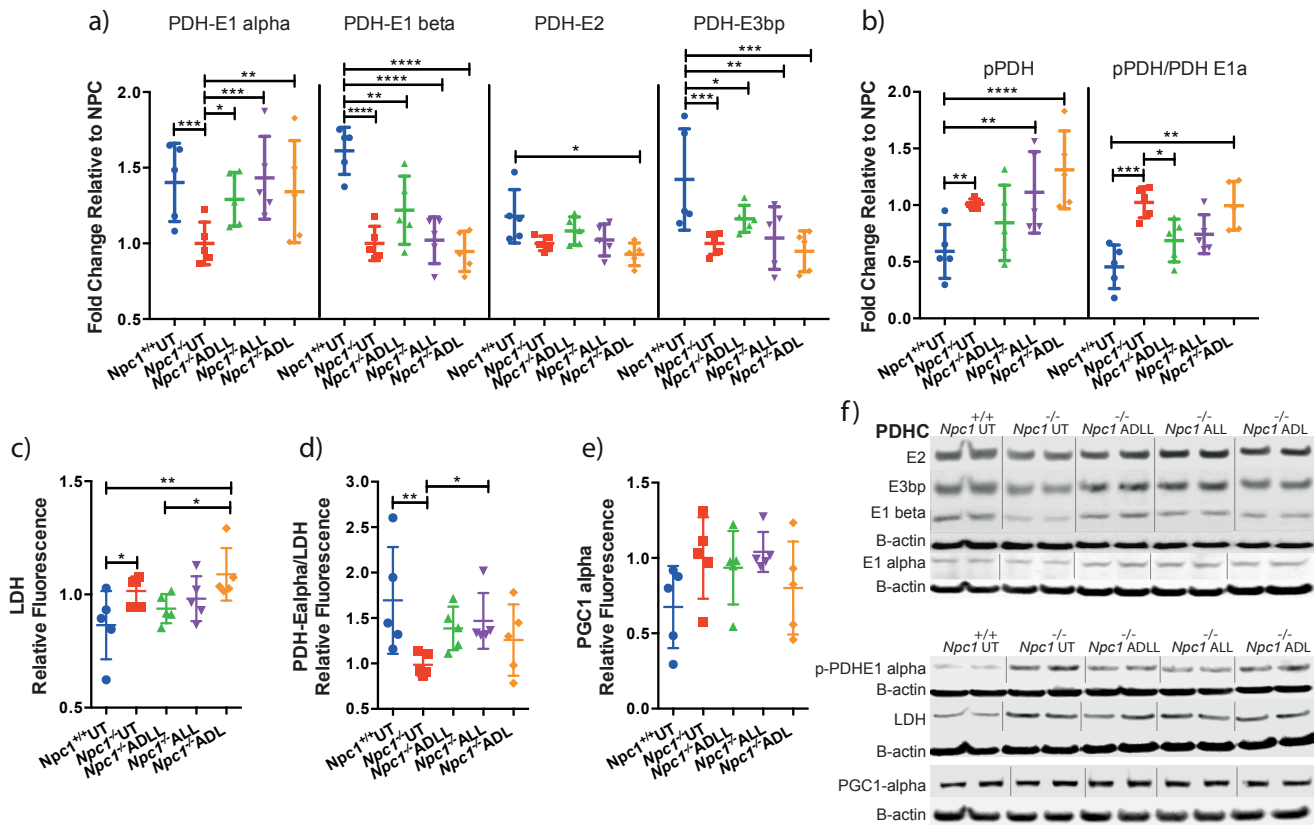


Figure 2.9. Effects of acetyl-leucine isomers on energy metabolism in NPC1 cerebellum at 59 days of age. **a)** PDHC protein expressions, Mean $\pm$ SD, * p <0.034, ** p <0.0045, *** p <0.001, **** p <0.0001 (2-way ANOVA). **b)** pPDH expressions and pPDH/PDH E1a ratios, Mean $\pm$ SD, * p =0.0333, ** p <0.0095, *** p =0.0006, **** p <0.0001 (2-way ANOVA). **c)** LDH expression, Mean $\pm$ SD, * p <0.034, ** p <0.0028 025 (One-way ANOVA). **d)** PDH E1 alpha/LDH ratios, Mean $\pm$ SD, * p =0.0494, ** p =0.0060 (One-way ANOVA). **e)** PGC 1 alpha expression, mean $\pm$ SD (One-way ANOVA). **f)** Western blot images of PDHC, pPDH, LDH, PGC-1alpha and β -actin loading controls.

ADLL decreases PDK2 protein expression

Pyruvate dehydrogenase activity is regulated by reversible phosphorylation; it is phosphorylated by pyruvate dehydrogenase kinases (PDK), which inactivates the complex, whilst pyruvate dehydrogenase phosphatase (PDP) dephosphorylates and activates the enzyme[167]. We therefore analyzed the cerebellar expression of three PDK isoforms (PDK1, PDK2 and PDK4) and a single isoform of PDP; PDP1[167]. Whilst levels of PDK1 and PDK4 in the

cerebella were not significantly different between 59 day old *Npc1*^{-/-} and *Npc1*^{+/+} animals, PDK2 was significantly lower (64.4%, $p < 0.0001$) (**Figure 2.10 a, b**). Furthermore, ADLL (which decreased the phosphorylation of PDH the most) decreased PDK2 to a significantly greater extent (42.3%, $p = 0.0022$) (**Figure 2.10 a, b**). PDP1 was unaltered in untreated *Npc1*^{-/-} cerebellum and perhaps surprisingly, it was only significantly increased by ADL (27.4%, $p = 0.0433$) (**Figure 2.10 a, b**).

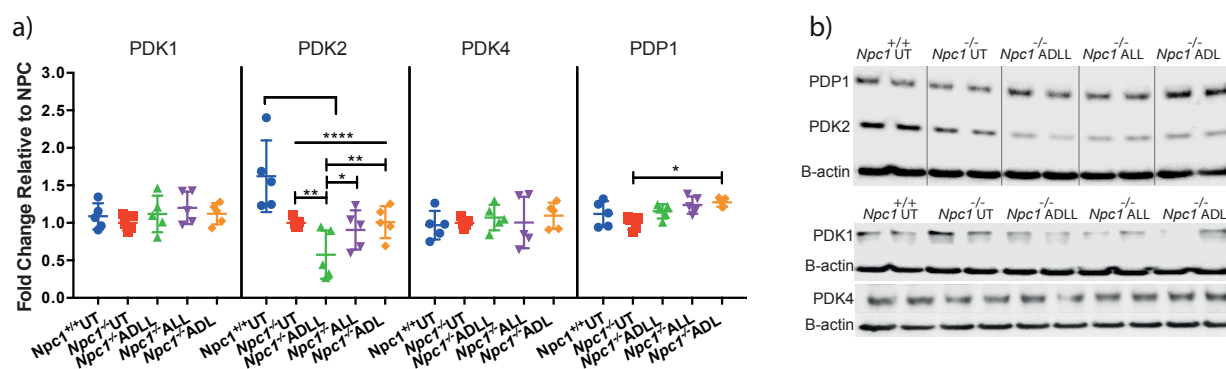


Figure 2.10. Effects of acetyl-leucine isomers on pyruvate dehydrogenase kinases and phosphatase in NPC cerebellum at 59 days of age. a) PDK 1-2-4 and PDP1 expression, Mean $\pm$ SD, * $p < 0.045$, ** $p < 0.0023$, **** $p < 0.0001$ (2-way ANOVA). **b)** Western blot images of PDKs and PDP1 along with their β -actin loading controls.

Differential effect with individual enantiomers on antioxidative system: ALL decreases SOD1 while ADL increases SOD2

The balance between reactive oxygen species (ROS) production and the anti-oxidant system activity is crucial for cellular viability[168]. Mitochondrial energy metabolism is a major source of cellular ROS production[168] and it has been recently shown that leucine can reduce ROS via its effects upon energy metabolism[169]. Superoxide dismutases are crucial components of the anti-oxidative system and are defective in *Npc1*^{-/-} brain [93]. We therefore assessed the impact of ALs on the anti-oxidative system by measuring the superoxide

dismutase 1 (SOD1) cytoplasmic and superoxide dismutase 2 (SOD2), a mitochondrial superoxide detoxifier. We were unable to detect a significant difference in SOD1 levels between *Npc1*^{-/-} and *Npc1*^{+/-} cerebella but also observed a significant reduction in SOD2 (25% $p=0.0061$) (**Figure 2.11 a, b**). While only ALL significantly decreased SOD1 levels in cerebellum (15.6%, $p=0.0472$), only ADL significantly elevated SOD2 levels compared to NPC untreated group (19.8%, $p=0.0214$) (**Figure 2.11 a, b**).

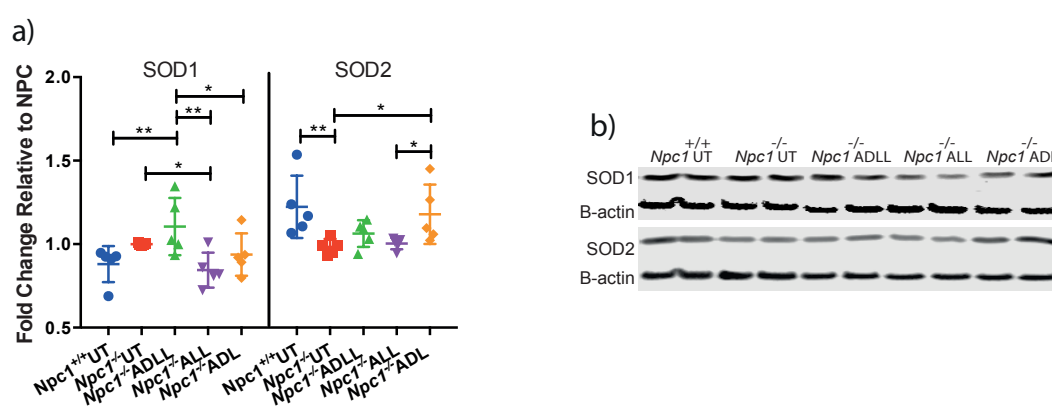


Figure 2.11. Effects of acetyl-leucine isomers on antioxidant system in NPC cerebellum at 59 days of age. a) SOD1 and SOD2 expression, Mean $\pm$ SD, * $p < 0.042$, ** $p < 0.0064$ (2-way ANOVA). **b)** Western blot images of SOD1 and SOD2 along with β -actin loading controls.

2.3.6. Acetyl-DL-Leucine synergises with miglustat in *Npc1*^{-/-} mice

Because acetyl-leucine isomers significantly reduced the accumulation of GSLs in *Npc1*^{-/-} mice *in vivo* we wished to examine whether this activity alone was responsible for the extended life span seen with the drug. We therefore treated animals with ADLL in combination with miglustat (an inhibitor of GSL biosynthesis) and compared to mice given monotherapy. We observed that mice provided with a combination of the two drugs had a statistically significant longer life span than animals on monotherapy (*Npc1*^{-/-} miglustat vs *Npc1*^{-/-} miglustat & ADLL $p=0.0063$; *Npc1*^{-/-} ADLL vs *Npc1*^{-/-} miglustat & ADLL $p=0.0016$;

median survivals; *Npc1*^{-/-} Untreated: 87, *Npc1*^{-/-} ADLL: 91; *Npc1*^{-/-} Miglustat: 117, *Npc1*^{-/-} Miglustat&ADLL: 138) (**Figure 2.12 a**), prevented the weight loss associated with miglustat treatment (**Figure 2.12 b**). Combination therapy significantly increased rotarod test performance, indicative of improved motor function relative to untreated mice (8 weeks: $p=0.0007$; 10,12 weeks $p<0.0001$) and mice receiving either miglustat (10 weeks: $p=0.0299$; 12 weeks: $p=0.0232$) or ADLL monotherapies (10,12 weeks: $p<0.0001$) (**Figure 2.12 c**). Gait analysis at 10 weeks of age demonstrated that hind Duty Cycle percentages were significantly improved with combination therapy (Compared to *Npc1*^{-/-} untreated, $p=0.0028$) whereas miglustat monotherapy showed no benefit (**Figure 2.12 d, e**). Hind stand Mean significantly improved in mice treated either with combination therapy ($p=0.0250$) or miglustat monotherapy ($p=0.0196$) relative to NPC1 (**Figure 2.12 d, f**).

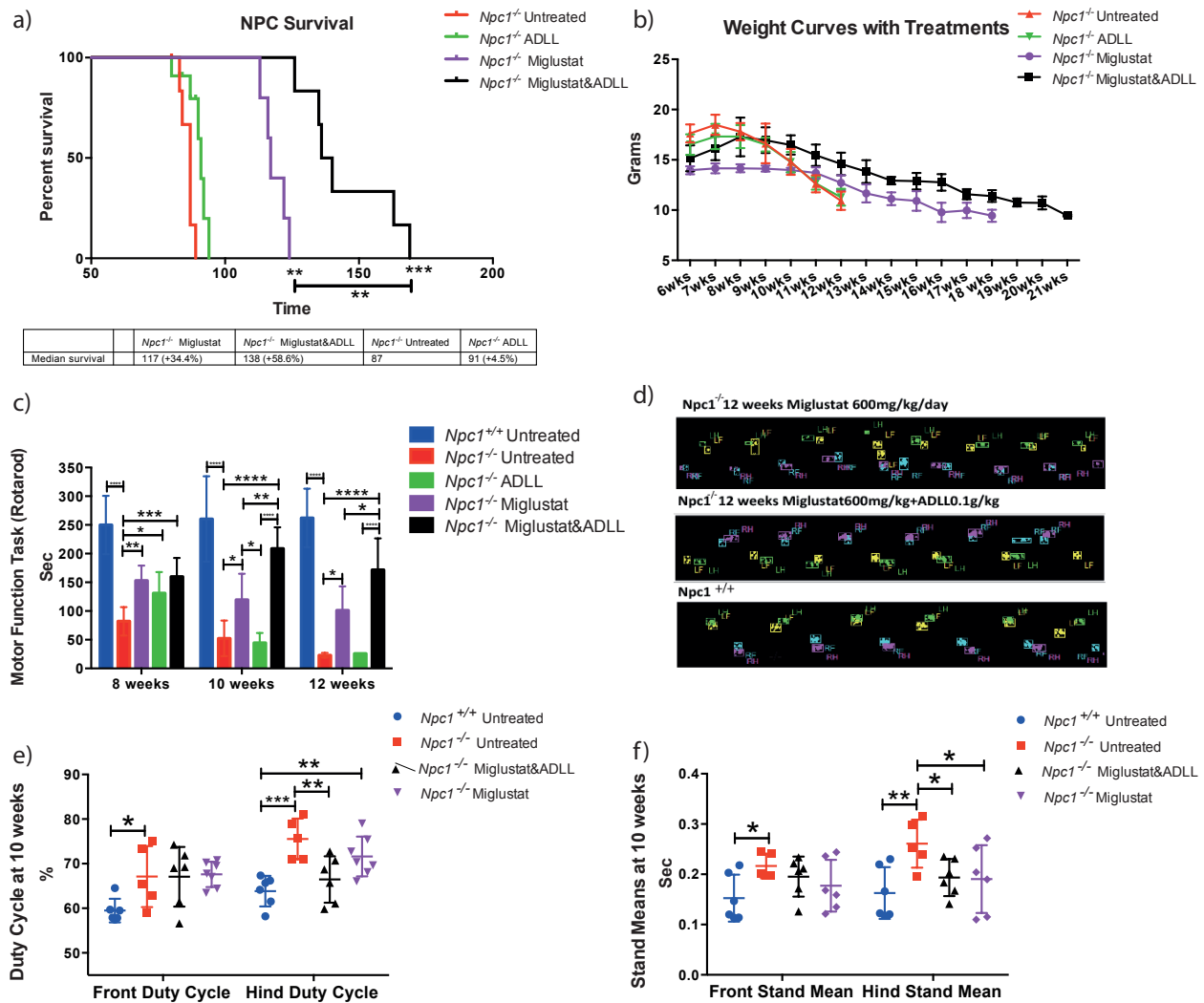


Figure 2.12. Effect of ADLL in combination with miglustat. For wild type untreated (*Npc1^{+/+}* UT), NPC1 untreated (*Npc1^{-/-}* UT), ADLL (*Npc1^{-/-}* ADLL), miglustat (*Npc1^{-/-}* MIG) and combination (*Npc1^{-/-}* Miglustat & ADLL) therapies min 5 animals for each group. Duty Cycle and Stand mean analyses were measured by 3 recorded runs per animal and quantitative results were obtained with Noldus CatWalk 10.5 software system. Motor function performance was measured with accelerating rotarod (1rpm per minute up to 10 rpm). **a)** Survival curves, Mean $\pm$ SD, $*p=0.0270$, $**p<0.0035$, $***p=0.0007$ (Gehan-Breslow-Wilcoxon test). **b)** Weight curves over time, Mean $\pm$ SD. **c)** Motor performance measurements at 8, 10 and 12 weeks of age, Mean $\pm$ SD, $*p<0.043$, $**p<0.0036$, $****p<0.0001$ (2-way ANOVA) **d)** Representative image of footprints of miglustat and combination therapy. **e)** Duty cycle measurements of front and hind paws at 10 weeks of age, Mean $\pm$ SD, $*p=0.011$, $**p<0.0053$, $***p=0.0002$ (2-way ANOVA) **f)** Stand mean measurements at of front and hind paws 10 weeks of age, Mean $\pm$ SD, $*p<0.033$, $**p<0.0016$ (2-way ANOVA)

2.4.Discussion

Acetyl-DL-leucine (ADLL) has been used as an anti-vertigo treatment for 60 years, although the mechanism underlying its clinical benefit remains unclear, but is believed to be related to correction of defects in neuronal membrane polarity [140]. Recent publications have also reported symptomatic relief with ADLL treatment in patients with monogenic cerebellar ataxias, including the rare LSD, Niemann-Pick disease type C [131].

A primary objective of this study was determine whether ADLL could have effects in the *Npc1*^{-/-} mouse comparable to those reported in patients that would therefore allow us to investigate its mode of action. We found that ADLL significantly improved ataxia when used acutely for one week in symptomatic NPC1 mice in a manner analogous to the clinical studies. We also confirmed that the individual enantiomers provided comparable benefit to the racemic mixture. However, surprisingly, when treatment was initiated pre-symptomatically it resulted in extension of life span, neuroprotection, and ameliorated biochemical hallmarks of the disease. Furthermore the ADLL provided synergistic benefit when combined with miglustat.

Phenotypic improvements resulting from early treatment with ADLL included reduced lipid storage in the liver and to a lesser extent in the brain, a significant increase in the number of surviving Purkinje cells in the cerebellum. These data are indicative of ADLL acting not only as a symptomatic modifier, but also being neuroprotective in NPC. In light of these observations the most probable explanation is acetyl-leucine isomers have activities in addition to their ability to influence membrane polarity.

The central question arising from these studies is the nature of the underlying mechanism(s) through which ADLL provides benefit in neurodegenerative conditions, such as NPC. The drugs tested are structural isomers of the amino acid, leucine. Leucine is known to play key roles in nutrient sensing pathways and also in mitochondrial energy metabolism. It was shown that leucine, potently activates of mTOR, and accordingly reduce lysosomal LC3 (LC3-II), therefore reduce LC3's substrate p62 in NPC cells[158]. However, we were unable to obtain experimental evidence that ADLL significantly affected either mTOR or aspects of the process of disrupted autophagy in our NPC mouse cerebellum. This might be due to acetylation of leucine blocking the interaction of leucine with mTOR in Hela cells[170]. In addition, leucine's metabolite Acetyl CoA has been shown to also activate mTOR and nutrient sensing pathways[171], yet NPC cerebellum has significantly decreased level of Acetyl CoA[97]. This lack of Acetyl CoA content in NPC might prevent nutrient sensing pathway activation.

In the CNS, branched chain amino acids, particularly leucine, are important for nitrogen donation and glutamine/glutamate homeostasis, insulin secretion and energy metabolism[172]. Catabolism of leucine generates acetyl CoA and up-regulates glutamate dehydrogenase, the enzyme that breaks down glutamate to alpha-ketoglutarate that feed into the TCA cycle[161][162]. Leucine also influences glucose metabolism and utilization via its effect on insulin secretion, mTOR and PI3K [172]. We therefore explored alterations to branched chain amino acid catabolism, glutamate, and glucose metabolism in the *Npc1*^{-/-} cerebellum and the capacity for leucine isomers to affect these aspects.

The previous studies show the hyperexcitability in NPC brain with an upregulation on glutamatergic system[173] and GDH mRNA levels displayed

gradual increase from 3 weeks on resulted statistically increased at 5 weeks of age in *Npc1*^{-/-} cerebellum [97]. We saw no significant alteration in expression levels of glutamate dehydrogenase protein in untreated NPC1 mice, also with AL treatments. Our data on protein expression levels might not be sensitive enough to pick up any changes in the disease and treatment groups.

On the other hand, we saw an upregulation of BCKADH activity (that catalyses the last step of amino acid catabolism to produce Acetyl CoA) in untreated NPC cerebellum, while we saw no change in enzyme activity with the treatments. These results display that AL compounds might be further nurturing Acetyl CoA production via amino acid catabolism, which was already active. AL isomers might be a compensatory mechanism to surge Acetyl CoA-TCA cycle derived energy production.

For energy production, glucose is converted to pyruvate, and pyruvate dehydrogenase complex (PDH) transforms pyruvate (obtained from glycolysis) to acetyl-coA, which will further be utilized in TCA cycle. In eukaryotic cells, TCA cycle products 3 NADH, which are used by aerobic respiration (OXPHOS) in mitochondria, producing 38 ATP [91]. Anaerobically, pyruvate is converted to lactate by lactate dehydrogenase (LDH). Increase in lactate and lactate dehydrogenase activation has been linked to several CNS diseases, such as Alzheimer's disease and aging phenotype [92].

Kennedy *et al.* have reported altered energy metabolism in the *Npc1*^{-/-} mouse cerebellum[93]. PDHE1 alpha was inactivated and there was a shift from pyruvate/pyruvate dehydrogenase dependency (upon which neurons rely for aerobic respiration) towards lactate/lactate dehydrogenase (i.e. anerobic respiration). The consequence of this would be progressive impairment of

energy generation[93]. These authors also confirmed alterations in cerebellar amino acids (including leucine), and low Acetyl CoA[97]. Interestingly, leucine was found to be elevated in *Npc1*^{-/-} mice at both 3 and 5 weeks of age (along with branched chain amino-acid transferase 1) in brain and cerebellum, which could be a compensatory response for defective pyruvate oxidation and subsequent deficient energy production in models of NPC disease [97].

In view of these findings of altered energy metabolism in NPC1 mice, we investigated the effects of AL treatments on glucose metabolism. We measured ADP/ATP and NAD/NADH ratios, as a sensitive measure of energy status [174], [175] and confirmed that ADLL treatment shifted the system towards a more glycolytic state, in the absence of significant changes to NAD-NADH coenzyme levels, which are lower in *Npc1*^{-/-} cerebellum, in agreement to previous findings in *Npc1*^{-/-} liver [176]. We then investigated whether the shift in glycolysis is towards pyruvate utilization to produce lactate (anaerobic) or to produce Acetyl CoA. We found that ADLL treatment increased pyruvate dependent mechanism-significantly increasing PDHE1alpha levels, and decreasing PDH enzyme inactivation (phosphorylation) in the cerebellum. Interestingly, PDH-deficient mice also display Purkinje cell degeneration and relapsing ataxia[177], suggesting the ability of ADLL to protect Purkinje cells against degeneration and rectify ataxia may be via activation of PDH. This intervention on PDH did not reflect a change on PGC1 alpha protein levels (indicator of OXPHOS), which might be due to the fact that up-regulating OXPHOS requires coenzyme NADH and ADLL treatment is not able to increase its level.

ADLL therapy activates PDH by decreasing pyruvate dehydrogenase kinase 2 (PDK2) protein, which was already decreased in NPC cerebellum. PDK2

is regulated by Acetyl CoA, NADH and glucose metabolism, and displays highly increased mRNA levels under diabetic conditions and low insulin levels in the cell, and is altered in Alzheimer and aging[99], [178], [179]. Having less PDK2 protein expression in NPC brain may result from reduced levels of NADH and Acetyl CoA in the system. The further decrease in PDK2 levels with ADLL treatment might be due to its leucine nature (acts as an insulin secretogoge), which leads to a reduction in PDK2[99].

Differential effects of AL enantiomers

ADLL is a racemic mixture of L and D enantiomers (ALL and ADL respectively) so we investigated the effects of the racemic mixture and the individual enantiomers, which have been reported to have differential effects on vestibular abnormalities with activity attributed to the L enantiomer in brain in models of acute vertigo[147], [180].

Acute treatment of NPC1 mice with ALL and ADL both improved ataxia in the same magnitude, but when the treatments applied chronically from weaning, enantiomers showed differential effect, suggesting that AL's mechanism of action differs in acute effect from compounds' chronic mechanistic targets.

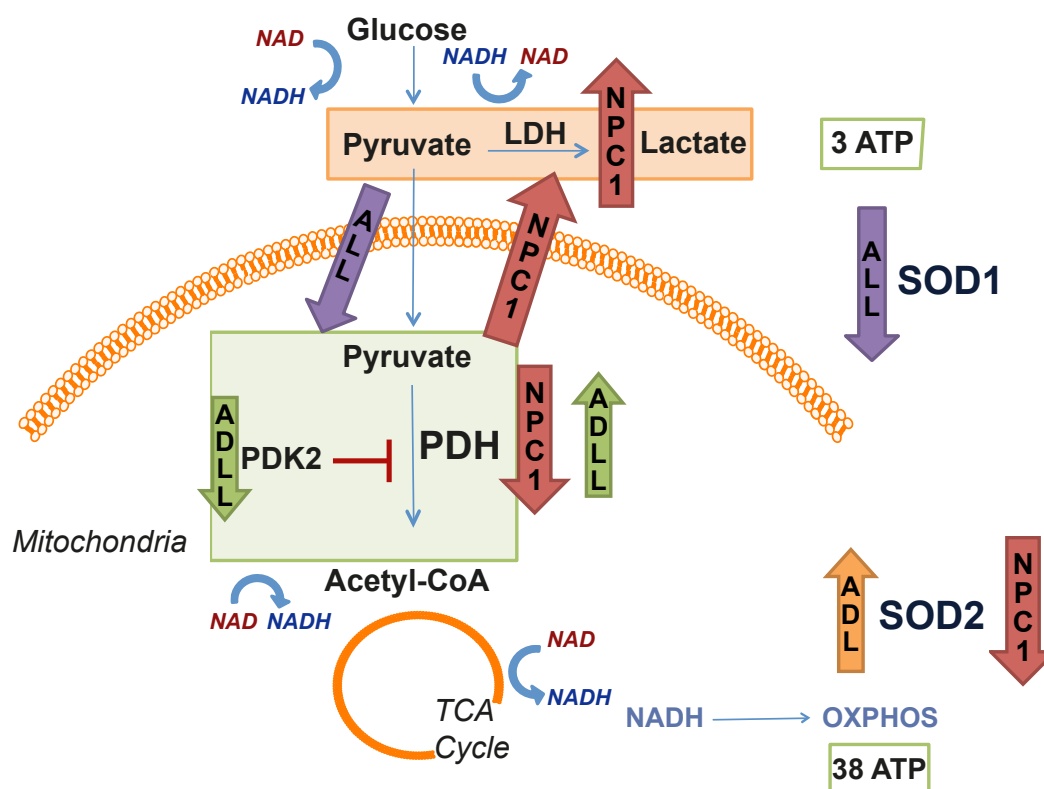
We found that Acetyl-L-Leucine (ALL) mediated the greatest reduction in stored glycosphingolipid levels, reduced inflammation in the cerebellum and shifted lactate dehydrogenase dependency towards pyruvate dehydrogenase (i.e. shifts metabolism from anaerobic to aerobic). We also found that ALL was able to reduce SOD1 levels, which is an important enzyme mediating the metabolic switch from oxidative phosphorylation to anaerobic glycolysis[181]. In NPC, the abnormal lysosomal accumulation of lipids leads to innate immune system

activation[42]. Inflammation has been shown to correlate with insulin signaling impairments[182], and increased lactate and lactate dehydrogenase enzyme levels are also seen in many inflammatory metabolic diseases, including diabetes, cancer [183] and obesity[184]. Interestingly, only ALL reduced GM3 lipid levels the most, which is known to impair insulin signaling[78]. Additionally, lactate dehydrogenase inhibitors were noted to alleviate this shift towards anaerobic respiration, occurring in inflammation, and suppress neoplastic proliferation, invasive growth and infection in colon cancer [185]. ALL attenuated regional cerebral glucose metabolism post vestibular damage in previous models[147]. In parallel to these findings, we hypothesize that ALL is the active enantiomer responsible for shifting the energy metabolism towards aerobic respiration, which may indirectly reduce inflammation[186]. *In vivo*, ALL treatment was associated with a significantly greater benefit (relative to ADL) in terms of motor function and lifespan, suggesting a possible connection between energy metabolism and motor function in NPC and suggests new approaches to pharmacological therapy for this and other neurodegenerative diseases.

Conversely, Acetyl-D-Leucine (ADL) normalized mitochondrial ROS scavenger MnSOD (SOD2) and increased PDP1 (activates pyruvate dehydrogenase complex) protein levels in the cerebellum and reduced sphinganine levels in mouse cerebellum and forebrain, to a greater extent than ADLL and ALL. SOD2 plays a pivotal role, with its loss in animals resulting in progressive seizures and an attenuated lifespan[187], which are features of NPC[4]. Its downregulation impairs oxidative phosphorylation, while its overexpression increases mitochondrial respiration [181]. Intriguingly, D-leucine previously has been reported to be antiepileptic (more so than L-leucine),

even reported to terminate ongoing seizures [188]. Normalization of SOD2 by ADL can be therefore potentially be relevant to both NPC patients with seizures and epilepsy more generally. Sphinganine (dihydrosphingosine) is reduced by ADL is a precursor of sphinglipids including ceramide, which also have been found to induce ROS production apoptosis and cell cycling arrest[189][190], [191]. We therefore hypothesize that ADL might improve antioxidative defenses and decrease pro-apoptotic elements i.e sphinganine (along with possibly dihydroceramide and ceramide). The order of ADL's impact on these system, and particularly the drug's effect on sphinganine metabolic pathways therefore merits further investigation.

The racemic mixture (ADLL) had an intermediate effect compared to single enantiomers on organ GSL levels (in almost all organs), on shift in metabolism to PDH from LDH, increased cerebellar SOD2 levels and improvements in behavioral parameters. However, ADLL was found to be the most beneficial (relative to individual enantiomers) on Purkinje cell survival and PDH E1 alpha activation, and this may implicate synergy between ALL (which corrects energy metabolism defects optimally) and ADL (which stimulates antioxidant mechanisms), with neurons being highly susceptible to defects in both these processes (Summary **Scheme 2.1**).



Scheme 2.1. Summary schematic of AL effect in the NPC1 mouse cerebellum.

Acetyl-Leucine isomers reduce lipid storage in liver

ADLL. ADL and ALL all significantly reduced lipid storage in the liver of *Npc1*^{-/-} mice with ALL being the most effective. This beneficial effect was not stereo selective. The differential effect of lipid lowering between liver and brain might be due to the bioavailability of AL's (oral bioavailability 45%[180]) Similarly, non-acetylated natural leucine found to be beneficial in acute and chronic liver injury and promote liver regeneration[192], also significantly improves energy metabolism and mitochondrial function in multiple mouse models of diabetes and obesity livers[182], [193].[155] Overall, these findings gives a possibility of leucine amino acid benefit in NPC livers.

Synergy between ADLL and Miglustat

The only approved therapy for NPC is the imino sugar drug miglustat that reversibly inhibits the first committed step in GSL biosynthesis. It has been found

to correct lipid-trafficking defects, and reduce accumulation of GSLs and storage of pathological lipids[194]. Furthermore, restoration of mitochondrial inner membrane complexes II and IV and subsequent protection against oxidative stress have also been shown in patients receiving miglustat treatment[194]. Beneficial effects on axonal degeneration, Purkinje cell survival and inflammation have also been shown in patients receiving miglustat[194]. In contrast, ATP levels and autophagy could not be corrected in NPC cell models following miglustat treatment[195]. The precise mechanism of action of miglustat explaining the clinical benefit resulting from inhibition of GSL biosynthesis remains poorly understood. It is interesting that ADLL and miglustat showed synergistic benefit and significantly ADLL corrected the weight loss associated with miglustat treatment. These outcomes suggest that ADLL and miglustat attenuate unique aspects of disease pathology and when combined result in synergy.

In conclusion, we have found that a well tolerated, over the counter drug ADLL slows disease progression and improves motor function in mouse models of NPC disease. ADLL helps restore aerobic metabolism and representing a new therapeutic target for this drug. Individual enantiomers have distinct effects in NPC cerebellum; while ALL normalizes altered PDH/LDH and SOD1 levels, ADL enhances the mitochondrial ROS scavenger SOD2 levels. Acetyl-DL-leucine also synergises with miglustat. Taken together, we have found unanticipated benefits of ADLL as a neuroprotective drug suggesting that this modified amino acid drug may have broader therapeutic utility than previously thought.

Chapter 3: Acetyl-DL-Leucine treatment of Sandhoff Disease mice

3.1.Introduction

Sandhoff disease is one of the GM2 gangliosidoses [196], a group of GSL lysosomal storage diseases that also includes Tay-Sachs disease and GM2 activator deficiency. Sandhoff disease is caused by mutations in *HEXB*, a gene that encodes the beta subunit of b-hexosaminidase, leading storage of GM2 and other substrates in the CNS resulting in progressive neurodegeneration and premature death [197]. Symptom onset can occur in infancy (classical form) or during adolescence or adulthood [198]. Early onset results in a more rapidly progressing disease course, while later- onset forms progress more slowly [198].

Due to the extremely low prevalence (1-9/1,000,000) and heterogeneity of the disease, diagnosis can be made very late even though patients exhibit symptoms. Juvenile-onset Sandhoff disease frequently presents as gait and

speech disturbances and lack of coordination, intellectual impairment and extrapyramidal signs [198], [199]. As the disease progresses, dysphagia, diarrhea/constipation, poor weight gain, behavioral or psychiatric problems, proximal-to-distal weakness and muscle wasting can occur[198], [200].

Although there is no approved specific treatment for Sandhoff disease, several experimental approaches have been developed over the years, ranging from substrate reduction therapy to gene therapy. Substrate reduction therapies, including miglustat, aim to use imino-sugars to inhibit glycosphingolipid biosynthesis[189],[190], which has shown some benefit in juvenile patients[191],[192]. Another interesting approach is using pharmacologic chaperones that inhibit hexosaminidase to stabilize its active site and increase b-hexosaminidase activity in lysosomes in patient fibroblasts[205].

In 2007, neuronal stem cells were administrated to Sandhoff disease mice (*Hexb*^{-/-}), led to an improvement in survival, motor function, and enzyme levels, along with reduced pathologic lipid accumulation and reduced immune activation [206]. Adeno associated virus (AAV) gene therapies in feline and murine models of Sandhoff disease resulted in a significant improvement in lifespan and pathology[207]–[209]. Recently, a heat-shock protein based therapy (arimoclomol, a small molecule inducer of HSP70), which can attenuate lysosomal pathologies, has been trialed in Sandhoff mice along with other LSD'S and improved lifespan and motor function [123].

The Sandhoff (*Hexb*^{-/-}) mouse model is pre-symptomatic up to 6-8 weeks of age. Subsequently, they develop tremor and their motor function begins to decline[202]. By the later stages of the disease (12-15 weeks) they are inactive

and are unable to complete motor function tests such as bar crossing [210]. They have progressive accumulation of GM2 and GA2 in the CNS and visceral organs, including the liver. In addition to GSL storage, there exhibit some shared pathologies with NPC; including inflammation, apoptotic cell death and motor function deterioration[202], [211].

Having shown that Acetyl-DL-leucine (ADLL) shows therapeutic benefit in the NPC1-null mouse model (**Chapter 2**), we next evaluated this treatment in the Sandhoff disease mouse model to determine whether its beneficial effect is disease specific to NPC or potentially a drug treatment that may have possible utility in multiple LSDs. We also explored ADLL's mechanism of action in this disease, and its impact in combination with miglustat to gain more insight into the pharmacology of ADLL.

3.2. Materials and Methods

3.2.1. Mice

Sandhoff mice[212] were bred as heterozygotes to generate affected mice (*Hexb*^{-/-}) and controls (*Hexb*^{+/+}), and were housed under non-sterile conditions, with food and water available *ad libitum*. All experiments were conducted using protocols approved by the UK Home Office Animal Scientific Procedures Act, 1986.

3.2.2. Drug treatments

ADLL (0.1g/kg/day) was administered by mixing the drug in a powdered chow. 1-5 mice were recruited to experimental groups.

3.2.3. Behavioural analysis

Gait analysis was performed as detailed in [Section 2.2.3](#). Bar crossing experiments were conducted to measure motor strength and coordination. The apparatus used was modified from reference[213]. Briefly, a metal bar (1.2 mm width and 26 cm in length) was suspended horizontally between two wooden supports 30 cm high over a cushioned surface and animals were allowed to grasp the center of the bar with forepaws only, the tail was released, and the clock was started. Latency to cross or fall from the bar was scored, with 120 sec, maximum, to termination of each trial. A score was given to each animal between +120 and -120. If crossing time was greater than 0, then score= 120- CT; if falling time was greater than 0, the score= FT-120

3.2.4. Tissue handling

As detailed in [Section 2.2.4](#).

3.2.5. Glycosphingolipid measurements

As detailed in [Section 2.2.6.](#)

3.2.6. Western Blotting

As detailed in [Section 2.2.9.](#)

3.2.7. Image Acquisition and Quantification

As detailed in [Section 2.2.10.](#)

3.2.8. Statistical Analyses

As detailed in [Section 2.2.13](#)

3.3.Results

3.3.1.Characterisation of the effect of ADLL in *Hexb*^{-/-} mouse

In light of the beneficial effect of ADLL on motor function and lipid storage in NPC, we subsequently tested ADLL in Sandhoff disease. *Hexb*^{-/-} animals were treated from weaning with ADLL (0.1mg/kg/day, the same dose used for treatment of *Npc1*^{-/-} mice). We observed extended survival, with a median increase in lifespan of 8 days (7.1% increase, $p=0.0157$) (**Figure 3.1 a**). Bar-crossing test performance significantly improved (12 weeks: $p=0.0343$; 13 weeks: $p=0.0058$), which is an indicative of delayed deterioration in motor function (**Figure 3.1 b, c**). Furthermore, ADLL significantly improved gait, specifically hind stand mean ($p=0.0323$), front ($p=0.0039$) and hind step cycle ($p=0.0062$) (**Figure 3.1 d**).

Animals were culled at 86 days of age for measuring changes in GSL levels in brain, cerebellum and liver. GSL levels were reduced in forebrain ($p=0.0354$) and cerebellum ($p=0.0445$), but not in the liver, with the treatment (**Figure 3.1 e**).

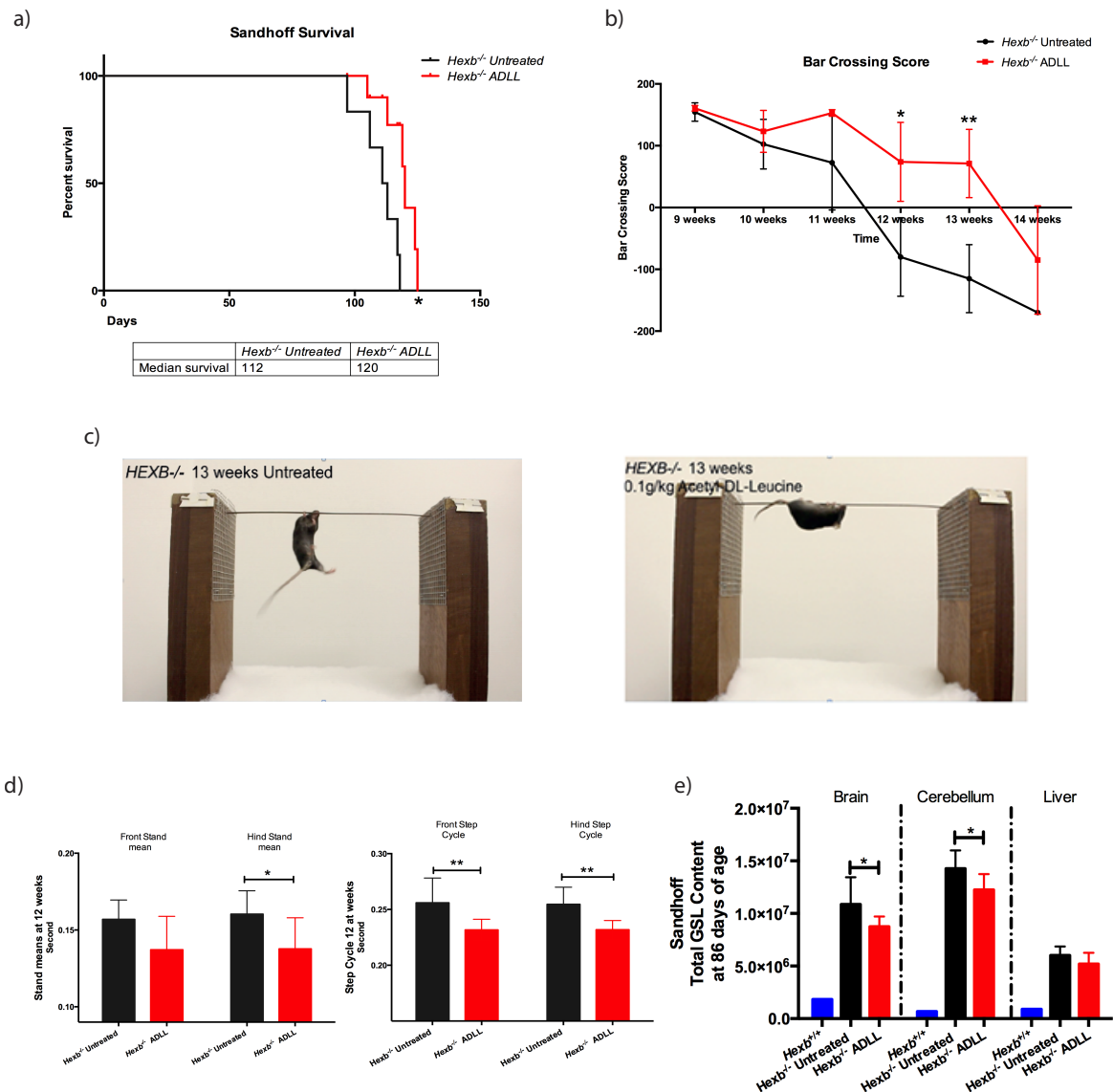


Figure 3.1. Effects of Acetyl-DL-Leucine on behavioural and biochemical parameters in 12 week old Sandhoff mouse. Wildtype untreated (*Hexb*^{+/+}), Sandhoff untreated (*Hexb*^{-/-} UT), ADLL (*Hexb*^{-/-} ADLL). 1 animal for *Hexb*^{+/+}, 5 animals for *Hexb*^{-/-} UT and *Hexb*^{-/-} ADLL a) Changes in life expectancy (%), mean $\pm$ SD, $*p=0.0157$ (Gehan-Breslow-Wilcoxon test) $n=5$ b) Bar crossing score, $*p=0.0343$, $**p=0.0058$, Mean $\pm$ SD, 2-way ANOVA. c) Representative images of mice bar crossing. d) Front-hind duty cycle and stand mean measurements of *Hexb*^{-/-} UT and *Hexb*^{-/-} ADLL, Mean $\pm$ SD, $*p=0.0323$, $**p<0.0063$ 2-way ANOVAs. e) GSL measurements of forebrain (brain), cerebellum and liver at 86 days of age. Mean $\pm$ SD, $*p=0.045$, 2-way ANOVA.

3.3.2. Targets of ADLL in *Hexb*^{-/-} mouse cerebellum

We next investigated whether the ADLL had the same mechanistic targets in *Hexb*^{-/-} cerebellum compared with *Npc1*^{-/-} cerebellum.

ADLL increases autophagy in *Hexb*^{-/-} mouse cerebellum

We first explored nutrient sensing and autophagy. Leucine is a known mTOR activator through increasing phosphorylation as recently reported in NPC fibroblasts[158]. Both mTOR and phosphorylated mTOR (pmTOR) levels were decreased in *Hexb*^{-/-} cerebellum (mTOR 41.2% $p=0.0404$, pmTOR 48.1%, $p=0.0178$) compared to *Hexb*^{+/+} cerebellum (**Figure 3.2 a, b**). But the ratio between phosphorylated and unphosphorylated forms (p/t) did not differ between *Hexb*^{+/+} and *Hexb*^{-/-} (**Figure 3.2 a, b**). ADLL treatment did not significantly alter the levels of mTOR and pmTOR proteins or the p/t ratio, in comparison to *Hexb*^{-/-} untreated mice (**Figure 3.2 a, b**). We measured the autophagy marker LC3I (cytosolic) and II (lysosomal), along with LC3 substrate p62. Both LC3I and LC3II were upregulated in *Hexb*^{-/-} untreated mice, compared to wild type mice (LC3I: 36.6%, $p=0.004$; LC3II: 50.3%, $p<0.0001$) (**Figure 3.2 c, d**), yet the ratio between lysosomal and cytosolic form, which indicates the autophagic flux did not vary between *Hexb*^{+/+} and *Hexb*^{-/-} (**Figure 3.2 c, d**). ADLL therapy significantly decreased LC3I levels (19.4% by mean, $p=0.0325$) but did not change LC3II levels, consequently II/I ratio notably increased (27.1% by mean, $p=0.0189$) in *Hexb*^{-/-} cerebellum (**Figure 3.2 c, d**). P62 levels were not different in *Hexb*^{+/+} and *Hexb*^{-/-} untreated cerebellums or ADLL treated *Hexb*^{-/-} mice (**Figure 3.2 e, f**).

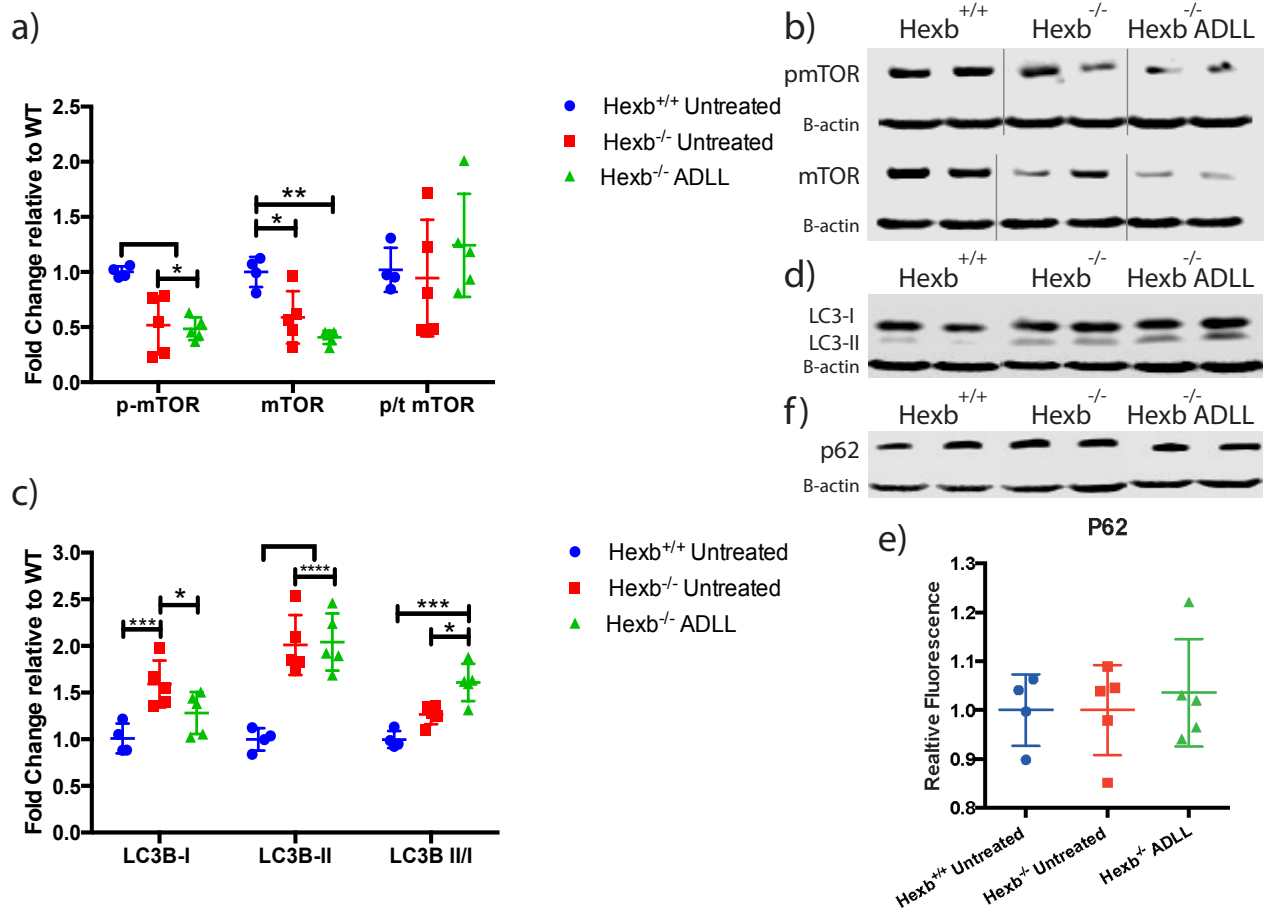


Figure 3.2. Effect of acetyl-DL-Leucine on autophagy in the cerebellum of 12 week old Sandhoff mouse. Wildtype untreated (*Hexb*^{+/+}), Sandhoff untreated (*Hexb*^{-/-} UT), ADLL (*Hexb*^{-/-}ADLL). 4 animals for *Hexb*^{+/+}, 5 animals for *Hexb*^{-/-} UT and *Hexb*^{-/-}ADLL. Proteins of interests were probed by western blotting and normalised to b-actin. Fluorescence values were obtained with ImageJ software and calculated relative to *Hexb*^{+/+}. a) mTOR, pmTOR expressions and mTOR p/t ratios, Mean $\pm$ SD **p*<0.05, ***p*=0.0043 (2-way ANOVA). b) Western blot images of pmTOR and mTOR proteins and their b-actin loading controls. c) LC3-I, LC3-II expressions, and Ratio of LC3B-II versus LC3-II. Mean $\pm$ SD, **p*<0.033 ****p*<0.0005, *****p*<0.0001 (2-way ANOVA). d) Western blot images of LC3s with their b-actin loading controls. e) p62 levels, Mean $\pm$ SD (One-way ANOVA). f) p62 expression on western blot with the loading control.

ADLL has differential effects on metabolic pathways in Sandhoff mouse compared to NPC1 mouse

We investigated ADLL's effect on metabolism in *Hexb*^{-/-} cerebellum. We found no difference in BCKADH protein levels between *Hexb*^{+/+} and *Hexb*^{-/-}, or with ADLL treatment (**Figure 3.3 a, b**). There was also no statistical difference

between healthy and disease group on the phosphorylated form of BCKADH and enzyme activation (ratio between total BCKADH and phosphorylated form: t/p) (**Figure 3.3 a, b**). Although ADLL decreased the phospho-BCKADH levels significantly (24.3%, $p=0.0193$), enzyme activity (t/p) did not alter (**Figure 3.3 a, b**).

Leucine up regulates glutamate dehydrogenase enzyme that catabolizes glutamate into alpha-ketoglutarate, which is an another element in the TCA cycle[162]. We found an upregulation of glutamate dehydrogenase levels in *Hexb*^{-/-}, in comparison to *Hexb*^{+/+} (34%, $p=0.0014$). ADLL supplementation resulted in a decrease in glutamate dehydrogenase by 26.6% in *Hexb*^{-/-} cerebellum ($p=0.0056$), back into the normal range of *Hexb*^{+/+} (**Figure 3.3 b, c**). We then tested its effect on energy metabolism by measuring PGC1 alpha levels. There was no detectable difference on PGC1 alpha protein expression in any group (**Figure 3.3 b, d**).

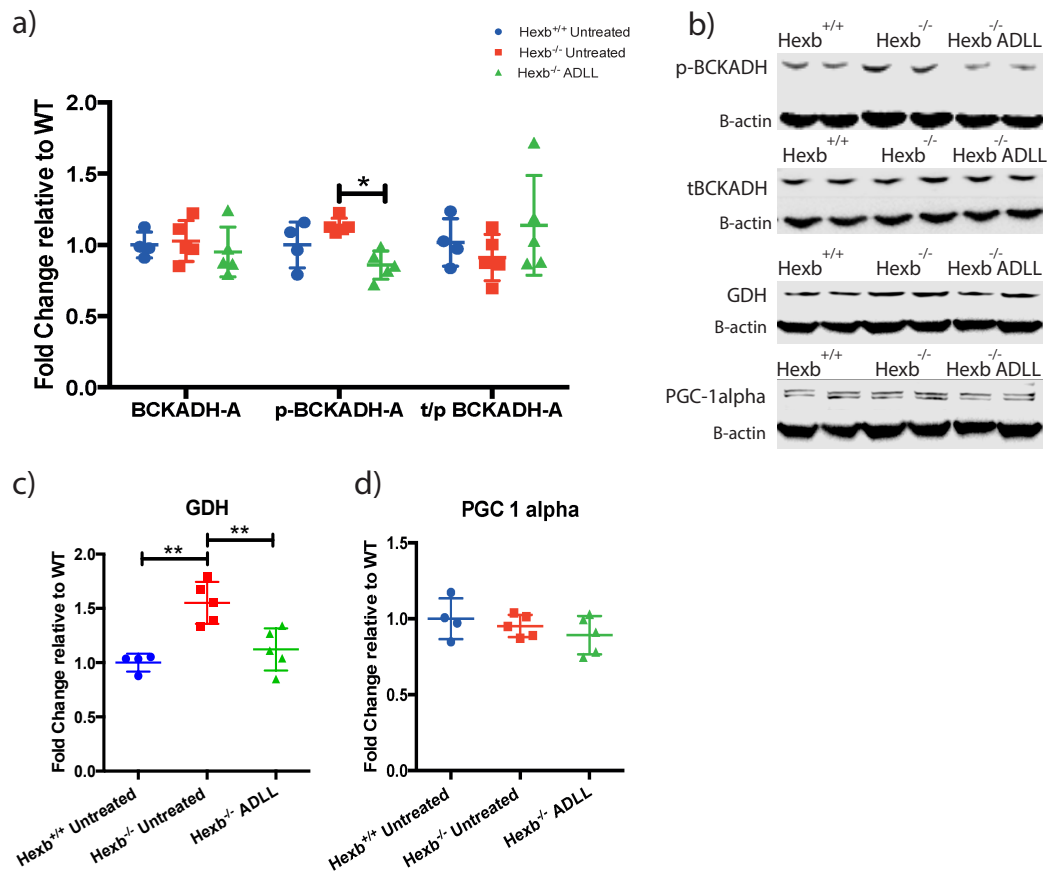


Figure 3.3. Effects of Acetyl-DL-Leucine on branched chain amino acid and glutamate catabolism, and PGC1-alpha levels in the cerebellum of 12-week-old Sandhoff mouse. Wildtype untreated (Hexb^{+/+}), Sandhoff untreated (Hexb^{-/-} UT), ADLL (Hexb^{-/-}ADLL). 4 animals for Hexb^{+/+}, 5 animals for Hexb^{-/-} UT and Hexb^{-/-}ADLL. Proteins of interests were measured by western blotting, normalised to b-actin. Fluorescence values were obtained with ImageJ software and calculated relative to Hexb^{+/+}. **a)** BCKADH, pBCKADH, p/t BCKADH expressions, Mean $\pm$ SD, *p=0.0193 (2-way ANOVA). **b)** BCKADH, pBCKADH, GDH and PGC-1 alpha western blot images with b-actin loading controls. **c)** GDH expression. Mean $\pm$ SD **p<0.0057 (One-way ANOVA). **d)** PGC 1 alpha expression, Mean $\pm$ SD (One-way ANOVA)

ADLL promotes anaerobic glycolysis via PDK4 upregulation

Leucine can also affect glucose metabolism via its effect on insulin[136]. We therefore tested ADLL's effect on glucose utilization by examining pyruvate dehydrogenase and lactate dehydrogenase enzyme profiles. Glucose metabolism enzymes were altered in Hexb^{-/-} cerebellum and also changed by ADLL treatment (**Figure 3.4**). For example, pyruvate dehydrogenase contains 3 subunits (E1, E2,

E3), which convert glycolysis product pyruvate to Acetyl CoA, the prime component of the TCA cycle[214]. Acetyl CoA is subjected to a series of reactions that forms the TCA cycle, and the product coenzyme NADH is later utilized by oxidative phosphorylation in mitochondria (38ATP)[215]. Lactate dehydrogenase, on the other hand, converts pyruvate to lactate in the cytosol, which produces 3 ATP[216].

Protein level measurements of the pyruvate dehydrogenase complex (PDHC) showed that the PDH subunits were not altered in the *Hexb*^{-/-} untreated cerebellum in comparison to WT (**Figure 3.4 a, f**). ADLL treated *Hexb*^{-/-} had increased E3 binding protein (E3bp) (62.7% increase relative to wild-type ($p=0.0105$) (**Figure 3.4 a, f**). In addition, E1alpha levels were increased by ADLL treatment compared to both *Hexb*^{+/+} (41.1%, $p=0.0097$) and *Hexb*^{-/-} (48.8%, $p=0.0010$) (**Figure 3.4 a, f**). PDH is regulated by phosphorylation (pPDH) of its E1 alpha subunit, which leads to the inactivation of the enzyme. Measurements of pPDH and the ratio of the phosphorylated form versus the non-phosphorylated form (p/t) (indicates the enzyme activity) showed that *Hexb*^{-/-} untreated cerebellum had significantly less pPDH than its *Hexb*^{+/+} counterpart (50.8% reduction $p=0.0050$) (**Figure 3.4 b, f**). The enzyme activation (p/t) increased in the *Hexb*^{-/-} untreated group compared to *Hexb*^{+/+} (55.9%, $p=0.0005$) (**Figure 3.4 b, f**). ADLL treatment of *Hexb*^{-/-} mice increased pPDH by 73.9% ($p=0.0270$) relative to untreated *Hexb*^{-/-} mice (**Figure 3.4 b, f**); however and ADLL treatment did not change enzyme activity levels in the *Hexb*^{-/-} cerebellum (**Figure 3.4 b, f**). LDH levels did not change between *Hexb*^{+/+} and *Hexb*^{-/-} groups, while ADLL significantly elevated the enzyme levels in the *Hexb*^{-/-} cerebellum (63.4%, $p=0.0071$) (**Figure 3.4 c, f**). Overall, the ratio between PDH's active site

E1 alpha and LDH showed that there was no significant shift in glucose metabolism preference both between healthy and disease group and with the treatment (**Figure 3.4 d, f**).

Pyruvate dehydrogenase enzyme phosphorylation is regulated by 4 pyruvate dehydrogenase kinases (PDK 1,2,3,4), which phosphorylate the enzyme (inactivation) and 2 pyruvate dehydrogenase phosphatases (PDP 1,2), which dephosphorylates the enzyme (activation)[216].

We measured the enzymes that are highly expressed in the cerebellum; PDK 1, 2, 4 and PDP1. Only PDK2 was significantly altered in the *Hexb*^{-/-} cerebellum, 42.2% lower than in the wild-type group ($p<0.0001$) (**Figure 3.4 e, f**). ADLL treatment did not have any impact on PDK2 levels in *Hexb*^{-/-} mice (**Figure 3.4 e, f**). Nevertheless, PDK4 protein levels were significantly raised in the *Hexb*^{-/-}ADLL treated group, in comparison to *Hexb*^{-/-} untreated (30%, $p<0.0001$) and in comparison to *Hexb*^{+/+} untreated (36.4%, $p<0.0001$) (**Figure 3.4 e, f**). Accordingly, PDP1 levels were slightly decreased following ADLL treatment, but only achieved statistical significance when compared with the *Hexb*^{+/+} group (24.3%, $p=0.0466$) (**Figure 3.4 e, f**).

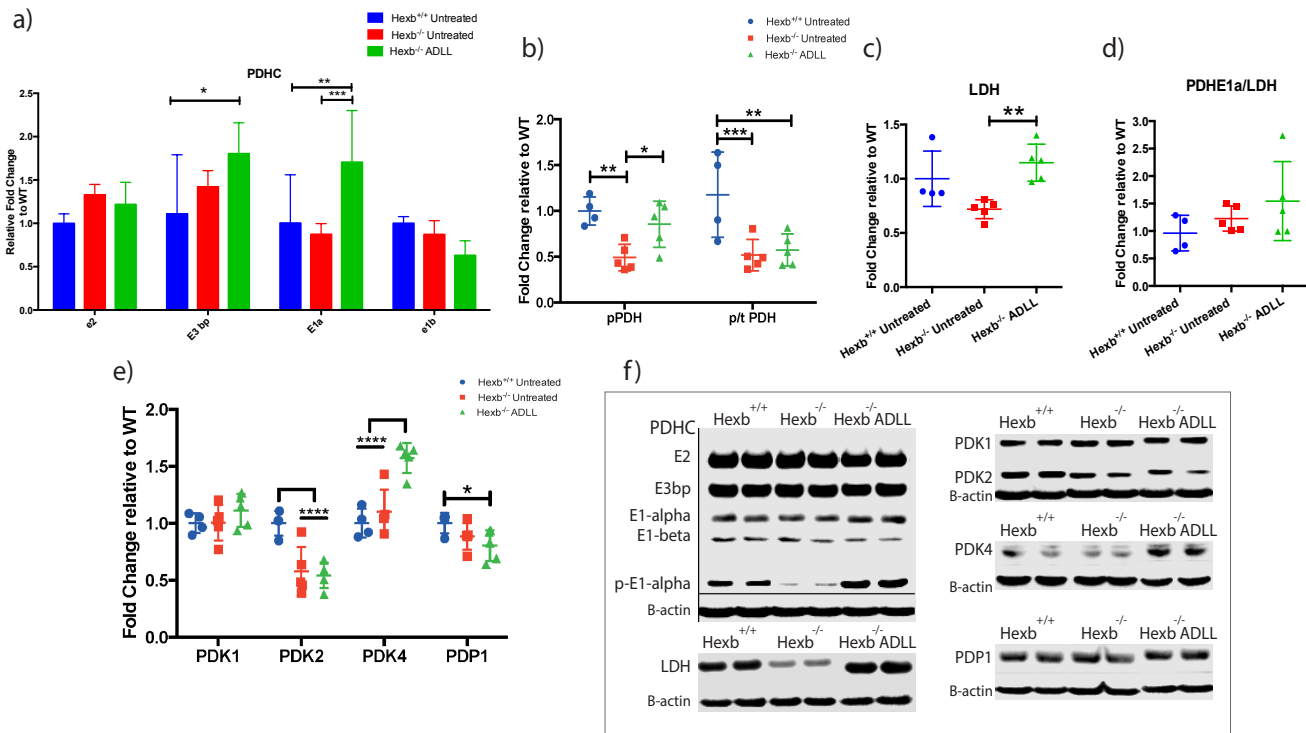


Figure 3.4. Effects of Acetyl-DL-Leucine on glucose metabolism in the cerebellum of 12-week-old Sandhoff mouse. **a)** PDHC protein expression, Mean $\pm$ SD, $*p=0.0105$, $**p=0.0097$, $***p=0.001$ (2-way ANOVA). **b)** pPDH expression and pPDH/PDHE1 alpha ratios, Mean $\pm$ SD, $*p=0.0270$, $**p<0.0052$, $***p=0.0005$ (2-way ANOVA). **c)** LDH expression, Mean $\pm$ SD, $**p=0.0071$ (One-way ANOVA). **d)** Ratio of PDHE1 alpha subunit and LDH, Mean $\pm$ SD, (One-way ANOVA). **e)** PDK 1-2-4 and PDP1 expression, Mean $\pm$ SD, $*p=0.0466$, $****p<0.0001$ (2-way ANOVA). **f)** Western blot images of PDHC, pPDH, LDH, PDKs and PDP1 along with their b-actin controls.

ADLL elevates SOD1 levels

Mitochondrial energy metabolism is a major source of cellular reactive oxygen species (ROS) production [168] and the balance between ROS levels and the activity of the anti-oxidant system is crucial to keep the system healthy. ROS can react directly with DNA, lipids, proteins and can cause cell death via disruption of anti-oxidative defenses. [168] Since leucine can reduce ROS via its effects on energy metabolism[169] and its reduction of superoxide dismutase levels (SOD) [217], we explored ADLL's impact on SOD1 and SOD2 levels in the cerebellum. SOD1 levels were significantly higher in *Hexb*^{-/-} mice than *Hexb*^{+/+}

(11.6%, $p=0.0216$), and ADLL treatment on Sandhoff mice increased its levels in the cerebellum (12.59% by mean, $p=0.0045$) (**Figure 3.5 a, b**). We saw no alteration in SOD2 levels in any of the experimental groups (**Figure 3.5 a, b**).

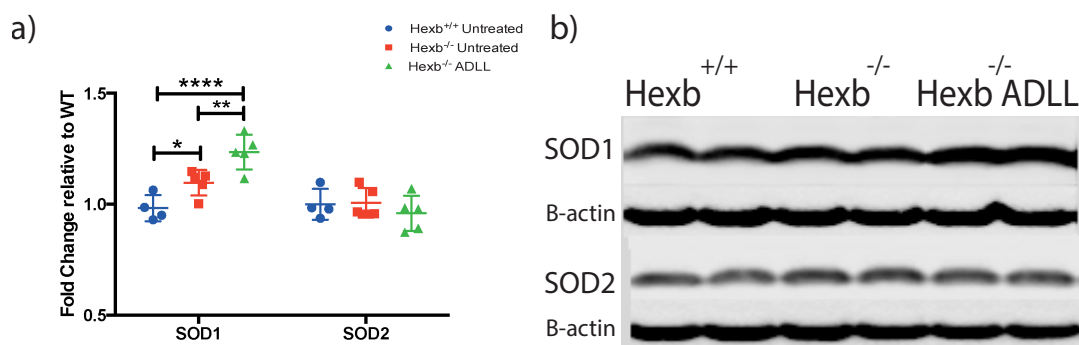


Figure 3.5. Effects of Acetyl-DL-Leucine on antioxidant metabolism in the cerebellum of 12-week-old Sandhoff mouse. a) SOD1 and SOD2 expression, Mean $\pm$ SD, $*p=0.0216$, $**p=0.0045$, $****p<0.0001$ (2-way ANOVA). **b)** SOD1 and SOD2 western blot images and their b-actin loading controls.

3.3.3. Miglustat combination does not improve the lifespan of Hexb^{-/-} mouse

Lastly, we combined same dose of ADLL (0.1g/kg/day) with Miglustat therapy and initiated the treatment from weaning (3 weeks of age). In accordance with previous studies [202], miglustat significantly increased the life expectancy of Hexb^{-/-} mice by 29.5 days in median (Median survival 141.5 days, $p=0.0088$)(**Figure 3.6**). Miglustat treated group lived also longer than ADLL monotherapy by 21.5 days in median ($p=0.0209$) (**Figure 3.6**). Miglustat and ADLL combination therapy resulted in 150 days survival time (in median), however it was not significantly different from miglustat-only therapy ($p=0.321$) (**Figure 3.6**).

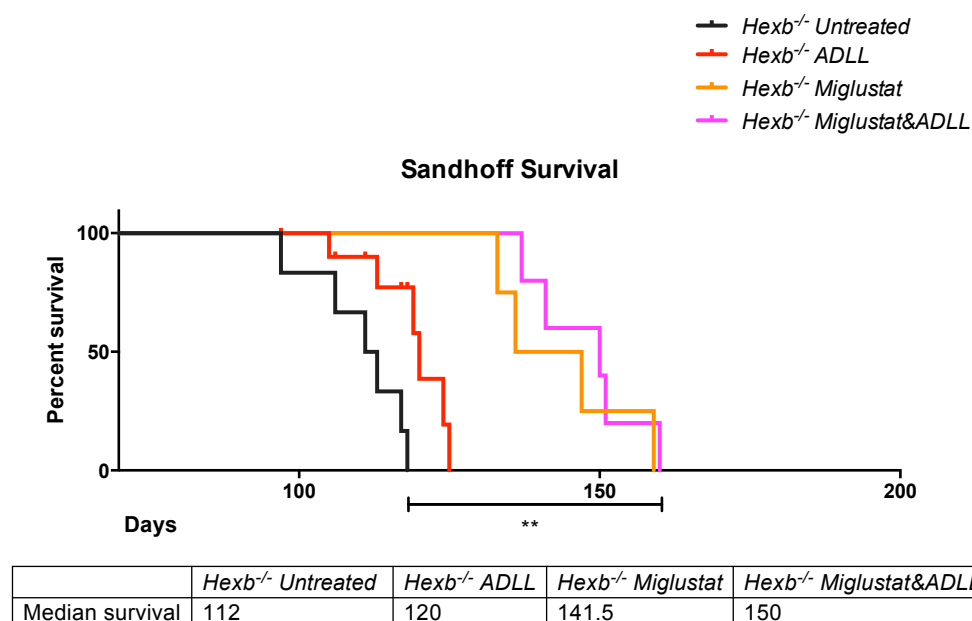


Figure 3.6. Lifespan evaluation of Miglustat vs Miglustat&ADLL therapies of $Hexb^{-/-}$ mouse. Sandhoff untreated ($Hexb^{-/-}$ UT) n=6, Sandhoff ADLL treated ($Hexb^{-/-}$ ADLL) n=6, Sandhoff Miglustat treated ($Hexb^{-/-}$ Miglustat) n=4, Sandhoff Miglustat&ADLL treated ($Hexb^{-/-}$ Miglustat&ADLL) n=5, Changes in life expectancy (%), Median, (Gehan-Breslow-Wilcoxon test).

3.4.Discussion

Sandhoff disease is one of the GM2 gangliosidoses, which shares some cellular pathologies and abnormalities with NPC [218]. Having shown the beneficial effect of Acetyl-DL-leucine in NPC1, we explored the drug's effect on another LSD, Sandhoff disease. We found that ADLL reduced lipid storage in Sandhoff disease and resulted in improvement in lifespan and behavioral parameters. However, ADLL differentially affects metabolic targets in Sandhoff disease cerebellum relative to NPC1.

A Proportional benefit with ADLL on Sandhoff mouse

Pre-symptomatic initiation of ADLL treatment (3 weeks of age), as observed in NPC (4 days in median), extended life expectancy in $Hexb^{-/-}$ mice by a median of 8 days (7.1% increase). Given that $Hexb^{-/-}$ mice live on average 3

We found that Sandhoff cerebellum had lowered mTOR and pmTOR protein levels, compared to *Hexb*^{+/+} mice; nevertheless no difference on mTOR activity (p/t mTOR), autophagic flux rate (LC3-II/LC3-I) and p62 levels were observed in untreated Sandhoff mice. Autophagy induction in *Hexb*^{-/-} mice in mid-brain and hippocampus, but not in the cerebellum was reported in previous studies[159], [219]. However, we found increased levels of both LC3-I and LC3-II in contrast to previous report in *Hexb*^{-/-} mice at end stage (15-16 weeks of age) [159], and also no change in mTOR activity. Given Sandhoff disease is a genetic disease that leads to progressive lipid storage, autophagic flux may alter throughout disease progression. ADLL treatment intriguingly resulted in an elevation of autophagic flux in *Hexb*^{-/-} cerebellum, which might have led to clearance of stored lipids and merits further investigation.

Autophagy is regulated by the cell's energy demands[220]. When there is a shortage of ATP, AMPK senses this reduction and inhibits mTORC1 both indirectly, through phosphorylation and activation of TSC2, and directly through the phosphorylation of Raptor[221]. Inactivation of mTOR upregulates autophagy, which lysosomes takes a major role with their degradatory enzymes to fulfill the cell's energy demands[220]. It has been shown that in *Hexb*^{-/-} brain abnormal autophagy system contributes to mitochondrial dysfunctions; such as increased oxidative stress, disruption of clearance of mitochondrial fragmentation and reduced SOD2 activity [219]. Recently, metabolomics studies of Sandhoff mice and patients samples showed the alterations of protein, lipid and energy metabolism[222]. They observed high protein and lipid catabolism, high levels of reactive oxygen species and oxidative stress markers, which they

argued to be an indication of increased respiratory chain activity[222]. Indeed, in our study, we found that the PDH enzyme, one of the indicators of aerobic respiration[223], was highly active (dephosphorylated) in *Hexb*^{-/-} compared to *Hexb*^{+/+} mice. This finding was consistent with down regulated PDK2 protein levels. Although the overall energy preference, which favors pyruvate utilization, is the opposite in Sandhoff cerebellum relative to NPC, low levels of PDK2 was a shared trait with NPC cerebellum. Acetyl CoA is one of the regulators of PDK2, and it is being utilized in the brain by four main pathways: cholesterol biosynthesis and fatty acid synthesis in glia, acetylcholine synthesis in cholinergic neurons, or entry into the TCA cycle[224]. Lipidomics of *Hexb*^{-/-} mice showed that lipid metabolism, including cholesterol esters and ceramide levels are altered[225], which is also seen in NPC[226]. Due to the accumulation of cholesterol in NPC, cholesterol biosynthesis and also Acetyl CoA levels are reduced[93], [224]. Therefore low PDK2 levels might result from low Acetyl CoA levels in the system, which is a result of down regulation of lipid biosynthesis pathways in Sandhoff disease mouse cerebellum.

ADLL treatment augmented PDH inactivation (phosphorylation) by increasing PDK4 levels, and increased LDH levels, shifting back to levels observed in *Hexb*^{+/+} mice. PDK4 is an essential regulator of glucose metabolism, and it is regulated by starvation, glucose, diabetes, high-fat feeding and nutrient availability[227], [228]. PDK4 overexpression resulted in increased autophagy (elevated LC3-II levels), while its knock out model showed a decrease in starvation-induced-Acetyl CoA-deprivation and autophagy in cells[229]. When glucose is scarce, PDK 4 is upregulated, and its up-regulation leads to fatty acid utilization[230]. PDK4's regulatory effect on glucose utilization in energy

metabolism also leads to an increase in lactate production, which is an indication of an alteration in glucose utilization[178]. Consistent with this, we observed an elevation and normalization in LDH enzyme levels with ADLL treatment. However, overall, PDHE1 alpha subunit and LDH enzyme ratios were similar to *Hexb*^{+/+} in both ADLL treated and untreated *Hexb*^{-/-} mice, in parallel with PGC-1alpha levels, which is a reliable indicator of energy metabolism[166]. All these results indicate that Sandhoff cerebellum has very active aerobic glucose utilization compared to WT cerebellum and ADLL decreases this state, most likely stimulating fat utilization and autophagy for energy production as a result of elevated PDK4 levels. However, how ADLL can trigger catabolic events; such as PDK4 upregulation, autophagy, and lipid oxidation remains unclear and merits further investigation.

ADLL's effect on BCAA and Glutamate catabolism

We found that activity of BCKADH (t/p) did not alter in *Hexb*^{-/-} cerebellum (activated in *Npc1*^{-/-}) while there was an elevation in glutamate dehydrogenase levels (unchanged in *Npc1*^{-/-}), which is in agreement with the recent metabolomics study of *Hexb*^{-/-} mice[222]. Because of the elevated protein catabolism, amino acid levels are highly increased in *Hexb*^{-/-}[222]. ADLL supplementation decreased phosphorylated BCKADH levels, (which activates the enzyme), but overall enzyme activity (t/p) did not change. This indicates that Acetyl-DL-Leucine did not trigger amino acid catabolism any further to produce Acetyl CoA for the TCA cycle.

Alterations in the glutamatergic system are accompanied by excitotoxicity in *Hexb*^{-/-} mice. [231]. Interestingly glutamate metabolism is highly linked with

glucose metabolism, either via albumin, which promotes glucose utilisation (via PDH activation) and glutamate release[232], or via glutamate dehydrogenase, which promotes glutamate entering into TCA cycle[233]. Interestingly, GDH elevation is associated with hyperinsulinism and hypoglycaemia[234]. We observed elevated GDH levels in *Hexb*^{-/-} mice, which might have led to increased insulin levels in the system that results in highly activated PDH enzyme. The insulin-GDH relationship in Sandhoff disease therefore merits further study.

ADLL's effect on the antioxidative system

Sandhoff disease is characterised by elevated ROS, elevated glutathione levels and decreased SOD2 levels[222]. We found elevated SOD1 levels in *Hexb*^{-/-} mice, and unaltered SOD2 levels. Increased SOD1 might be a compensatory mechanism to counteract increased inflammation and to activate the antioxidant systems[235], which is also seen in NPC [93]. Further increase in SOD1 levels with ADLL treatment might occur due to elevated lactate production and JNK activation, which might increase cytoplasmic anti-oxidative players.

ADLL & Miglustat combination is not synergistic on Sandhoff mouse

Combination of ADLL with miglustat, which is a known agent to extend life span[202], did not show any additional benefit on Sandhoff mice, as it was seen in NPC1 (**Chapter 2**). The most prominent metabolic difference between NPC1 and Sandhoff disease is the “opposite” glucose metabolism. Intervention with ADLL treatment to highly inactive PDH in NPC1, a crucial enzyme for neuron survival because of its strong reliance on the oxidative metabolism of glucose[236], might have resulted a substantial life span increase when ADLL combined with lipid lowering drug miglustat in this animal model.

In summary, we found that ADLL treatment attenuates lipid storage, improves behavioral parameters and modestly extends lifespan in Sandhoff mice. However, its mechanism of action is different from NPC and merits further investigation.

Chapter 4: Assessment of the efficacy of leucine amino acid derivatives in NPC1

4.1.Introduction

L-leucine (LL) is one of ten essential amino acids that the human body is unable to synthesize and must therefore be a part of the daily diet, primarily in the form of protein[237]. L-Leucine (LL) serves not only as a building block for protein synthesis but is also a potent activator of the mammalian target of rapamycin (mTOR), involved in many cellular processes, including protein synthesis, cell growth, and metabolism [237]. LL supplementation has been found to be beneficial in many disorders. Increasing LL intake in the diet reduced diet-induced obesity with a decrease in weight gain and adiposity, and also improved insulin sensitivity by alleviating high-fat-diet-induced hypoglycaemia [155]. LL as a known insulin secretagogue [137], [138], which lowers haemoglobin A1C levels and improves glycemic control and alleviates

inflammation in adipose tissue in a mouse models of type 2 diabetes and obesity [182]. LL is widely used as a muscle-building supplement and its effect on increasing muscle protein synthesis is extensively discussed in reference [238]. Branched chain amino acid mixture therapies, containing leucine, was trialled in liver diseases. A mixture of leucine, isoleucine and valine was found to improve cirrhosis complications in a large randomised trial [239]. Xu *et. al.* showed LL supplementation can also be a therapy option for a genetic disease; Roberts Syndrome (RBS)[240]. RBS is an autosomal recessive disease caused by mutations in ESCO2 gene, manifesting prenatal growth retardation (ranging from mild to severe), limb malformations, craniofacial abnormalities and mental retardation[241]. P53 activation and mTOR suppression was associated with ESCO2 mutation and LL relived proliferation and developmental defects linked to RBS phenotype in zebra fish model[240]. LL's direct mTOR activatory effect was also found to be beneficial in another congenital disorder; Diamond-Blackfan anemia (DBA)[242]. DBA is a haploinsufficiency of ribosomal proteins and LL treatment greatly protected against ribosomal protein loss in a zebra fish model[242].

L-Leucine can pass the blood-brain barrier faster than other amino acids. Nozomi *et al.* showed that branched chain amino acids act as neuroprotective agents for cerebellar neurons by giving Aminoleban (L-amino acid cocktail) to patients who have spinocerebellar degeneration, which degeneration or loss of granule cells and Purkinje cells is the main cause, and measuring ataxia in their study [135], [139], [243].

The D enantiomer of leucine (DL) is also found in food and is produced by bacteria, has no known physiological function has been ascribed to it in

eukaryotes [244]. It is ingested from dietary sources and is present in several brain regions. However, it does not incorporate into mammalian proteins, and its mTOR activation potency is weak [245]. *In vivo* studies in horse, mouse and human indicate that D-leucine has analgesic effects via opioid-related mechanisms, inhibiting transport of enkephalins across the blood-brain barrier, and naloxone substantially reverses these analgesic effects [246]. On the other hand, both D and L forms of Leucine separately have been found to be potent in terms of anti-seizure effects in mice, but the D form was more potent, being able to terminate on-going seizures [188], [247].

In this chapter, we evaluated the efficacy of non-N-acetylated leucine amino acids' in NPC1 murine and cellular models. We tested racemic mixture of leucine (DL Leucine-DLL), L Leucine (LL), D-Leucine (DL), also an ester form of Leucine (ethyl leucinate- Et-Leu) with an aim to illuminate whether the beneficial effects of AL compounds (discussed in **Chapter 2**) are due to the N-acetyl modification of leucine amino acid. We also evaluated whether another chemical modification, namely an esterification that is a known drug modification approach to improve the drug's efficacy, can improve the benefit of Leucine amino acid treatment.

4.2. Materials and Methods

4.2.1.Mice

As detailed in [Section 2.2.1.](#)

4.2.2.Cells

Chris Wassif (NIH) kindly provided mycoplasma-free wild type and NPC1 null Chinese Hamster Ovary (CHO) cells.

4.2.3.Drug treatments

DLL (Fluorochem, #080309), LL (Aldrich #L8000), DL (Aldrich, #855448) and Et-Leu (Sigma-Aldrich #61850) (0.1g/kg/day) were administered by mixing the drug in powdered chow. Four to 6 mice were recruited to experimental groups. *In vitro* studies were conducted with each compound dissolved in PBS at 50mM stock solution and diluted to 1mM working concentration in media (DMEM High Glucose-Gibco #10566016).

4.2.4. Behavioural analysis

As detailed in [Section 2.2.3.](#)

4.2.5.Tissue and Cell handling

As detailed in [Section 2.2.4.1](#)

4.2.6.Sphingosine and Sphinganine measurements

[As detailed in Section 2.2.5.](#)

4.2.7.Glycosphingolipid measurements

As detailed in [Section 2.2.6.](#)

4.2.8. Microscopy

As detailed in Section 2.2.8.

4.2.8.1. Filipin Staining of CHO cells

Free cellular cholesterol was visualized with Filipin complex (from *Streptomyces filipinensis* (Sigma)). Cells fixed with 4% paraformaldehyde were washed 3xPBS and incubated with 1.5mg/mL glycine for 10min to quench auto-fluorescence. Cells were incubated with filipin (0.05mg/mL in PBS/10% FBS/0.2% Triton x100) for 2 hours, washed 3xPBS and visualized.

4.2.9. Quantifications by FACS

4.2.9.1. LysoTracker Green and Propidium Iodide Double Staining

In vitro FACS experiments were performed in order to measure relative acidic compartment volume staining live cells with LysoTracker™ Green DND-26 (Thermo Fisher-L7526) at 250nM for 10minutes in PBS at RT, centrifuged at 1200rpm for 10mins and cells resuspended in FACS buffer (PBS, 1% BSA, 0.1% NaN₃ sodium azide). Cells were stained with propidium iodide (20nM) (Invitrogen-P3566) immediately prior to analysis on the FACS instrument for dead cell discrimination. FACS analyses were performed collecting data on 10,000-recorded cells using FACS Canto (Becton Dickinson, (BD)) with BD software.

4.2.9.2. Mitotracker Green and MitoSOX Red Double Staining and FACS analysis

Mitochondrial volume was measured using MitoTracker Green (Invitrogen #M7514), and mitochondrial reactive oxygen species (MitoSOX Red-

Invitrogen #M36008). Live cells were incubated in media with 50nM MitoTracker Green and 5uM MitoSOX Red for 10 minutes at 37°C. Cells then washed two times with warm HBSS containing 5% FCS. FACS analysis was performed on 10,000- cells as described (4.2.9.1). MitoTracker green was detected in the FITC channel and MitoSox-red dye was detected at PE channel.

4.2.10. Image Acquisition and Quantification

As detailed in [Section 2.2.10](#).

4.2.11. Statistical Analyses

As detailed in [Section 2.2.13](#)

4.3.Results

4.3.1.Leucine treatments differentially affect behavioural parameters and lifespan in *Npc1*^{-/-} mice

As discussed in **Chapter 2**, The *Npc1*^{nih} (BALB/cNctr-*Npc1*^{m1N/J}) mouse strain (hereafter referred to as *Npc1*^{-/-}) was used due to it recapitulating the main pathologies of the clinical disorder [151]. Symptoms present at 6-7 weeks of age, including ataxia, gait abnormalities, motor function deterioration, tremor and gradual weight loss. Eventually mice reach a humane endpoint at 10-12 weeks of age[151].

Leucine compounds (DLL-LL-DL-EtLeu) were orally administered to mice from 3-weeks of age (0.1/kg/day in mice) comparable to the dosing regimen used with acetylated leucine compounds in **Chapter 2**.

Untreated *Npc1*^{-/-} mice lived for a median of 87 days; DLL treatment increased the median life span by 7 days (8%, $p=0.0136$) (**Figure 4.1 a**). Other leucine isomers did not have a significant impact on life expectancy (median survivals for LL=88 days, DL=84 days, Et-Leu=82 days) (**Figure 4.1 a**). We then evaluated treatments' performances on behavioural parameters at 8 weeks of age (comparable to the studies in **Chapter 2**). Ataxia measurements at 8 weeks of age in *Npc1*^{-/-} showed gait was normal and comparable to *Npc1*^{+/+}, and leucine treatments did not show any significant impact ($0.21 < p < 0.70$) (**Figure 4.1 b, c**). However, automated gait analysis revealed that untreated *Npc1*^{-/-} mice had increased hind paw duty cycle ($p=0.0071$)[123] (percentage of hind paws in contact with the platform relative to the elapsed time between two consecutive contacts of the same paw) and only DL treatment significantly lowered this parameter, returning back to *Npc*^{+/+} levels ($p=0.0028$) (**Figure 4.1 d**). The speed

of *Npc1*^{-/-} mice was reduced at 8 weeks of age compared to wild type mice ($p=0.0384$) (**Figure 4.1 e**). None of the treatment groups had a significant impact on speed in *Npc1*^{-/-} mice ($p<0.97$) (**Figure 4.1 e**). We also evaluated the mice on the accelerating rotarod to test motor strength, function and coordination[152]. *Npc1*^{-/-} mice fell in a significantly shorter time then their *Npc1*^{+/+} counterparts ($p<0.0001$) (**Figure 4.1 f**). LL and Et-Leu treatments increased the duration of time *Npc1*^{-/-} mice stayed on the accelerating rotarod (*Npc1*^{-/-} LL $p=0.0039$, *Npc1*^{-/-} Et-Leu $p=0.0087$) (**Figure 4.1 f**).

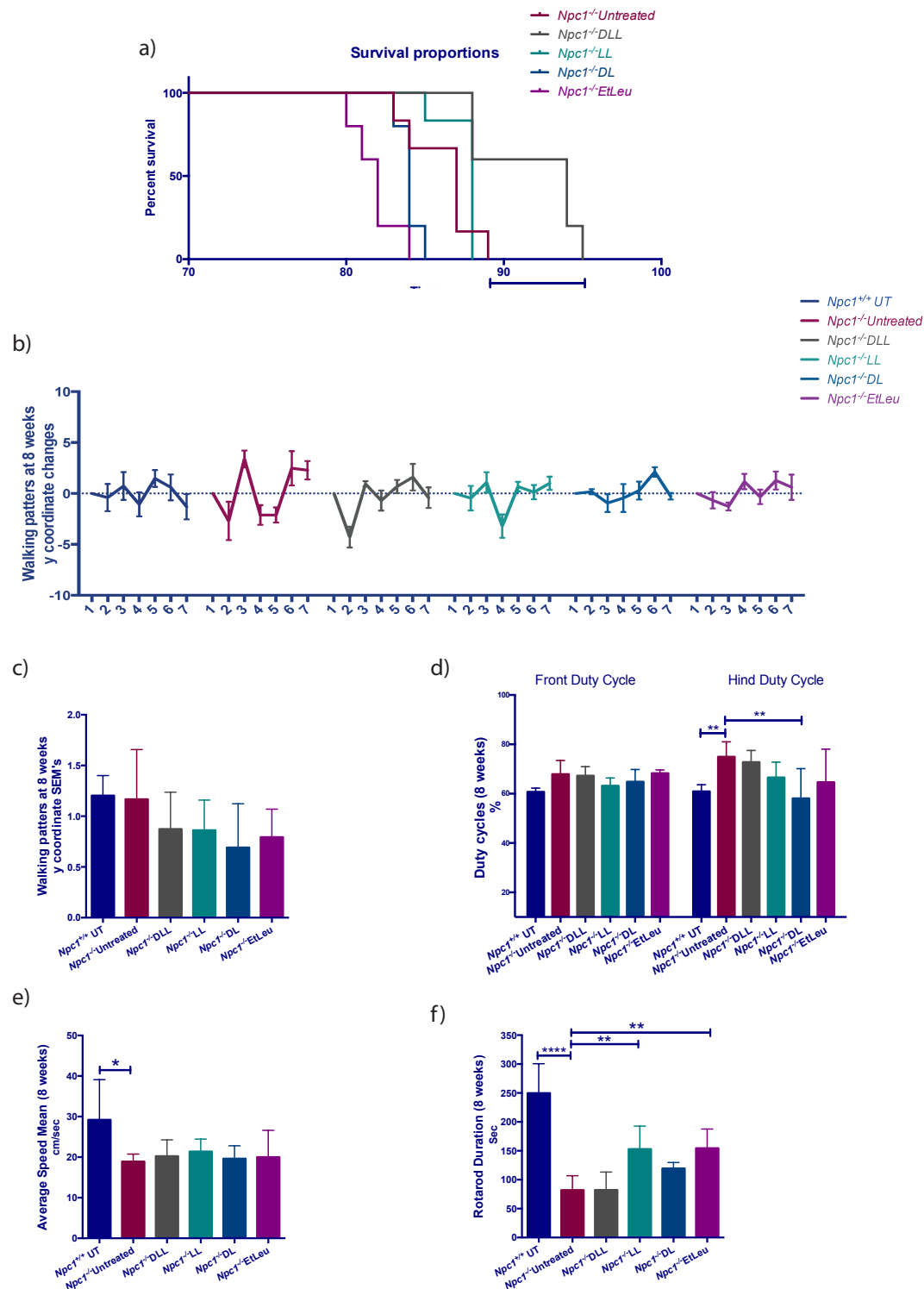


Figure 4.1. The impact of Leucine amino acids on behavioural parameters in NPC1 mice at 8 weeks of age. For wild type untreated (*Npc1*^{+/-} UT), NPC1 untreated (*Npc1*^{-/-} UT), DLL (*Npc1*^{-/-} DLL), LL (*Npc1*^{-/-} LL), ADL (*Npc1*^{-/-} DL), Et-Leu (*Npc1*^{-/-} EtLeu), treatments minimum 4 animals, maximum 6 per group. **a)** Life expectancy, Mean $\pm$ SD, **p*=0.0136 (Gehan-Breslow-Wilcoxon test) *n*=5 **b)** Leucine treatments Y coordinates displacement of each consecutive paw from a straight-line trajectory. (Mean $\pm$ SD, *n*=4)

c) Leucine treatments SEMs of Y coordinate changes, n=4. **d)** Front and hind duty cycle measurements, min 4 animals each group Mean $\pm$ SD, $**p<0.0071$ (2-way ANOVA) **e)** Average speed means, min 5 animals in each group, Mean $\pm$ SD, $*p=0.0384$ (One-way ANOVA) **f)** Motor performance measurements by Rotarod, min 5 animals each group, Mean $\pm$ SD, $**p<0.0088$, $***p<0.0001$ (One-way ANOVA).

4.3.2. Effects of Leucine treatments on biochemical storage: benefit of DL-Leucine in the brain

NPC1 loss of function results in sphingosine and its *de novo* precursor sphinganine being stored, which leads to the secondary accumulation of glycosphingolipids (GSL) and cholesterol [16]. In accordance with **Chapter 2**, we evaluated the effects of leucine isomers on *Npc1*^{-/-} mice on lipid storage in brain and liver at 59 days of age (8.3 weeks). As expected, at 8.3 weeks untreated *Npc1*^{-/-} mice display biochemical and histological pathologies indicative of disease [13]. In the liver, *Npc1*^{-/-} mice displayed substantial accumulation of lipids compared to *Npc*^{+/+} mice: 96.3% more sphingosine ($p<0.0001$) (**Figure 4.2 a**), 99.1% more sphinganine ($p<0.0001$) (**Figure 4.2 b**), 90.4% more total GSL ($p<0.0001$) (**Figure 4.2 c**), and 61.6% more cholesterol content ($p<0.0001$) (**Figure 4.2 d**). None of the isomers significantly lowered lipid storage in the liver (**Figure 4.2 a-d**).

Biochemical analyses on brain showed that *Npc1*^{-/-} untreated brain displayed a significant accumulation of sphingosine (45.5%, $p<0.0001$), sphinganine (42.5%, $p<0.0001$) and total GSL (52.4%, $p=0.045$) in comparison to wild type mouse brain, and only DLL treatment was able to significantly decrease sphingosine and sphinganine, while other leucine treatments failed to show a significant effect (**Figure 4.2 e-g**). *Npc1*^{-/-} DLL group had 19.4% less sphingosine content than *Npc1*^{-/-} UT group ($p=0.0263$) (*Npc1*^{-/-}LL 13.36% decrease,

$p=0.1781$; $Npc1^{-/-}$ DL 11.77% decrease, $p=0.2786$; $Npc1^{-/-}$ Et-Leu 5.45% decrease, $p=0.9056$) (**Figure 4.2 e**). Likewise, sphinganine levels of DLL treated brains were 26.1% lower than $Npc1^{-/-}$ UT group with a statistical significance ($p=0.0074$) ($Npc1^{-/-}$ LL 1.7% increase, $p>0.9999$; $Npc1^{-/-}$ DL 9.3%, $p=0.6455$; $Npc1^{-/-}$ EtLeu 9.9% average decrease, $p=0.5859$) (**Figure 4.2 f**). None of the leucine treatments significantly affected total GSL levels ($Npc1^{-/-}$ DLL 1.7% increase, $p=0.9995$; $Npc1^{-/-}$ LL 19.6% increase, $p=0.7674$; $Npc1^{-/-}$ DL 13.4% decrease, $p=0.7641$, $Npc1^{-/-}$ Et-Leu 3.5% decrease, $p=0.9987$) (**Figure 4.2 g**).

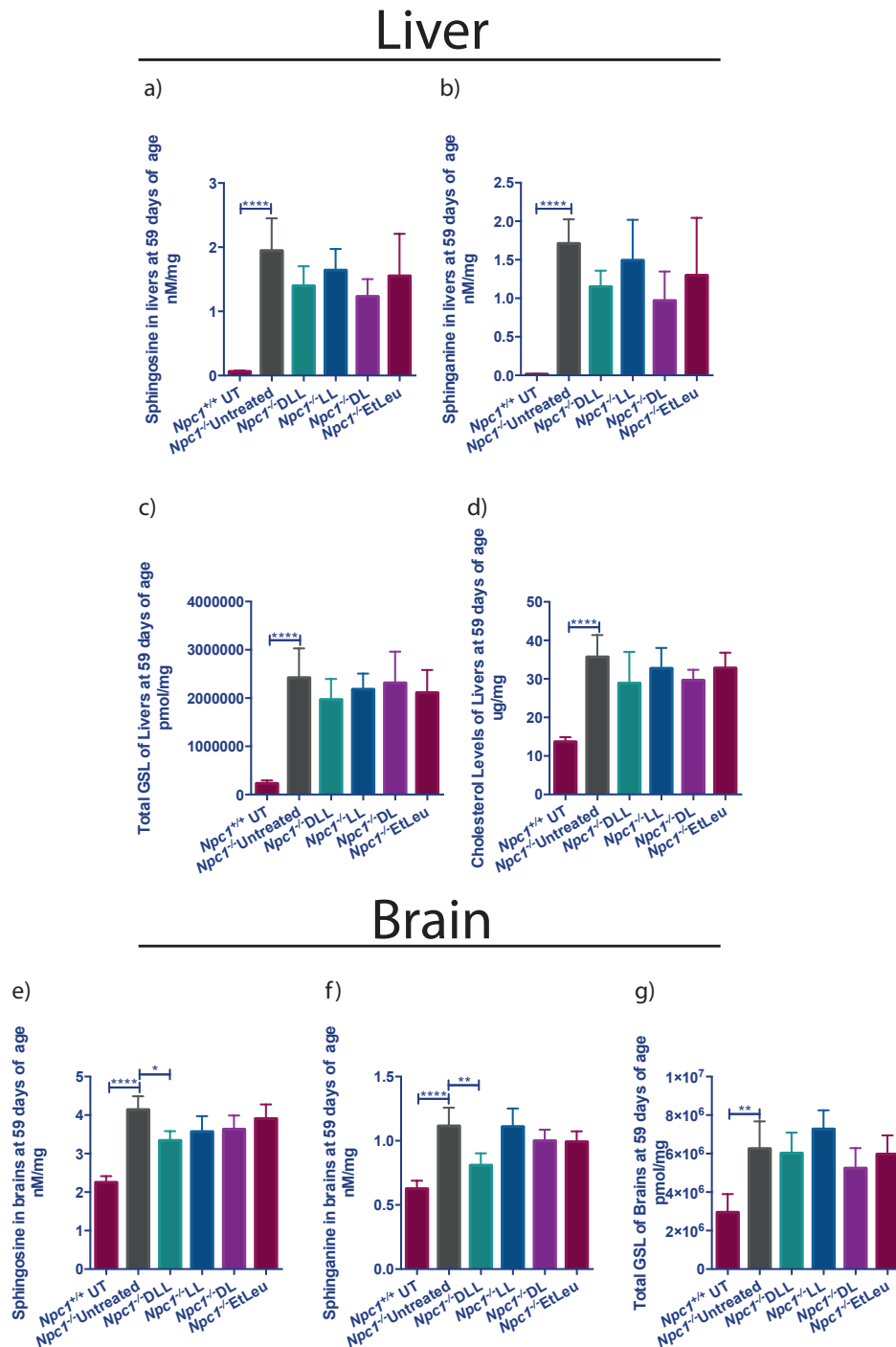


Figure 4.2. Leucine amino acid treatments' efficacy on lipid storage in NPC at 59 days of age. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), DLL (*Npc1*^{-/-} DLL), LL (*Npc1*^{-/-} LL), ADL (*Npc1*^{-/-} DL), EtLeu (*Npc1*^{-/-} EtLeu), min 4 max 6 animals each group. **a)** Sphingosine measurements in livers. Livers were homogenised in water and sphingoid bases were extracted, purified with SPE NH2 column and eluted in acetone. Samples then labelled with OPA. Mean $\pm$ SD, **** p <0.0001 (One-way ANOVA) **b)** Sphinganine levels in livers. Sphinganine measured along with sphingosine in the same HPLC separation. Mean $\pm$ SD, **** p <0.0001 (One-way ANOVA). **c)** Total glycosphingolipid (GSL) measurement in liver. GSLs were extracted from water-homogenised tissues with Svennerholm extraction, followed by recombinant EGCase

enzyme digestion and anthralinic acid labelling. Mean $\pm$ SD, **** p <0.0001 (2-way ANOVA). **d)** Cholesterol levels in liver. Cholesterol was extracted from homogenates (in water) with Folch extraction and assayed with Amplex Red Cholesterol Assay Kit (Invitrogen). Mean $\pm$ SD, **** p <0.0001 (One-way ANOVA). **e)** Sphingosine levels in brain. Mean $\pm$ SD, * p = 0.0263, **** p <0.0001(One-way ANOVA). (Performed by Judy Yau) **f)** Sphinganine levels in in brain. Mean $\pm$ SD, ** p =0.0074, **** p <0.0001(One-way ANOVA). (Performed by Judy Yau) **g)** Total GSL profiles in brain. Mean $\pm$ SD, ** p =0.0045 (One-way ANOVA).

4.3.3. Leucine treatments on cerebellar pathology: L-Leucine and Et-Leucinate were beneficial

Npc1^{-/-} mice exhibit progressive neurodegeneration, with cerebellar Purkinje cell loss progressing from anterior to posterior lobes [153]. Microglial activation is also a feature of CNS pathology (**Figure 4.3 a**). We therefore tested leucine treatments on neurodegeneration and glial cell activation at 59 days of age. Only Et-Leu was able to significantly enhance Purkinje cell survival (123% at lobule 1 and 2) (p =0.0354) (**Figure 4.3 a, b**). CD68 activated microglia cell density (cell number/area) decreased with LL treatment by 16.6% (p =0.0263), and with Et-Leu treatment by 19.3% (p =0.0351) (**Figure 4.3 a, c**).

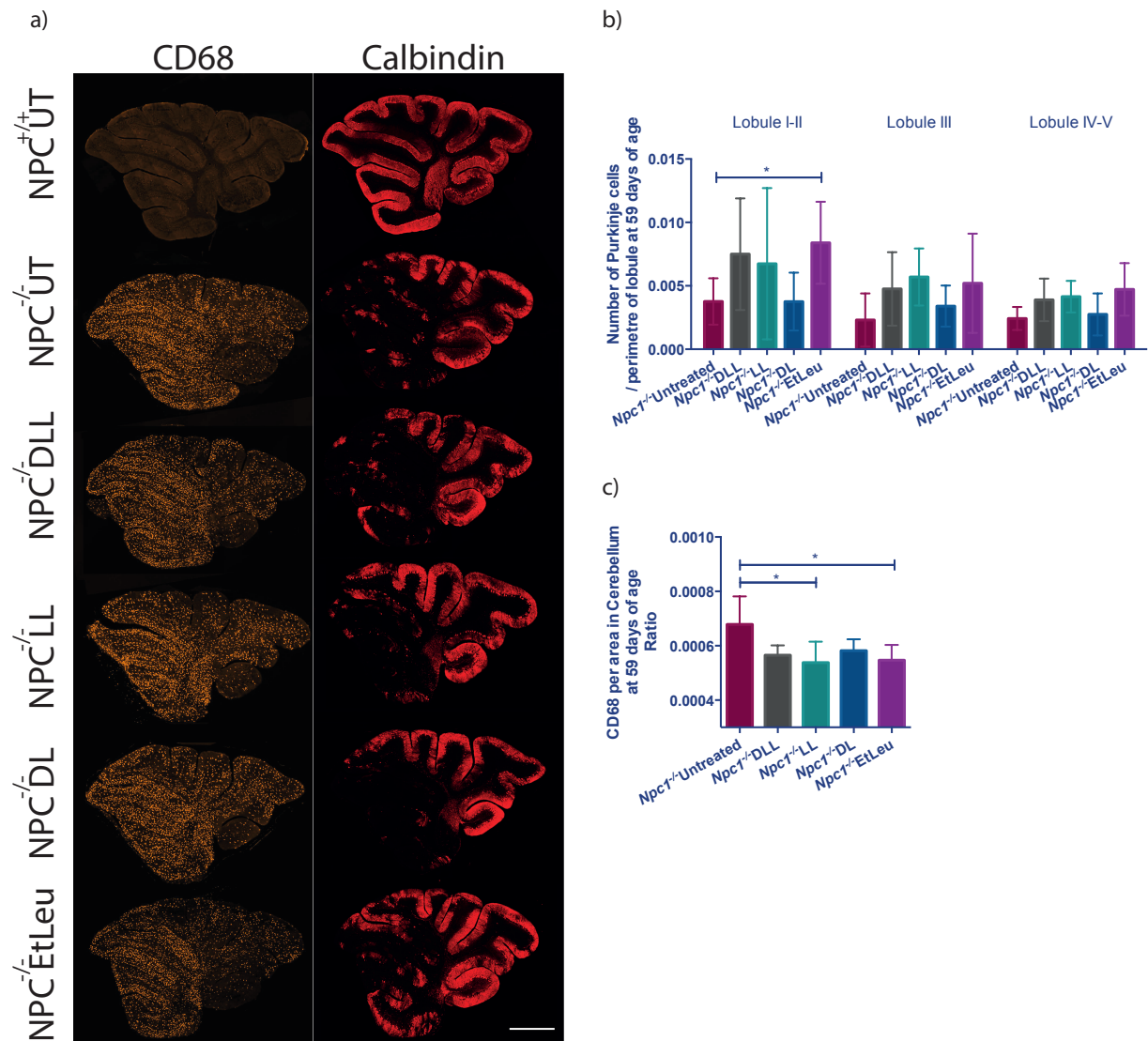


Figure 4.3. Effects of leucine amino acids in NPC cerebellum at 59 days of age. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), DLL (*Npc1*^{-/-} DLL), LL (*Npc1*^{-/-} LL), ADL (*Npc1*^{-/-} DL), EtLeu (*Npc1*^{-/-} EtLeu), treatments n=4 animals for each group. **a)** Fluorescence images showing changes between wild type and NPC and with treatments. Sections were stained with either calbindin-b (Purkinje cells, red) or CD68 (activated microglia, orange). Scale bar, 800 um. (Performed by Judy Yau) **b)** Purkinje cell density in lobule I-II, III and IV-V at 59 days of age relative to NPC untreated. Mean $\pm$ SD, * $p < 0.011$, ** $p < 0.028$ (One-way ANOVA) (Performed by Judy Yau) **c)** CD68 activated glial cell density in cerebellum at 59 days of age. Mean $\pm$ SD, * $p < 0.046$, *** $p < 0.0009$ (One-way ANOVA). (Performed by Judy Yau)

4.3.4. Leucine treatments in vitro: effects on CHO cells

We tested leucine treatments (1mM for 72 hours) on NPC1 deficient Chinese Hamster Ovary (CHO) cells to gain further insight on cellular impact of the treatments, utilising lysosomal and mitochondrial probes as well as measurements of pathologic storage lipids..

As predicted, total acidic compartment volume (measured with LysoTracker) in NPC1 CHO cells was 43% higher than wild type (WT) CHO cells ($p<0.0001$) (**Figure 4.4 a**)[248]. Due to the disruption of the clearance of mitochondrial fragments and the production of superoxide in the NPC1 cell [249], mitochondrial volume (MitoTracker) in NPC1 cells was 29.3% larger and superoxide content was 28% higher than in WT CHO cells ($p<0.0001$) (**Figure 4.4 a**). 72-hour 1mM treatment with leucines resulted a significant decrease in acidic compartment volume (23% by DLL, 22% by LL, 24% by DL, 26% by Et-Leu, $p<0.0001$), but not at WT levels (**Figure 4.4 a**). On the other hand, all treatments lowered mitochondrial volume (35% by DLL, 32% by LL, 37% by DL, 28% by Et-Leu, $p<0.0001$), normalising to WT levels (**Figure 4.4 a**). Mitochondrial superoxide levels were reduced by all the treatments (16% by DLL, $p=0.0012$; 21% by LL, $p<0.0001$; 16% by DL, $p=0.0011$; 23% by Et-Leu, $p<0.0001$), yet only LL and Et-Leu treatments were able to return the levels back to wild type levels (**Figure 4.4 a**).

Assessments of known biochemical storage lipids showed that NPC1 CHO cells had substantial accumulation of sphingosine (61%, $p<0.0001$), GSL (53%, $p<0.0001$) and free cholesterol (100%, $p<0.0001$) compared to WT (**Figure 4.4 b**). Cellular sphingosine levels were decreased with a statistical significance by

DLL (45%, $p=0.0025$ -normalised to WT levels), DL (32%, $p=0.0161$) and Et-Leu (45%, $p=0.0010$ -normalised to WT levels), but not with LL. Total GSL levels meaningfully decreased with only DL (28%, $p=0.0392$) and Et-Leu (28%, $p=0.0370$) treatments, being statistically indifferent from WT group (**Figure 4.4 b**). Finally, stored cholesterol decreased with all leucine treatments, (38% by DLL, 32% by LL, 28% by DL, 34% by Et-Leu, $p<0.0001$), however they did not normalise cholesterol levels (**Figure 4.4 b, c**).

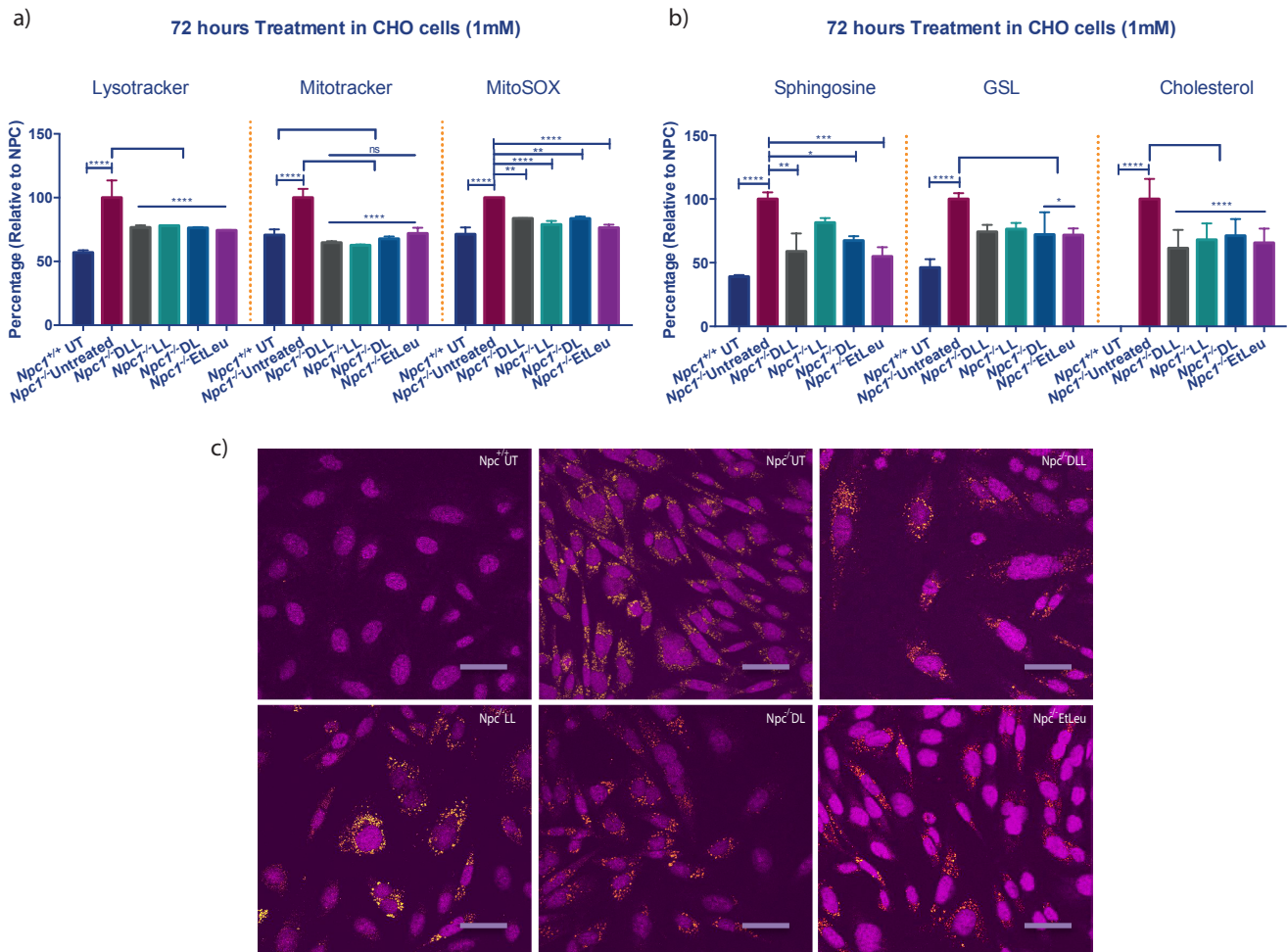


Figure 4.4. Effects of leucine amino acid treatments on NPC CHO cells. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), DLL (*Npc1*^{-/-} DLL), LL (*Npc1*^{-/-} LL), ADL (*Npc1*^{-/-} DL), EtLeu (*Npc1*^{-/-} EtLeu), treatments n=3 for each group. **a)** Lysotracker, Mitotracker, MitoSOX fluorescence % values after 72h treatments, relative to NPC1 UT, Mean $\pm$ SD, * $p < 0.0013$, **** $p < 0.0001$ (2-way ANOVA). **b)** Sphingosine, total GSL and free Cholesterol percentages, relative to NPC1 UT, Mean $\pm$ SD, * $p < 0.04$, ** $p < 0.004$, *** $p = 0.001$ **** $p < 0.0001$ (2-way ANOVA). **c)** Representative images of CHO cells with and without treatments. Filipin for cholesterol (yellow) and propidium iodide for nucleus (pink), scale bar 20μm.

4.4.Discussion

In this chapter, we evaluated essential amino acid leucine's effects in models of NPC disease, utilising both NPC1 deficient mice and CHO cells. We trialled L and D enantiomers as well as the racemic mixture (DL). We also evaluated an esterified Leucine (results summarised in **Table 4.1** and **Table 4.2**).

4.4.1.Racemic mixture performed better than individual enantiomers

The evaluation of leucine isomers in NPC1 deficient mice (**Table 4.1**) showed that DL-Leucine (DLL) significantly improved a number of parameters more than individual enantiomers (LL, DL). Life expectancy of the NPC1 mice significantly increased with DLL, while individual enantiomer-only therapies were comparable to the untreated group. Interestingly, although LL therapy improved motor function and reduced CD68 microglial staining the DL therapy improved some gait parameters whereas the racemic mixture treated mice failed to show any improvement in these parameters. On the other hand, DLL lowered the pathologic accumulation of sphingosine and sphinganine, but the individual enantiomers did not alter these aspects. These results indicate that L and D forms have different targets and synergise when combined resulting in life span extension and a reduction in sphinganine levels. However, activity loss occurred in several parameters that LL and DL individual therapies improved, possibly due to the dose reduction of L and D leucine in DLL therapy (50% LL+ 50%DL).

	<i>In vivo</i> Parameters	DLL	LL	DL	ETLEU
1	Survival	+	0	0	0
2	Gait Duty cycle	0	0	+	0
3	Average Speed	0	0	0	0
4	Rotarod	0	+	0	+
5	Liver Sphingosine	0	0	0	0
6	Liver Sphinganine	0	0	0	0
7	Liver GSL	0	0	0	0
8	Liver Cholesterol	0	0	0	0
9	Brain Sphingosine	+	0	0	0
10	Brain Sphinganine	+	0	0	0
11	Brain GSL	0	0	0	0
12	Cerebellum CD68	0	+	0	+
13	Cerebellum Purkinje cells	0	0	0	+

Table 4.1. Summary of leucine treatments in *Npc1* knock-out mouse model. (+) indicates statistically significant improvement, (0) indicates no effect was seen with treatments.

At the cellular level (**Table 4.2**), the racemic mixture and individual enantiomers reduced total acidic compartment volume and free cholesterol equally, yet none of the treatments were able to normalise to WT levels. Sphingosine accumulation was lowered with DL, did not change with LL, but normalised with DLL. GSL storage was decreased (not normalised) in cells with DL, but not with LL and DLL. Total mitochondrial volume was returned back to WT levels with DLL, LL and DL. Mitochondrial superoxide build-up was rescued with LL, significantly lowered with DL and DLL. All these data strengthen the hypothesis that the D and L enantiomers have different mechanistic targets and the most robust organelle target of leucine (both L and D form and the racemic mixture) was mitochondria in the NPC cell model. It is known that L-Leucine supplementation effects several mitochondrial parameters; increases total energy expenditure and increases mitochondrial biogenesis in skeletal muscle cells and adipocytes and in liver [250], [251]. It also reduces antioxidant

enzymes (superoxide dismutases) when rats are challenged with high cholesterol diet[217]. In addition, our results on alleviating ROS in cells with leucine amino acid by using MitoSOX as an indicator was compatible with previous studies performed on epithelial cells [169]. We hypothesise that the mitochondrial aspects that were rescued and alleviated with Leucine therapies might have a secondary beneficial impact on lysosome-derived abnormalities in NPC1. Further studies are needed to discover the actual targets and how these targets affect lysosomal pathology and the relationship between the acetylated and non-acetylated leucine derivatives.

	<i>In vitro</i> Parameters	DLL	LL	DL	ETLEU
1	Acidic compartment volume	+	+	+	+
2	Mitochondrial volume	=	=	=	=
3	Mitochondrial Superoxide	+	=	+	=
4	Sphingosine	=	0	+	=
5	GSL	0	0	+	+
6	Cholesterol	+	+	+	+

Table 4.2. Summary of leucine treatments in *Npc1* knock out CHO cells. (=) indicates a normalisation to wild type level, (+) indicates statistically significant improvement compared to NPC1 untreated level, (0) indicates no effect was seen with treatments.

L and D leucine synergy in DL-Leucine therapy might have led to a decrease in sphingosine and sphinganine, which might increase the life expectancy in NPC1 mouse model. Sphinganine (dihydrosphingosine) is the *de novo* precursor of sphingosine and dihydroceramide, which further produces ceramide. Dihydroceramide has been found to induce apoptosis and cell cycle arrest[189]. Similarly, accumulation of ceramide has been found to associate

with an induction of ROS production and cell death[190], [191]. Both ceramide and sphingosine independently have been found to lead to mitochondrial apoptotic pathway activations (BCL2 BCL-XL families) apart from their effect on protein kinase C [252]. It has been shown that long chain sphingoid bases (dihydrosphingosine and pytosphingosine) accumulation mediates an increase in oxidative stress markers and mitochondrial fragmentation and dysfunction in the NPC1 yeast model, results in decrease in chronological lifespan [253]. Thus, sphingosine-lowering effects of DLL (shown in both murine and the cellular model) might be resulting in mitochondrial rescue, improving life expectancy.

4.4.2.Esterification of L-Leucine improves L-Leucine efficacy

Our studies showed that L-Leucine ethyl ester (Et-Leu) therapy improved more parameters that were improved in *Npc1*^{-/-} mice and in NPC1 CHO cells than L-leucine therapy. Et-Leu treated animals showed significantly improved motor performance (rotarod), and decreased the number of active microglial and increased the number of surviving Purkinje cells in the cerebellum, while L-leucine only improved motor function and lowered activated microglia numbers (**Table 4.1**). At the cellular level, Et-leu rescued mitochondrial volume, mitochondrial superoxide comparable to LL, yet it also normalized sphingosine levels (no effect was seen with LL). In addition, ethyl esterification of leucine resulted in decreased GSLs, reduced lysosomal volume and lower cholesterol levels (**Table 4.2**).

Esterification of drugs is a common modification used to enhance delivery and efficacy, and to prevent drugs undergoing some in vivo biotransformation through chemical or enzymatic cleavage[254]. The ester (ethyl) form of the

amino acid tryptophan has been used to treat another genetic disease, Hartnup disease, to overcome the defective intestinal transport (which arises from defective lysosomal transport systems) of tryptophan therapy[255]. Overall the data suggest that ethyl esterification is able to increase the efficacy of native L-leucine amino acid.

In summary, we trialed leucine amino acid and its isomers, and its ethyl ester form in NPC1 deficient murine and cell models and found that they have therapeutic potential in several abnormalities in NPC disease. Racemic mixture of leucine (DLL) was able to extend the survival in mice and shows great promise as a noninvasive supplementary therapy. Esterification of L-Leucine (Et-Leu) resulted in increase in potency of amino acid, which suggests an availability of drug-design approaches for leucine amino acid for NPC.

Chapter 5: Exploring Acetyl-Leucine's protein targets in NPC1 cells

5.1.Introduction

Acetyl-DL-leucine (ADLL) is a racemic (1:1) mixture of enantiomers and a derivative of the branched chain amino acid leucine, which was discussed in previous chapters. There are different hypotheses about the target of ADLL including effects on ion channels[134], glutamate neurotransmission in the cerebellum via the branched-chain amino acid transferases [256] and neural metabolism via binding to membrane phospholipids [147]. In **Chapter 2**, we showed ALs effects on energy and antioxidant metabolism in NPC1 cerebellum, with no detectable effect on known leucine amino acid interactor mTOR [257]. Therefore to better understand the targets of AL we initiated proteomic-binding studies to determine their protein “interactome”.

In this chapter, we investigated the effects of ALs in NPC CHO cells and conducted a “drug target” study employing streptavidin-biotin interaction on NPC murine fibroblasts[258]. We used biotin conjugated ALL and acetyl-D-leucine (ADL) and found the interacting proteins of the compound using a discovery proteomics approach.

5.2. Materials and Methods

5.2.1.Cells

Chris Wassif (NIH) kindly provided wild type and NPC1null Chinese Hamster Ovary (CHO) cells. Primary mouse fibroblasts were derived from BALBc/NPC^{nih} mice (*Npc1*^{-/-}) and (*Npc1*^{+/+}). Mice were bred as heterozygotes to generate affected mice and control genotypes, and were housed under non-sterile conditions, with food and water available *ad libitum*. All experiments were conducted using protocols approved by the UK Home Office Animal Scientific Procedures Act, 1986.

5.2.2.Drug treatments

ADLL and enantiomers (ADLL: Molekula #73891210, ALL: Sigma Aldrich #441511, ADL: Sigma #A0876) were dissolved in PBS at 50 mM stock solution and diluted to 1mM working concentration in media (DMEM High Glucose-Gibco #10566016).

5.2.3. Cell handling

As detailed in [Section 2.2.4.1](#)

5.2.4.Sphingosine and Sphinganine measurements

As detailed in Section 2.2.5.

5.2.5.Glycosphingolipid measurement

As detailed in Section 2.2.6.

5.2.6.Cholesterol measurement

As detailed in Section 2.2.7.

5.2.7.Quantifications by FACS

5.2.7.1.Lysotracker Green and Propium Iodide Double Staining

As detailed in Section 4.2.9.1

5.2.7.2.Mitotracker Green and MitoSOX Red Double Staining

As detailed in Section 4.2.9.2

5.2.8.Sample Preparation for Mass Spectroscopy

Cells were lysed in 1% Digitonin for 30 minutes on ice, and spun down at 13,000 rpm for 10 minutes. Pellets were discarded. 30ul Streptavidin beads (Thermo Fisher Pierce Streptavidin Agarose Resins, #20361) were washed three times with 1% Digitonin (1mL) and spun down at 500g for 2 minutes. Biotin-ALL stock solutions (in PBS) were diluted in 1% Digitonin at the sufficient concentration to coat the beads (Binding capacity: 1uL resin-10ug biotin compound). Streptavidin beads were incubated with Biotin-ALL compounds for 15 minutes at room temperature by vigorously shaking. The excess of drug solution was removed from the beads by spinning the beads at 500g for 2 minutes, followed by washing the beads with PBS (1ml) two times. Protein

lysates (with protease inhibitor) were incubated on the beads in protein lysate for 30 minutes (with constant agitation) at 4 °C. Beads were spun down at 500g and washed with 0.1% Digitonin (1mL) (x3). Protein elution from the beads was achieved by boiling with the addition of 30ul Tris-HCl (pH 6), followed by addition of Lithium borate buffer (LB buffer) (with DTT) and boiled at 96 °C for 6 minutes. Protein elution with D-Biotin (50mM aqueous solution, Invitrogen #B20656) was performed by adding "D-Biotin" solution (2.5mM Biotin solution 150mM NaCl+100mM Tris HCl+1mM EDTA pH=8) with 1:3 ratio (1-part volume of beads: 3 part volume of D-Biotin sol.) by vigorously shaking at RT for 20mins. Beads were spun down at 2000rpm for 5 minutes, and supernatants were obtained. LB buffer (with DTT) was added on supernatant samples were boiled at 96 °C for 6 minutes.

Denatured protein elutions were then loaded on 10% acrylamide gel and Coomassie stained.

5.2.9.Mass Spectroscopy

The gel stained with Coomassie Brilliant Blue R-250 was cut into pieces and every lane was divided into five or six parts that were separately injected for MS analysis. Gel pieces were destained with 50 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN) and dehydrated with ACN, for 10 minutes at room temperature. Mass spectroscopy protocol was obtained from Stefana Petrescu Lab (Romanian Academy, Bucharest).

Reduction and alkylation of proteins.

Disulfide bonds were reduced with 10 mM dithiothreitol (DTT) in 100 mM ABC, by incubation for 45 minutes in the dark at 56°C with occasional vortexing. The alkylation of the free cysteines was achieved in the presence of 50 mM iodoacetamide (IAA) in 100 mM ABC, by incubation for 50 minutes in the dark with occasional vortexing. Gel pieces were washed with 50 mM ABC in 50% ACN, and ACN by incubation for 15 minutes at room temperature, followed by the drying stage in the Speed-Vac concentrator.

Enzymatic digestion.

For proteins trypsinization, gel pieces were hydrated with 5.6-6.5 ng/μL trypsin solution and incubated 30 minutes on ice, followed by gradual addition of trypsin solution depending on the volume of solution absorbed. The samples were further incubated overnight at 37°C.

Peptide extraction.

The peptides resulted after trypsinization were extracted with 5% formic acid (FA) and 50% ACN (in 1:2 ratio digestion volume/extraction volume), by incubation for 15 minutes at 37°C (2 times) and 5% FA in ACN, for 30 minutes at 37°C (1 time) with moderate agitation. All solutions containing mixtures of peptides were combined and dried in Speed-Vac concentrator, and used further for LC-MS/MS analysis.

LC-MS/MS analysis.

The dried peptides were solubilized in 20 μL mobile phase A (0.1% FA + 2% ACN) and 5 μL from each sample were injected into a nano-liquid chromatograph (nano LC) Easy nano LC II (Proxeon Biosystems) coupled online to a LTQ Orbitrap Velos Pro instrument (Thermo Fisher Scientific). For peptide

separation a 2-30% gradient of mobile phase B (0.1% FA + 98% ACN) was applied to a dual system composed of a C18 trap column 2 cm × 100 µm (Proxeon Biosystems) and a C18 analytical column, 10 cm × 75 µm (Proxeon Biosystems) connected to a Stainless Steel emitter (Thermo Scientific) for electrospray ionization. The mass spectrometer performed by acquiring full MS scan between 300-1800 m/z in the Orbitrap at a resolution of 30000 at m/z4, followed by 10 consecutive MS/MS scans in the ion trap using Collision Induced Dissociation (CID) for peptide fragmentation.

Peptide and protein identification.

The spectrum files were searched using the SEQUEST HT algorithm, integrated into Proteome Discoverer v1.4 against a human version of Swiss prot database (20238 sequences as of 08/2013) with the following settings: precursor mass tolerance of 20 ppm, ion fragment mass tolerance of 0.6 Da, carbamidomethylation of Cys residues as a static modification, and oxidation of methionine and deamidated of asparagine and glutamine as the dynamic modifications. For peptide confidence assignment a decoy database was searched in parallel containing the reversed sequences from the initial database. Only peptides with a precursor mass tolerance of maximum 10 ppm and a 99% estimated confidence were kept in the final results. At least 1 unique peptide was required to validate the identification of a protein group.

5.2.10. Statistical Analyses

As detailed in [Section 2.2.13](#)

5.3.Results

As discussed in **Chapter 4**, NPC1 protein deletion/mutation results in an overall acidic compartment volume increase (measured by LysoTracker), overall mitochondrial compartment increase (measured by MitoTracker-membrane potential independent), mitochondrial superoxide increase (measured by MitoSOX); and biochemical accumulation of sphingoid bases sphingosine and sphinganine, GSL and cholesterol. First, we evaluated these parameters to assess the efficacy of acetyl-leucine compounds (ADLL, ALL, ADL), using a CHO cell model (**Figure 5.1**). CHO cells were treated with 1mM ALs for 24 hours and resulted in normalisation of MitoSOX staining with all three drugs ($p<0.0001$, compared to NPC1 UT), significant decrease in MitoTracker Green ($p=0.0009$ ADLL; $p=0.0002$ ALL; $p=0.0005$ ADL) but reduction in acidic compartment volume only occurred with ADLL ($p=0.0009$) and ALL ($p=0.0014$), not with ADL ($p=0.2288$) (**Figure 5.1 a**). Sphingosine levels were significantly lower than NPC UT by 22% with ADLL ($p=0.0009$), 29% with ALL ($p<0.0001$) and 14% with ADL ($p=0.0149$) (**Figure 5.1 b**). Total GSL levels reduced significantly with all treatments in similar magnitudes (26% with ADLL $p=0.0007$; 25% with ALL $p=0.0010$; and 22% with ADL $p=0.0024$) (**Figure 5.1 b**). Likewise, cholesterol content was significantly lower than untreated NPC1 CHO cells (18% with ADLL $p=0.0043$; 13% with ALL $p=0.0263$; and 14% with ADL $p=0.0212$) (**Figure 5.1 b**).

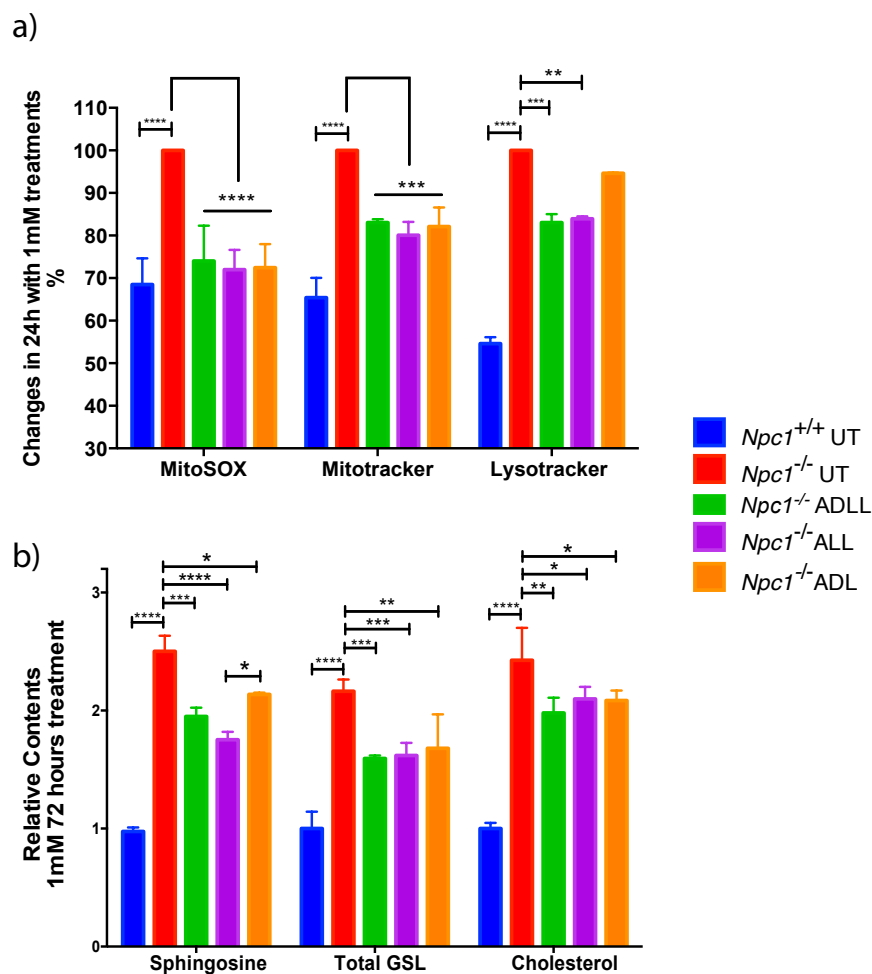


Figure 5.1 Effects of acetyl-leucine treatments on NPC CHO cells. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT), ADLL (*Npc1*^{-/-} ADLL), ALL (*Npc1*^{-/-} ALL), ADL (*Npc1*^{-/-} ADL), treatments n=3 for each group. **a)** Lysotracker, Mitotracker, MitoSOX fluorescence % values after 24h treatments, relative to NPC1 UT, Mean $\pm$ SD, ** $p < 0.0013$, *** $p < 0.001$ **** $p < 0.0001$ (2-way ANOVA). **b)** Sphingosine, total GSL and free Cholesterol percentages after 72h treatments, relative to NPC1 UT, Mean $\pm$ SD, * $p < 0.04$, ** $p < 0.004$, *** $p < 0.001$ **** $p < 0.0001$ (2-way ANOVA).

Having shown the storage-reducing effects of acetyl-leucine compounds (Acetyl-DL-Leucine: ADLL, Acetyl-L-Leucine: ALL, Acetyl-D-Leucine: ADL) in *in vitro* NPC1 model, we expended our research into understanding which protein/proteins acetyl-leucine compounds (ALs) can interact with.

We generated biotin conjugated ALs (John Spencer lab, University of Sussex), attaching biotin to the nitrogen atom of AL and produced enantiopure versions (Biotin-ALL: Compound 13-AM013 and Biotin-ADL: Compound 14-

AM014) (**Figure 5.2 a**). As a second chemical variation of biotin-AL compounds, we attached to *tert*-butyl to the carboxylic acid site of acetyl-leucine (Biotin ALL: Compound 1-AM001 and Biotin ADL: Compound 2-AM002) (**Figure 5.2 a**). Lastly, Compound RJ, which includes a biotin moiety, yet does not contain acetyl-leucine was included as a negative control (**Figure 5.2 a**). Our approach to understanding AL's protein targets was conducted by measuring the biological activity of biotin-AL compounds, followed by coating streptavidin beads with active and inactive biotin-AL, which are in the same chemical confirmation (**Figure 5.2 b**).

When CHO cells were treated with biotin-AL compounds for 72h and relative acidic volume changes (as an indication of therapeutic effect) measured (LysoTracker), we found only Compound 13-AM013 (Biotin ALL with no *tert*-butyl modification) was able to significantly reduce the acidic compartment volume ($p=0.0008$), similar to ADLL ($p=0.0227$), compared to NPC1 (**Figure 5.2 c**). We therefore selected Compound 13 as "Active drug" and another ALL form Compound 1-AM001 (Biotin ALL with *tert*-butyl modification) as "Inactive drug" to continue our discovery research of ALL's protein targets.

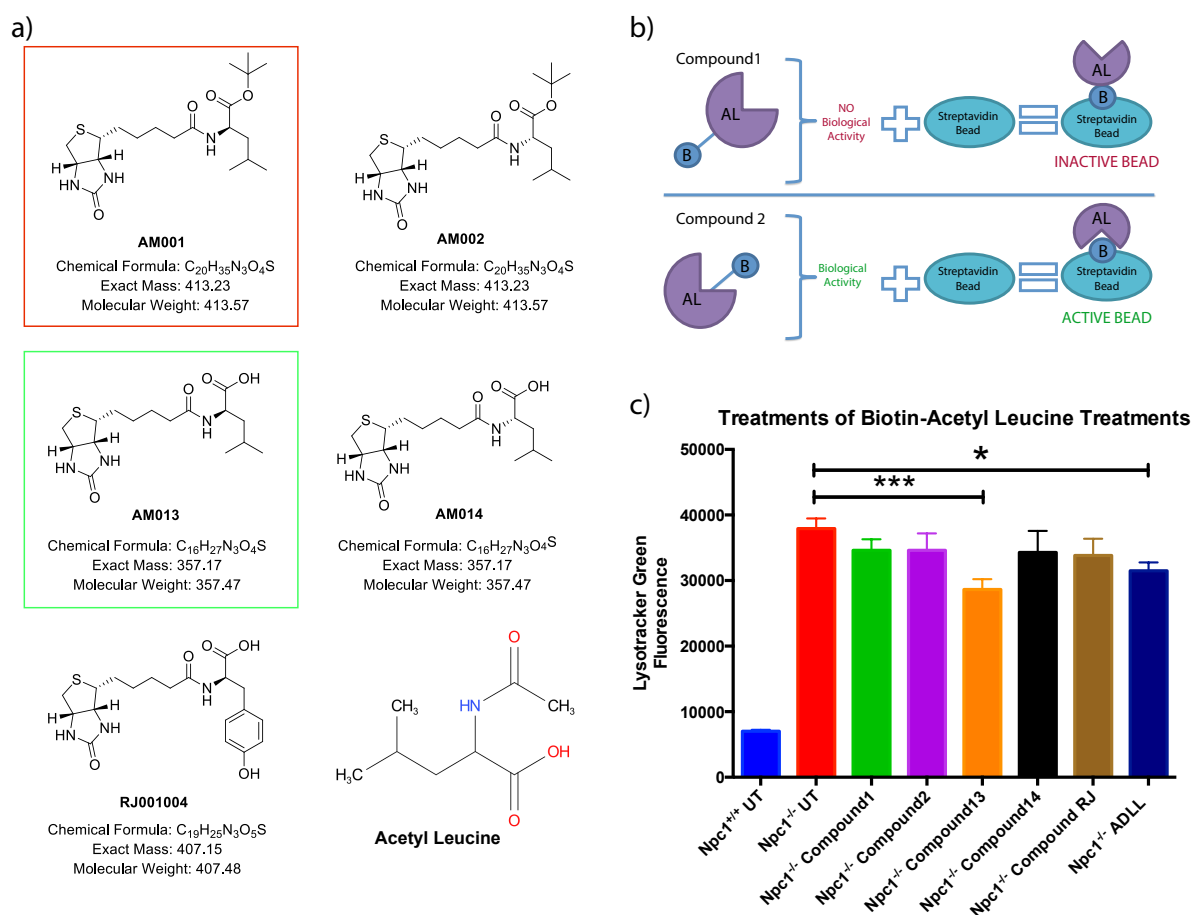


Figure 5.2. Biotinylated Acetyl-Leucine compounds' potency and utilisation for drug-protein interaction study. For wild type untreated (*Npc1*^{+/+} UT), NPC1 untreated (*Npc1*^{-/-} UT); and treatments *Npc1*^{-/-} compound 1-12-13-14-RJ-ADLL). **a)** Biotin-Acetyl-leucine compounds tested. Biologically active drug shown in green, and its corresponding inactive pair was shown in red box. Biotin-AL compounds were produced by Andrew McGown/John Spencer, University of Sussex. **b)** Study design. Active and inactive compounds were selected depending on biological activity (measuring relative acidic compartment volume changes). Streptavidin beads were incubated in either active or inactive Biotin-AL to be employed for drug-protein interaction studies. **c)** Relative lysosomal/acidic compartment volume changes with Biotinylated acetyl-leucine compounds. Negative control: Compound RJ, Positive control: Acetyl-DL-Leucine. * $p=0.0277$, *** $p=0.0008$, Mean $\pm$ SD, One-way ANOVA.

We performed 4 protein-drug interaction experiments with slightly different protocols in order to optimise the experimental conditions. The approach was to coat streptavidin beads and incubate them in protein homogenates obtained from NPC1 mouse fibroblasts (**Figure 5.3**). In the first experiment, we used 1mg cell homogenates, incubated them with first inactive

beads for 30 minutes. Flow-through was obtained and incubated with active beads for 30 minutes. Then we washed the beads and extracted the proteins by boiling at 97°C for 6 minutes, and performed gel protein separation (**Figure 5.3**). In the second experiment, we used the same amount of protein and incubation time, but we performed D-biotin elution (**Figure 5.3**). The third experiment was done with same incubation and elution method, but 0.6mg protein homogenate was used (**Figure 5.3**). Finally, the last experiment (4th) was performed with 0.4mg protein homogenate (**Figure 5.3**). All experiments included inactive and active drug coated beads but in the 2nd experiment we introduced naked beads; in 3rd and 4th experiments we added D-biotin coated beads as negative control groups.

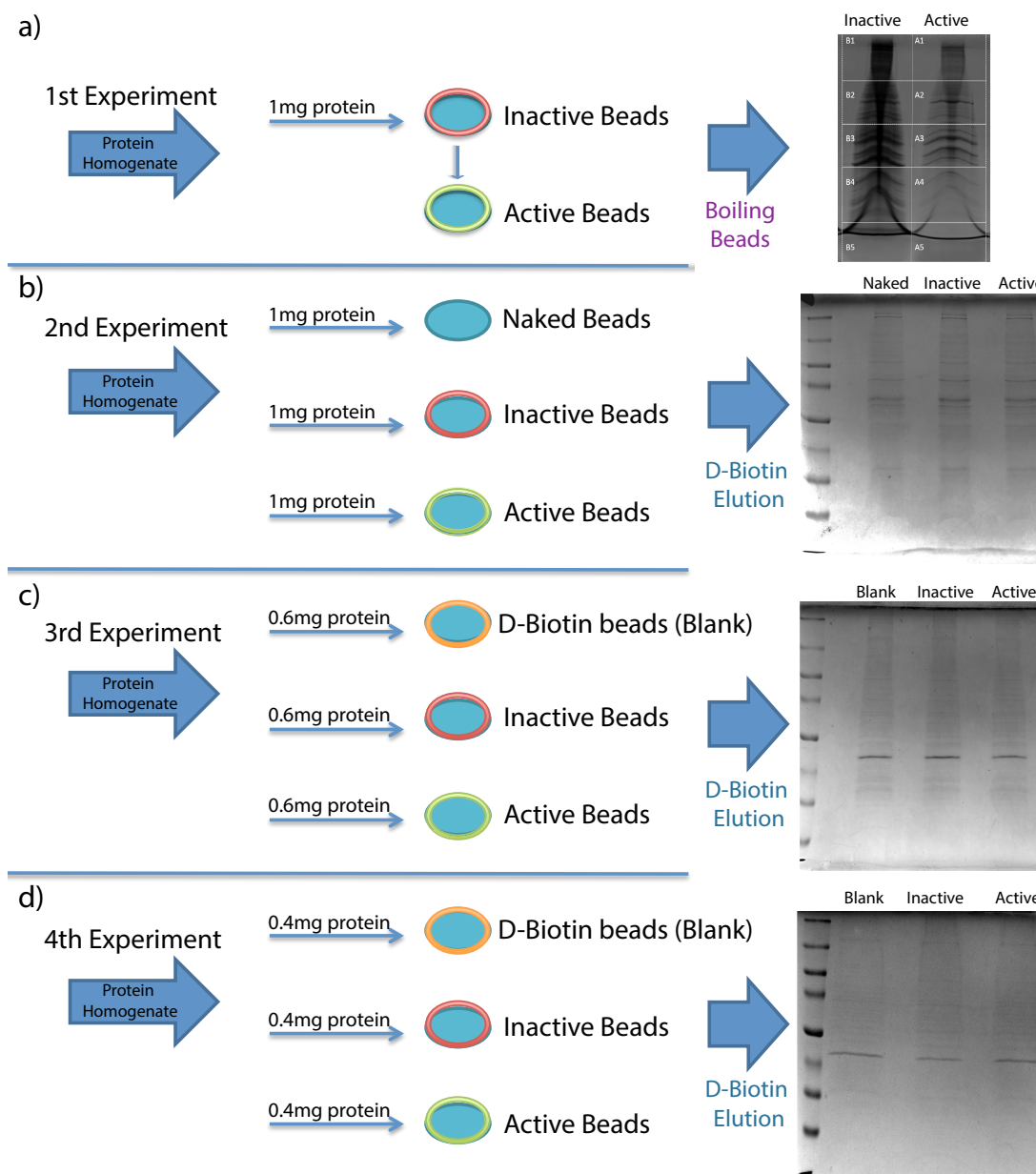


Figure 5.3. Protein-drug interaction study summary. Protein homogenates were incubated with streptavidin beads, which were uncoated (naked beads), D-biotin coated (blank beads), inactive Biotin-ALL coated (inactive beads) or active Biotin-ALL coated (active beads). Proteins were eluted from beads with either boiling or competitive D-biotin incubation. Eluates were run on 10% SDS Page gels and Coomassie stained. Finally, samples were run on LC-MS-MS with in-gel digestion and extraction method. **a)** 1st experiment; 1mg protein homogenates, 30 min protein-beads incubation, boiling elution. **b)** 2nd experiment: 1mg protein homogenates, 30 min protein-beads incubation, D-biotin elution. **c)** 3rd experiment: 0.6mg protein homogenates, 30 min protein-beads incubation, D-biotin elution. **d)** 4th experiment: 0.4mg protein homogenates, 30 min protein-beads incubation, D-biotin elution.

The sample preparation for proteomics was identical for all four experiments after protein incubation with beads (detailed in methods). Exclusion criteria to detect the protein target of Biotin-ALL active compound comprised: 1) excluding proteins, which had less than 2 unique peptides and PSM values in the dataset, 2) Excluding proteins retained on inactive/blank/naked columns. This approach gave us 4 tables (from experiments 1-4) that only active drug was able to interact/bind. **Table 5.1** shows 1st, **table 5.2** shows 2nd, **table 5.3** shows 3rd and **table 5.4** shows the 4th experiment in order from higher PSM values (The total number of identified peptide sequences (peptide spectrum matches) for the protein-indication of semi quantitative analysis), to lower values.

Table 5.1. Protein interactors obtained from the first experiment results.

Accession	Description	Σ# Unique Peptides	1 st exp. # PSM1mg Active Drug (boiled)
Q6TX D4	Dynamin-binding protein OS=Mus musculus GN=Dnmbp PE=1 SV=2 - [DNMBP_MOUSE]	16	19
Q6VN B8	WD repeat and FYVE domain-containing protein 3 OS=Mus musculus GN=Wdfy3 PE=1 SV=1 - [WDFY3_MOUSE]	13	16
Q8CD G3	Deubiquitinating protein VCIP135 OS=Mus musculus GN=Vcpi1 PE=2 SV=1 - [VCIP1_MOUSE]	9	15
P596 72	Ankyrin repeat and SAM domain-containing protein 1A OS=Mus musculus GN=Anks1a PE=1 SV=3 - [ANS1A_MOUSE]	11	14
O888 11-2	Isoform 2 of Signal transducing adapter molecule 2 OS=Mus musculus GN=Stam2 - [STAM2_MOUSE]	7	14
Q9Z0 R6-2	Isoform 2 of Intersectin-2 OS=Mus musculus GN=Itsn2 - [ITSN2_MOUSE]	8	13
G3UZ D6	Ubiquitin conjugation factor E4 B OS=Mus musculus GN=Ube4b PE=4 SV=1 - [G3UZD6_MOUSE]	8	13
A2A W19	Endophilin-B2 OS=Mus musculus GN=Sh3glb2 PE=4 SV=1 - [A2AWI9_MOUSE]	6	13
Q623 83	Transcription elongation factor SPT6 OS=Mus musculus GN=Supt6h PE=1 SV=2 - [SPT6H_MOUSE]	9	10
Q6PA R5-5	Isoform 5 of GTPase-activating protein and VPS9 domain-containing protein 1 OS=Mus musculus GN=Gapvd1 - [GAPD1_MOUSE]	6	9
Q6P5 49	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 OS=Mus musculus GN=Inpp1 PE=1 SV=1 - [SHIP2_MOUSE]	6	9
P056 22	Platelet-derived growth factor receptor beta OS=Mus musculus GN=Pdgfrb PE=1 SV=1 - [PGFRB_MOUSE]	5	9
E9QN Q4	Rap guanine nucleotide exchange factor 2 OS=Mus musculus GN=Rapgef2 PE=4 SV=1 - [E9QNQ4_MOUSE]	5	9
E9Q3	Protein Pi4ka OS=Mus musculus GN=Pi4ka PE=4 SV=1 -	7	8

L2	[E9Q3L2_MOUSE]		
B2RU J2	ErbB2ip protein OS=Mus musculus GN=ErbB2ip PE=2 SV=1 - [B2RUJ2_MOUSE]	5	8
Q99 MR6- 3	Isoform C of Serrate RNA effector molecule homolog OS=Mus musculus GN=Srrt - [SRRT_MOUSE]	5	8
E9Q3 I8	Intersectin-1 OS=Mus musculus GN=Itsn1 PE=4 SV=1 - [E9Q3I8_MOUSE]	6	7
Q6P5 E6-2	Isoform 2 of ADP-ribosylation factor-binding protein GGA2 OS=Mus musculus GN=Gga2 - [GGA2_MOUSE]	6	7
E9Q6 61	Uncharacterized protein OS=Mus musculus GN=Sympk PE=4 SV=1 - [E9Q661_MOUSE]	5	7
Q80U K0	SEC14 domain and spectrin repeat-containing protein 1 OS=Mus musculus GN=Sestd1 PE=2 SV=1 - [SESD1_MOUSE]	4	7
E9PX 53	Serine/threonine-protein phosphatase 4 regulatory subunit 1 OS=Mus musculus GN=Ppp4r1 PE=4 SV=1 - [E9PX53_MOUSE]	3	7
A2A MY5	Ubiquitin-associated protein 2 OS=Mus musculus GN=Ubp2 PE=4 SV=1 - [A2AMY5_MOUSE]	3	7
Q8VD P3-2	Isoform 2 of Protein-methionine sulfoxide oxidase MICAL1 OS=Mus musculus GN=Mical1 - [MICA1_MOUSE]	5	6
Q99P 88	Nuclear pore complex protein Nup155 OS=Mus musculus GN=Nup155 PE=2 SV=1 - [NU155_MOUSE]	5	6
Q5PR F0	HEAT repeat-containing protein 5A OS=Mus musculus GN=Heatr5a PE=2 SV=2 - [HTR5A_MOUSE]	4	6
Q99J X3-2	Isoform 2 of Golgi reassembly-stacking protein 2 OS=Mus musculus GN=Gorasp2 - [GORS2_MOUSE]	4	6
E9Q3 G8	Protein Nup153 OS=Mus musculus GN=Nup153 PE=4 SV=1 - [E9Q3G8_MOUSE]	4	6
Q8K4 Z5	Splicing factor 3A subunit 1 OS=Mus musculus GN=Sf3a1 PE=1 SV=1 - [SF3A1_MOUSE]	4	6
Q8CI 08	SLAIN motif-containing protein 2 OS=Mus musculus GN=Slain2 PE=1 SV=2 - [SLAI2_MOUSE]	3	6
E9Q0 A3	Protein Arhgef11 (Fragment) OS=Mus musculus GN=Arhgef11 PE=4 SV=1 - [E9Q0A3_MOUSE]	4	5
D3Z2 J3	Putative ATP-dependent RNA helicase DHX30 OS=Mus musculus GN=Dhx30 PE=4 SV=1 - [D3Z2J3_MOUSE]	4	5
A3KG B4	TBC1 domain family member 8B OS=Mus musculus GN=Tbc1d8b PE=2 SV=1 - [TBC8B_MOUSE]	4	5
Q8R1 T1	Charged multivesicular body protein 7 OS=Mus musculus GN=Chmp7 PE=1 SV=1 - [CHMP7_MOUSE]	3	5
D3Z3 T5	Choline-phosphate cytidyltransferase A OS=Mus musculus GN=Pcyt1a PE=4 SV=1 - [D3Z3T5_MOUSE]	3	5
A6H8 H2	Dennd4c protein OS=Mus musculus GN=Dennd4c PE=2 SV=1 - [A6H8H2_MOUSE]	3	5
Q3TJ D7	PDZ and LIM domain protein 7 OS=Mus musculus GN=Pdlm7 PE=2 SV=1 - [PDLI7_MOUSE]	3	5
D3Z0 M2	Transmembrane protein 106B (Fragment) OS=Mus musculus GN=Tmem106b PE=4 SV=1 - [D3Z0M2_MOUSE]	3	5
Q0VG Y8-2	Isoform 2 of Protein TANC1 OS=Mus musculus GN=Tanc1 - [TANC1_MOUSE]	2	5
Q91Y D3	mRNA-decapping enzyme 1A OS=Mus musculus GN=Dcp1a PE=1 SV=1 - [DCP1A_MOUSE]	2	5
E9PV 69	Protein 2700078E11Rik OS=Mus musculus GN=2700078E11Rik PE=4 SV=1 - [E9PV69_MOUSE]	2	5
Q8C2 C6	Protein Tfg OS=Mus musculus GN=Tfg PE=2 SV=1 - [Q8C2C6_MOUSE]	2	5
Q8VC H8	UBX domain-containing protein 4 OS=Mus musculus GN=Ubxn4 PE=1 SV=1 - [UBXN4_MOUSE]	2	5
F8WI I0	Breast cancer anti-estrogen resistance protein 1 OS=Mus musculus GN=Bcar1 PE=4 SV=1 - [F8WII0_MOUSE]	4	4

Q9CS H3	Exosome complex exonuclease RRP44 OS=Mus musculus GN=Dis3 PE=2 SV=4 - [RRP44_MOUSE]	4	4
B9EJ R8	HEAT repeat containing 2 OS=Mus musculus GN=Heatr2 PE=2 SV=1 - [B9EJR8_MOUSE]	4	4
Q8K1 20-2	Isoform 2 of Nuclear factor of activated T-cells, cytoplasmic 4 OS=Mus musculus GN=Nfatc4 - [NFAC4_MOUSE]	4	4
Q8CG F7-2	Isoform 2 of Transcription elongation regulator 1 OS=Mus musculus GN=Tcerg1 - [TCRG1_MOUSE]	4	4
Q9EP 53-4	Isoform 4 of Hamartin OS=Mus musculus GN=Tsc1 - [TSC1_MOUSE]	4	4
Q8C4 Q6	Axin interactor, dorsalization-associated protein OS=Mus musculus GN=Aida PE=1 SV=1 - [AIDA_MOUSE]	3	4
Q8BX C6	COMM domain-containing protein 2 OS=Mus musculus GN=Commd2 PE=1 SV=1 - [COMD2_MOUSE]	3	4
Q99L J0	CTTNBP2 N-terminal-like protein OS=Mus musculus GN=Cttnbp2nl PE=1 SV=1 - [CT2NL_MOUSE]	3	4
Q8VD 75	Huntingtin-interacting protein 1 OS=Mus musculus GN=Hip1 PE=1 SV=2 - [HIP1_MOUSE]	3	4
Q9DB Q7-3	Isoform 3 of Protein-associating with the carboxyl- terminal domain of ezrin OS=Mus musculus GN=Scyl3 - [PACE1_MOUSE]	3	4
Q9Z2 I0	LETM1 and EF-hand domain-containing protein 1, mitochondrial OS=Mus musculus GN=Letm1 PE=2 SV=1 - [LETM1_MOUSE]	3	4
D3Z6 37	MCG10134, isoform CRA_c OS=Mus musculus GN=Tpd52 PE=4 SV=1 - [D3Z637_MOUSE]	3	4
D3YU G5	MKL/myocardin-like protein 1 (Fragment) OS=Mus musculus GN=Mkl1 PE=4 SV=1 - [D3YUG5_MOUSE]	3	4
Q9D1 X0	Nucleolar protein 3 OS=Mus musculus GN=Nol3 PE=1 SV=1 - [NOL3_MOUSE]	3	4
F8VP U2	Protein Farp1 OS=Mus musculus GN=Farp1 PE=4 SV=1 - [F8VPU2_MOUSE]	3	4
O352 39	Tyrosine-protein phosphatase non-receptor type 9 OS=Mus musculus GN=Ptpn9 PE=2 SV=2 - [PTN9_MOUSE]	3	4
Q6PA 06	Atlastin-2 OS=Mus musculus GN=Atl2 PE=1 SV=1 - [ATLA2_MOUSE]	2	4
Q3U3 08	Cytoplasmic tRNA 2-thiolation protein 2 OS=Mus musculus GN=Ctu2 PE=2 SV=1 - [CTU2_MOUSE]	2	4
Q80 WC7- 2	Isoform 2 of Arf-GAP domain and FG repeat-containing protein 2 OS=Mus musculus GN=Agfg2 - [AGFG2_MOUSE]	2	4
Q9C WR0- 2	Isoform 2 of Rho guanine nucleotide exchange factor 25 OS=Mus musculus GN=Arhgef25 - [ARHGP_MOUSE]	2	4
Q611 27	NGFI-A-binding protein 2 OS=Mus musculus GN=Nab2 PE=1 SV=2 - [NAB2_MOUSE]	2	4
Q8BH Z0	Protein FAM49A OS=Mus musculus GN=Fam49a PE=2 SV=1 - [FA49A_MOUSE]	2	4
G5E8 A7	Protein Smaug homolog 2 OS=Mus musculus GN=Samd4b PE=4 SV=1 - [G5E8A7_MOUSE]	2	4
D3Z1 X8	SWI/SNF complex subunit SMARCC1 OS=Mus musculus GN=Smarrcc1 PE=4 SV=1 - [D3Z1X8_MOUSE]	2	4
Q9Z0 H7	B-cell lymphoma/leukemia 10 OS=Mus musculus GN=Bcl10 PE=2 SV=1 - [BCL10_MOUSE]	3	3
D3Z7 D5	Collagen alpha-2(VI) chain OS=Mus musculus GN=Col6a2 PE=4 SV=1 - [D3Z7D5_MOUSE]	3	3
F6SX 81	G-protein-signaling modulator 1 (Fragment) OS=Mus musculus GN=Gpsm1 PE=4 SV=1 - [F6SX81_MOUSE]	3	3
Q67F Y2-2	Isoform 2 of B-cell CLL/lymphoma 9-like protein OS=Mus musculus GN=Bcl9l - [BCL9L_MOUSE]	3	3
A2BE 28-2	Isoform 2 of Ribosomal biogenesis protein LAS1L OS=Mus musculus GN=Las1l - [LAS1L_MOUSE]	3	3

Q920 I9-2	Isoform 2 of WD repeat-containing protein 7 OS=Mus musculus GN=Wdr7 - [WDR7_MOUSE]	3	3
Q9QX L2-3	Isoform 3 of Kinesin-like protein KIF21A OS=Mus musculus GN=Kif21a - [KI21A_MOUSE]	3	3
Q612 92	Laminin subunit beta-2 OS=Mus musculus GN=Lamb2 PE=2 SV=2 - [LAMB2_MOUSE]	3	3
Q9JIH 2	Nuclear pore complex protein Nup50 OS=Mus musculus GN=Nup50 PE=1 SV=3 - [NUP50_MOUSE]	3	3
E9Q6 V7	Protein Diap2 OS=Mus musculus GN=Diap2 PE=4 SV=1 - [E9Q6V7_MOUSE]	3	3
D3Z7 01	Protein Ube4a OS=Mus musculus GN=Ube4a PE=4 SV=1 - [D3Z701_MOUSE]	3	3
Q80Y 56	Rabenosyn-5 OS=Mus musculus GN=Zfyve20 PE=2 SV=1 - [RBNS5_MOUSE]	3	3
Q8R4 H2	Rho guanine nucleotide exchange factor 12 OS=Mus musculus GN=Arhgef12 PE=1 SV=2 - [ARHGC_MOUSE]	3	3
E9QL 52	Ribosomal RNA-processing protein 1 homolog A OS=Mus musculus GN=Rrp1 PE=4 SV=1 - [E9QL52_MOUSE]	3	3
F6SJ W2	Uncharacterized protein (Fragment) OS=Mus musculus GN=Dstyk PE=3 SV=1 - [F6SJW2_MOUSE]	3	3
Q99P M9	Uridine-cytidine kinase 2 OS=Mus musculus GN=Uck2 PE=1 SV=1 - [UCK2_MOUSE]	3	3
Q9DC 71	28S ribosomal protein S15, mitochondrial OS=Mus musculus GN=Mrps15 PE=2 SV=2 - [RT15_MOUSE]	2	3
G5E8 68	Alsin OS=Mus musculus GN=Als2 PE=4 SV=1 - [G5E868_MOUSE]	2	3
A2RR K7	Bcr protein OS=Mus musculus GN=Bcr PE=2 SV=1 - [A2RRK7_MOUSE]	2	3
Q9CQ 02	COMM domain-containing protein 4 OS=Mus musculus GN=Commd4 PE=2 SV=1 - [COMD4_MOUSE]	2	3
O350 75	Down syndrome critical region protein 3 homolog OS=Mus musculus GN=Dscr3 PE=2 SV=1 - [DSCR3_MOUSE]	2	3
P0C6 F1	Dynein heavy chain 2, axonemal OS=Mus musculus GN=Dnah2 PE=2 SV=1 - [DYH2_MOUSE]	2	3
Q8QZ S3	Folliculin OS=Mus musculus GN=Flcn PE=1 SV=1 - [FLCN_MOUSE]	2	3
Q9CZ J2	Heat shock 70 kDa protein 12B OS=Mus musculus GN=Hspa12b PE=1 SV=1 - [HS12B_MOUSE]	2	3
Q922 D4-2	Isoform 2 of Serine/threonine-protein phosphatase 6 regulatory subunit 3 OS=Mus musculus GN=Ppp6r3 - [PP6R3_MOUSE]	2	3
O890 32-3	Isoform 3 of SH3 and PX domain-containing protein 2A OS=Mus musculus GN=Sh3pxd2a - [SPD2A_MOUSE]	2	3
E9Q3 M0	Mothers against decapentaplegic homolog 2 OS=Mus musculus GN=Smad2 PE=4 SV=1 - [E9Q3M0_MOUSE]	2	3
Q922 L6	Negative elongation factor D OS=Mus musculus GN=Th11 PE=2 SV=2 - [NELFD_MOUSE]	2	3
F7CC X1	Protein-tyrosine kinase 2-beta (Fragment) OS=Mus musculus GN=Ptk2b PE=4 SV=1 - [F7CCX1_MOUSE]	2	3
Q8CE E7	Retinol dehydrogenase 13 OS=Mus musculus GN=Rdh13 PE=2 SV=1 - [RDH13_MOUSE]	2	3
Q921 I6	SH3 domain-binding protein 4 OS=Mus musculus GN=Sh3bp4 PE=2 SV=1 - [SH3B4_MOUSE]	2	3
P703 62	Ubiquitin fusion degradation protein 1 homolog OS=Mus musculus GN=Ufd1l PE=1 SV=2 - [UFD1_MOUSE]	2	3
Q8R1 05	Vacuolar protein sorting-associated protein 37C OS=Mus musculus GN=Vps37c PE=2 SV=1 - [VP37C_MOUSE]	2	3
Q8CI 33-2	Isoform 2 of CWF19-like protein 1 OS=Mus musculus GN=Cwf19l1 - [C19L1_MOUSE]	3	2
B1B1 D8	39S ribosomal protein L2, mitochondrial OS=Mus musculus GN=Mrpl2 PE=4 SV=1 - [B1B1D8_MOUSE]	2	2
O359	39S ribosomal protein L23, mitochondrial OS=Mus	2	2

72	musculus GN=Mrpl23 PE=2 SV=1 - [RM23_MOUSE]		
Q9CQ N7	39S ribosomal protein L41, mitochondrial OS=Mus musculus GN=Mrpl41 PE=2 SV=1 - [RM41_MOUSE]	2	2
O888 45	A-kinase anchor protein 10, mitochondrial OS=Mus musculus GN=Akap10 PE=1 SV=3 - [AKA10_MOUSE]	2	2
Q91 W96	Anaphase-promoting complex subunit 4 OS=Mus musculus GN=Anapc4 PE=2 SV=1 - [APC4_MOUSE]	2	2
Q6PE 80	Axl protein OS=Mus musculus GN=Axl PE=2 SV=1 - [Q6PE80_MOUSE]	2	2
Q8CII 2	Cell division cycle protein 123 homolog OS=Mus musculus GN=Cdc123 PE=2 SV=2 - [CD123_MOUSE]	2	2
Q9D7 S9	Charged multivesicular body protein 5 OS=Mus musculus GN=Chmp5 PE=2 SV=1 - [CHMP5_MOUSE]	2	2
O352 18	Cleavage and polyadenylation specificity factor subunit 2 OS=Mus musculus GN=Cpsf2 PE=1 SV=1 - [CPSF2_MOUSE]	2	2
Q8VE H6	COBW domain-containing protein 1 OS=Mus musculus GN=Cbwd1 PE=2 SV=1 - [CBWD1_MOUSE]	2	2
P081 22	Collagen alpha-2(IV) chain OS=Mus musculus GN=Col4a2 PE=2 SV=4 - [CO4A2_MOUSE]	2	2
Q91Y Z2	C-terminal binding protein 2, isoform CRA_b OS=Mus musculus GN=Ctbp2 PE=2 SV=1 - [Q91YZ2_MOUSE]	2	2
Q8VD R9	Dedicator of cytokinesis protein 6 OS=Mus musculus GN=Dock6 PE=1 SV=4 - [DOCK6_MOUSE]	2	2
Q6PN C0	DmX-like protein 1 OS=Mus musculus GN=Dmxl1 PE=1 SV=1 - [DMXL1_MOUSE]	2	2
P974 65	Docking protein 1 OS=Mus musculus GN=Dok1 PE=1 SV=2 - [DOK1_MOUSE]	2	2
Q8JZ Y1	FtsJ homolog 1 (E. coli) OS=Mus musculus GN=Ftsj1 PE=2 SV=1 - [Q8JZY1_MOUSE]	2	2
Q8VD V3	Guanine nucleotide exchange factor for Rab-3A OS=Mus musculus GN=Rab3il1 PE=2 SV=1 - [R3GEF_MOUSE]	2	2
P702 27	Inositol 1,4,5-trisphosphate receptor type 3 OS=Mus musculus GN=Itpr3 PE=1 SV=3 - [ITPR3_MOUSE]	2	2
Q91 WG4- 2	Isoform 2 of Elongator complex protein 2 OS=Mus musculus GN=Elp2 - [ELP2_MOUSE]	2	2
O704 58-2	Isoform 2 of Oncostatin-M-specific receptor subunit beta OS=Mus musculus GN=Osmr - [OSMR_MOUSE]	2	2
Q8BN E1-2	Isoform 2 of Protein FAM115A OS=Mus musculus GN=Fam115a - [F115A_MOUSE]	2	2
Q7TN 29-2	Isoform 2 of Stromal membrane-associated protein 2 OS=Mus musculus GN=Smap2 - [SMAP2_MOUSE]	2	2
Q6ZP Z3-2	Isoform 2 of Zinc finger CCCH domain-containing protein 4 OS=Mus musculus GN=Zc3h4 - [ZC3H4_MOUSE]	2	2
Q9Z0 H3-2	Isoform B of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 OS=Mus musculus GN=Smarcb1 - [SNF5_MOUSE]	2	2
Q8K0 C4	Lanosterol 14-alpha demethylase OS=Mus musculus GN=Cyp51a1 PE=2 SV=1 - [CP51A_MOUSE]	2	2
Q9QX D8	LIM domain-containing protein 1 OS=Mus musculus GN=Limd1 PE=1 SV=2 - [LIMD1_MOUSE]	2	2
G3U WE1	MCG11048, isoform CRA_c OS=Mus musculus GN=Tecr PE=4 SV=1 - [G3UWE1_MOUSE]	2	2
E9P WX8	Microtubule-associated serine/threonine-protein kinase 4 (Fragment) OS=Mus musculus GN=Mast4 PE=4 SV=1 - [E9PWX8_MOUSE]	2	2
Q6ZQ 33	MKIAA0857 protein (Fragment) OS=Mus musculus GN=Rab11fip5 PE=2 SV=1 - [Q6ZQ33_MOUSE]	2	2
Q6KA U4	Multivesicular body subunit 12B OS=Mus musculus GN=Fam125b PE=2 SV=2 - [F125B_MOUSE]	2	2
Q8CC 86	Nicotinate phosphoribosyltransferase OS=Mus musculus GN=Naprt1 PE=2 SV=1 - [PNCB_MOUSE]	2	2

A2AC M0	Novel protein containing six WD40 domains at C-terminus OS=Mus musculus GN=Rptor PE=4 SV=1 - [A2ACM0_MOUSE]	2	2
Q8BT S4	Nuclear pore complex protein Nup54 OS=Mus musculus GN=Nup54 PE=1 SV=1 - [NUP54_MOUSE]	2	2
O354 65	Peptidyl-prolyl cis-trans isomerase FKBP8 OS=Mus musculus GN=Fkbp8 PE=1 SV=2 - [FKBP8_MOUSE]	2	2
Q3UQ 28	Peroxidasin homolog OS=Mus musculus GN=Pxdn PE=2 SV=2 - [PXDN_MOUSE]	2	2
Q69Z Q2	Pre-mRNA-splicing factor ISY1 homolog OS=Mus musculus GN=Isy1 PE=1 SV=2 - [ISY1_MOUSE]	2	2
H7BX 23	Prolyl 4-hydroxylase subunit alpha-3 OS=Mus musculus GN=P4ha3 PE=4 SV=1 - [H7BX23_MOUSE]	2	2
D3Z0 A8	Protein 5730469M10Rik (Fragment) OS=Mus musculus GN=5730469M10Rik PE=4 SV=1 - [D3Z0A8_MOUSE]	2	2
Q9D1 R2	Protein KTI12 homolog OS=Mus musculus GN=Kti12 PE=1 SV=1 - [KTI12_MOUSE]	2	2
D3YY U8	Protein Obsl1 OS=Mus musculus GN=Obsl1 PE=4 SV=1 - [D3YYU8_MOUSE]	2	2
F7AY W2	Protein PRRC2B (Fragment) OS=Mus musculus GN=Prrc2b PE=4 SV=1 - [F7AYW2_MOUSE]	2	2
F2Z3 U3	Protein Raph1 (Fragment) OS=Mus musculus GN=Raph1 PE=4 SV=1 - [F2Z3U3_MOUSE]	2	2
Q9D2 C2	Protein SAAL1 OS=Mus musculus GN=Saal1 PE=1 SV=1 - [SAAL1_MOUSE]	2	2
Q8K0 G5	Protein TSSC1 OS=Mus musculus GN=Tssc1 PE=1 SV=2 - [TSSC1_MOUSE]	2	2
Q9JM 13	Rab5 GDP/GTP exchange factor OS=Mus musculus GN=Rabgef1 PE=1 SV=1 - [RABX5_MOUSE]	2	2
F6XV P7	RING finger protein 31 (Fragment) OS=Mus musculus GN=Rnf31 PE=4 SV=1 - [F6XVP7_MOUSE]	2	2
Q8CD J8	Stonin-1 OS=Mus musculus GN=Ston1 PE=2 SV=2 - [STON1_MOUSE]	2	2
Q3TI R3	Synembryn-A OS=Mus musculus GN=Ric8a PE=1 SV=2 - [RIC8A_MOUSE]	2	2
Q9D0 I4	Syntaxin-17 OS=Mus musculus GN=Stx17 PE=2 SV=1 - [STX17_MOUSE]	2	2
Q9Z0 U1	Tight junction protein ZO-2 OS=Mus musculus GN=Tjp2 PE=1 SV=2 - [ZO2_MOUSE]	2	2
Q91 W67	Ubiquitin-like protein 7 OS=Mus musculus GN=Ubl7 PE=1 SV=2 - [UBL7_MOUSE]	2	2
Q8CE C2	Uncharacterized protein OS=Mus musculus GN=Arnt PE=2 SV=1 - [Q8CEC2_MOUSE]	2	2
P973 90	Vacuolar protein sorting-associated protein 45 OS=Mus musculus GN=Vps45 PE=1 SV=1 - [VPS45_MOUSE]	2	2

Table 5.2. Protein interactors obtained from the second experiment results.

Accession	Description	Σ Unique Peptides	2 nd exp. # PSM 1mg Active Drug Biotin elution
A0A0R4J0I9	Low density lipoprotein receptor-related protein 1 OS=Mus musculus OX=10090 GN=Lrp1 PE=1 SV=1 - [A0A0R4J0I9_MOUSE]	4	7
E9PVA8	eIF-2-alpha kinase activator GCN1 OS=Mus musculus OX=10090 GN=Gcn1 PE=1 SV=1 - [GCN1_MOUSE]	23	6
Q8BX70-3	Isoform 3 of Vacuolar protein sorting-associated protein 13C OS=Mus musculus OX=10090 GN=Vps13c - [VP13C_MOUSE]	4	5
Q9D880	Mitochondrial import inner membrane translocase subunit TIM50 OS=Mus musculus OX=10090 GN=Timm50 PE=1 SV=1 -	4	5

	[TIM50_MOUSE]		
E9QA D4	Coiled-coil domain-containing protein 93 OS=Mus musculus OX=10090 GN=Ccdc93 PE=1 SV=1 - [E9QAD4_MOUSE]	2	5
P630 37	DnaJ homolog subfamily A member 1 OS=Mus musculus OX=10090 GN=Dnaja1 PE=1 SV=1 - [DNJA1_MOUSE]	3	4
Q9EQ C5	N-terminal kinase-like protein OS=Mus musculus OX=10090 GN=Scyl1 PE=1 SV=1 - [SCYL1_MOUSE]	3	4
A2AJ 72	Far upstream element (FUSE)-binding protein 3 OS=Mus musculus OX=10090 GN=Fubp3 PE=1 SV=1 - [A2AJ72_MOUSE]	7	3
Q70FJ 1-3	Isoform 3 of A-kinase anchor protein 9 OS=Mus musculus OX=10090 GN=Akap9 - [AKAP9_MOUSE]	4	3
Q624 18-3	Isoform 3 of Drebrin-like protein OS=Mus musculus OX=10090 GN=Dbnl - [DBNL_MOUSE]	3	3
Q4FE 56	Probable ubiquitin carboxyl-terminal hydrolase FAF-X OS=Mus musculus OX=10090 GN=Usp9x PE=1 SV=1 - [Q4FE56_MOUSE]	15	2
P105 18	Delta-aminolevulinic acid dehydratase OS=Mus musculus OX=10090 GN=Alad PE=1 SV=1 - [HEM2_MOUSE]	6	2
P067 45	Glucose-6-phosphate isomerase OS=Mus musculus OX=10090 GN=Gpi PE=1 SV=4 - [G6PI_MOUSE]	6	2
Q070 76	Annexin A7 OS=Mus musculus OX=10090 GN=Anxa7 PE=1 SV=2 - [ANXA7_MOUSE]	5	2
Q2YD W2-2	Isoform 2 of Protein misato homolog 1 OS=Mus musculus OX=10090 GN=Msto1 - [MSTO1_MOUSE]	5	2
A0A0 R4J1Y 7	Thioredoxin domain-containing protein 5 OS=Mus musculus OX=10090 GN=Txndc5 PE=1 SV=1 - [A0A0R4J1Y7_MOUSE]	5	2
Q3U MG6	Non-metastatic cells 7, protein expressed in, isoform CRA_c OS=Mus musculus OX=10090 GN=Nme7 PE=1 SV=1 - [Q3UMG6_MOUSE]	4	2
Q91 W86	Vacuolar protein sorting-associated protein 11 homolog OS=Mus musculus OX=10090 GN=Vps11 PE=1 SV=3 - [VPS11_MOUSE]	4	2
A0A0 G2JD V8	Calponin OS=Mus musculus OX=10090 GN=Cnn3 PE=1 SV=1 - [A0A0G2JDV8_MOUSE]	3	2
Q9JIG 7	Coiled-coil domain-containing protein 22 OS=Mus musculus OX=10090 GN=Ccdc22 PE=1 SV=1 - [CCD22_MOUSE]	3	2
Q9R1 11	Guanine deaminase OS=Mus musculus OX=10090 GN=Gda PE=1 SV=1 - [GUAD_MOUSE]	3	2
Q3V1 T4-3	Isoform 3 of Prolyl 3-hydroxylase 1 OS=Mus musculus OX=10090 GN=P3h1 - [P3H1_MOUSE]	3	2
A0A0 87WS I8	Acyl-CoA dehydrogenase family member 11 OS=Mus musculus OX=10090 GN=Acad11 PE=1 SV=2 - [A0A087WSI8_MOUSE]	2	2
H7B WY2	AP-3 complex subunit mu-1 OS=Mus musculus OX=10090 GN=Ap3m1 PE=1 SV=1 - [H7BWY2_MOUSE]	2	2
H3BK 90	ATPase family, AAA domain-containing 3A (Fragment) OS=Mus musculus OX=10090 GN=Atad3a PE=1 SV=1 - [H3BK90_MOUSE]	2	2
F6W M66	Endothelin-converting enzyme 2 (Fragment) OS=Mus musculus OX=10090 GN=Ece2 PE=4 SV=1 - [F6WM66_MOUSE]	2	2
P702 27	Inositol 1,4,5-trisphosphate receptor type 3 OS=Mus musculus OX=10090 GN=Itp3 PE=1 SV=3 - [ITPR3_MOUSE]	2	2
Q91 WG4- 2	Isoform 2 of Elongator complex protein 2 OS=Mus musculus OX=10090 GN=Elp2 - [ELP2_MOUSE]	2	2
Q920 Q4-2	Isoform 2 of Vacuolar protein sorting-associated protein 16 homolog OS=Mus musculus OX=10090 GN=Vps16 - [VPS16_MOUSE]	2	2
P341 52-3	Isoform 3 of Focal adhesion kinase 1 OS=Mus musculus OX=10090 GN=Ptk2 - [FAK1_MOUSE]	2	2
A0A2 86YD 77	Neurolysin, mitochondrial (Fragment) OS=Mus musculus OX=10090 GN=Nln PE=1 SV=1 - [A0A286YD77_MOUSE]	2	2

Table 5.3. Protein interactors obtained from the third experiment results.

Accession	Description	Σ # Unique Peptides	3 rd exp. # PSM 0.6 mg Active Drug Biotin elution
Q9DBJ1	Phosphoglycerate mutase 1 OS=Mus musculus OX=10090 GN=Pgam1 PE=1 SV=3 - [PGAM1_MOUSE]	2	5
Q9JLV1	BAG family molecular chaperone regulator 3 OS=Mus musculus OX=10090 GN=Bag3 PE=1 SV=2 - [BAG3_MOUSE]	5	4
Q7TMB8	Cytoplasmic FMR1-interacting protein 1 OS=Mus musculus OX=10090 GN=Cyfp1 PE=1 SV=1 - [CYFP1_MOUSE]	24	3
P17047	Lysosome-associated membrane glycoprotein 2 OS=Mus musculus OX=10090 GN=Lamp2 PE=1 SV=2 - [LAMP2_MOUSE]	2	3
Q3UH60	Disco-interacting protein 2 homolog B OS=Mus musculus OX=10090 GN=Dip2b PE=1 SV=1 - [DIP2B_MOUSE]	15	2
P51125-3	Isoform 3 of Calpastatin OS=Mus musculus OX=10090 GN=Cast - [ICAL_MOUSE]	12	2
P51660	Peroxisomal multifunctional enzyme type 2 OS=Mus musculus OX=10090 GN=Hsd17b4 PE=1 SV=3 - [DHB4_MOUSE]	9	2
A0A1D5RLY6	Microtubule-associated protein 1S OS=Mus musculus OX=10090 GN=Map1s PE=1 SV=1 - [A0A1D5RLY6_MOUSE]	5	2
Q9JIG7	Coiled-coil domain-containing protein 22 OS=Mus musculus OX=10090 GN=Ccdc22 PE=1 SV=1 - [CCD22_MOUSE]	3	2
F6QCP8	Angiotensin-converting enzyme (Fragment) OS=Mus musculus OX=10090 GN=Ace PE=1 SV=1 - [F6QCP8_MOUSE]	2	2
Q9Z0P7-2	Isoform 2 of Suppressor of fused homolog OS=Mus musculus OX=10090 GN=Sufu - [SUFU_MOUSE]	2	2
A0A1C7CYV0	Plastin-3 (Fragment) OS=Mus musculus OX=10090 GN=Pls3 PE=1 SV=1 - [A0A1C7CYV0_MOUSE]	2	2
P17751	Triosephosphate isomerase OS=Mus musculus OX=10090 GN=Tpi1 PE=1 SV=4 - [TPIS_MOUSE]	2	2
Q8VDP2	UPF0428 protein CXorf56 homolog OS=Mus musculus OX=10090 PE=1 SV=1 - [CX056_MOUSE]	2	2
Q9JHF5	V-type proton ATPase subunit a OS=Mus musculus OX=10090 GN=Tcirg1 PE=1 SV=1 - [Q9JHF5_MOUSE]	2	2

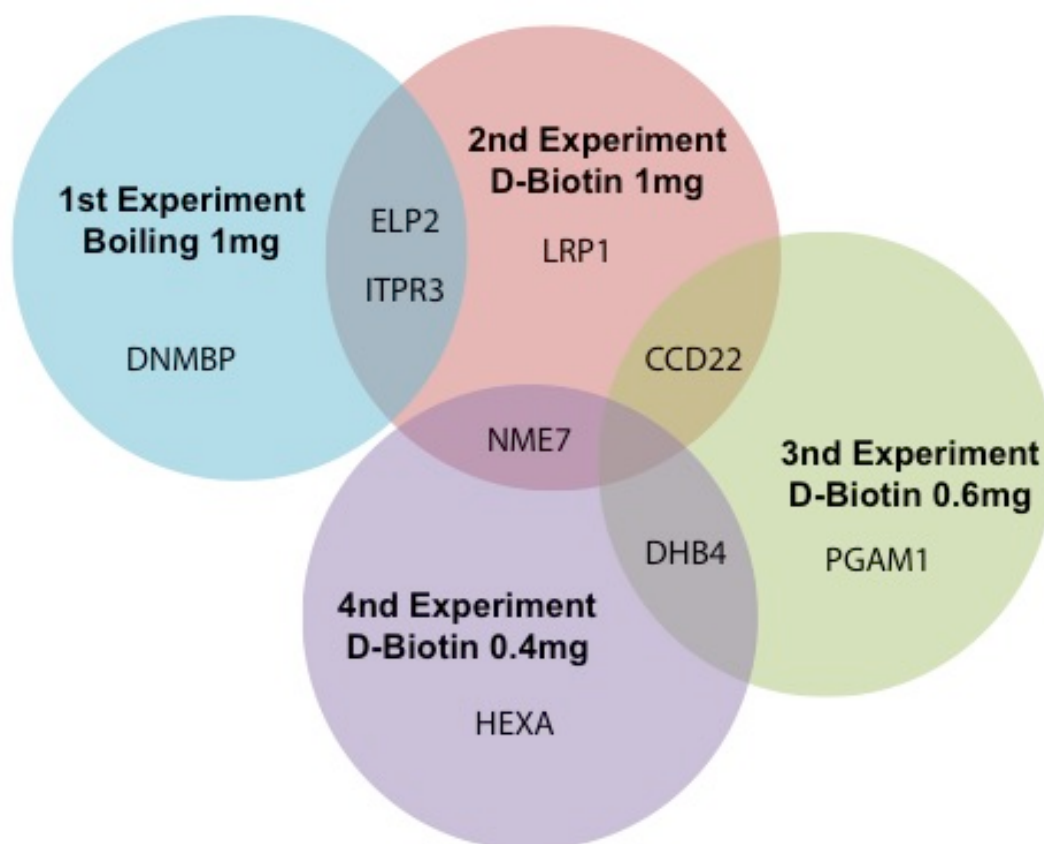
Table 5.4. Protein interactors obtained from the forth experiment results.

Accession	Description	Σ # Unique Peptides	4 th exp. # PSM 0.4mg Active Drug Biotin elution
P29416	Beta-hexosaminidase subunit alpha OS=Mus musculus OX=10090 GN=Hexa PE=1 SV=2 - [HEXA_MOUSE]	13	7
Q9WUD1	STIP1 homology and U box-containing protein 1 OS=Mus musculus OX=10090 GN=Stub1 PE=1 SV=1 - [CHIP_MOUSE]	3	4
Q5ND52	rRNA methyltransferase 3, mitochondrial OS=Mus musculus OX=10090 GN=Mrm3 PE=2 SV=1 - [MRM3_MOUSE]	3	3
Q8R0A0	General transcription factor IIF subunit 2 OS=Mus musculus OX=10090 GN=Gtf2f2 PE=1 SV=1 - [T2FB_MOUSE]	2	3
Q9WUK4	Replication factor C subunit 2 OS=Mus musculus OX=10090 GN=Rfc2 PE=1 SV=1 - [RFC2_MOUSE]	2	3

Q3TIX9	U4/U6.U5 tri-snRNP-associated protein 2 OS=Mus musculus OX=10090 GN=Usp39 PE=1 SV=2 - [SNUT2_MOUSE]	2	3
Q5SSW2	Proteasome activator complex subunit 4 OS=Mus musculus OX=10090 GN=Psme4 PE=1 SV=1 - [PSME4_MOUSE]	10	2
Q9CZ30	Obg-like ATPase 1 OS=Mus musculus OX=10090 GN=Ola1 PE=1 SV=1 - [OLA1_MOUSE]	9	2
P51660	Peroxisomal multifunctional enzyme type 2 OS=Mus musculus OX=10090 GN=Hsd17b4 PE=1 SV=3 - [DHB4_MOUSE]	9	2
Q04857	Collagen alpha-1(VI) chain OS=Mus musculus OX=10090 GN=Col6a1 PE=1 SV=1 - [CO6A1_MOUSE]	7	2
Q8K1G9	DEAH (Asp-Glu-Ala-His) box polypeptide 35 OS=Mus musculus OX=10090 GN=Dhx35 PE=1 SV=1 - [Q8K1G9_MOUSE]	6	2
Q9DBR7-2	Isoform 2 of Protein phosphatase 1 regulatory subunit 12A OS=Mus musculus OX=10090 GN=Ppp1r12a - [MYPT1_MOUSE]	6	2
B9EHJ3	Tight junction protein ZO-1 OS=Mus musculus OX=10090 GN=Tjp1 PE=1 SV=1 - [B9EHJ3_MOUSE]	6	2
E9Q179	G-rich sequence factor 1 OS=Mus musculus OX=10090 GN=Grsf1 PE=1 SV=1 - [E9Q179_MOUSE]	4	2
Q3UMG6	Non-metastatic cells 7, protein expressed in, isoform CRA_c OS=Mus musculus OX=10090 GN=Nme7 PE=1 SV=1 - [Q3UMG6_MOUSE]	4	2
Q05BH3	DEAH (Asp-Glu-Ala-His) box polypeptide 16 OS=Mus musculus OX=10090 GN=Dhx16 PE=1 SV=1 - [Q05BH3_MOUSE]	3	2
Q9ESX5	H/ACA ribonucleoprotein complex subunit DKC1 OS=Mus musculus OX=10090 GN=Dkc1 PE=1 SV=4 - [DKC1_MOUSE]	3	2

Highest retention occurred on Dynamin Binding Protein (DNMBP) in the first experiment, lipoprotein receptor-related protein 1 (LRP1) in the second experiment, Phosphoglycerate mutase 1 (PGAM1) in the third experiment, and finally, Beta hexosaminidase a (HEXA) in the fourth experiment (**Scheme 5.1**). The analyses of 4 experiment showed that there was no common protein target that was shared by all 4 experiments. However, Isoform 2 of elongator complex protein (ELP2) and Inositol 1,4,5-trisphosphate receptor type 3 (ITPR3) were shared in 1st and 2nd experiment in which 1mg protein used, yet different method implemented for protein elution. D-Biotin eluted 2nd and 3rd experiments both retained D-Coiled-coil domain-containing protein 22 (CCD22). 3rd and 4th experiment results shared peroxisomal multifunctional enzyme type 2 retention

(DHB4). Lastly, 2nd (1mg) and 4th experiment (0.4 mg) both showed Nucleoside diphosphate kinase 7 (NME7) (**Scheme 5.1**).



Scheme 5.1. Results of 4 proteomics experiments. The proteins with the highest PSM values and shared proteins were displayed.

5.4.Discussion

In this chapter, we tested AL isomers in cells and confirmed their beneficial effect in NPC1 CHO cells. As a second step, we utilised biotin-avidin interaction to explore interacting proteins of one of ALs, ALL by conjugating ALL with biotin, coating streptavidin bead surface to allow lysed NPC1 mouse fibroblast proteins' to interact with the active site of the compound. Consequently, we implemented a discovery proteomics technique on eluted proteins from the beads and explore the most robustly interacting proteins, which ALL potentially targets for beneficial effect.

Acetyl-Leucine isomers correct NPC mouse fibroblasts

Our studies done on CHO cells showed that acetyl-leucine isomers were able to correct NPC lysosomal and mitochondrial pathologies, confirming the finding of ALs reducing effect in biochemical storage in non-neuronal cells e.g. liver (**Chapter 2**). Twenty-four hour treatments with three acetyl-leucine isomers normalised burden of superoxide species, and highly reduced mitochondrial volume. On the other hand, lysosomal volume was reduced with ADLL and ALL, but not with ADL; in lesser extend than mitochondrial parameters. These trends might be due to, as discussed in **Chapter 4**, leucine's mitochondrial effect in the first instance. Similarly, having less beneficial impact on lysosomal volume, and achieving a significant reduction of sphingosine, cholesterol and GSL levels only post 72 hours treatment strengthen the hypothesis of having secondary impact on lysosome with both leucine and acetyl-leucine treatments.

However, 24-hour treatment provided a differentiation of AL's in cells, having no effect on lysosomal volume with ADL. Also, same biotinylation site of Acetyl-leucine's individual enantiomers showed potency in L form (Compound 13), but not in D form (Compound 14) might be a strong hint of the existence of different active sites of conformational isomers of acetyl-leucine. In the body, L form of enantiomers are favoured; leucine amino acid not being an exception[188]. In **Chapter 2**, we explored the individual enantiomers' effect and showed that L and D isoform have distinct affinity/target towards several metabolism aspects. This trend might be further straightened with the observations we demonstrated in this chapter on having different potency on lysosomal volume at 24h and having different potency when the biotin was

added to amide site of acetyl-leucine. Blockage of amide resulted in no activity in ADL might indicate this is the active site of this D enantiomer.

Drug-Protein interaction: Method evaluation

We performed drug-protein interaction study exploiting the high affinity of streptavidin-biotin binding. Our purpose was to explore the proteins that can specifically and effectively bind to active drug that was linked to the streptavidin column. The methodology of the study was ligand-protein enrichment, which is highly dependent on following parameters:

1. The total protein concentration
2. Lysis buffer
3. Binding time, wash time/volume/number/stringency
4. Elution method

We maintained the same lysis buffer (1% Digitonin), binding and washing times and volumes across our experiments. Digitonin, as a mild detergent, creates pores in biological membranes by getting replaced of cholesterol[259]. It allows an efficient, yet not invasive lysis, without denaturing the proteins[260], which in principle allows the detection of as many protein interactors as possible, but also it can allow us to detect proteins that could potentially interacting with drug-interacting proteins.

On the other hand, we trialled different protein concentrations and two elution methods to find the optimum experimental conditions with minimum unspecific binding. We tested two elution methods on 1 mg protein homogenates, and found that boiling the beads as a harsh elution method [261], resulted in a high number of protein (154 proteins) eluted from the beads (**Table 5.1**). The

second experiment was done using a D-Biotin competitive elution method[262], which reduced the elution of nonspecific proteins. When the same experiment was done using D-Biotin elution, the numbers of proteins significantly reduced (31 proteins) (**Table 5.2**). Boiling versus D-Biotin elution resulted in only 2 shared protein interactors, suggesting that boiling beads might not be sufficiently specific for this study. When we gradually reduced the concentration of protein lysates, keeping the D-elution method constant, we saw a predicted reduction in the detection of interacting proteins (0.6mg lysate: 15 proteins; 0.4mg lysate: 17 proteins) (**Table 5.3 and 5.4**). However, it is noteworthy that lowering the protein concentration from 0.6mg to 0.4mg did not yield a proportional reduction in protein numbers. These results might indicate that optimum protein concentration to achieve sufficient yield with high specificity is around 0.6-0.4mg. However, this study requires a positive control drug (i.e. miglustat), whose interacting proteins are known, is needed to determine the optimum protein concentrations and elution methods.

Drug-Protein interaction: Targets of Acetyl-L-Leucine

Our 4 proteomics experiments identified different proteins that were retained with the highest affinity on Biotin-ALL coated streptavidin beads (**Scheme 5.1**). 1mg lysate with boiling showed the highest interaction with ALL was with Dynamin Binding Protein (DNMBP-Tuba). Tuba is a multidomain scaffolding protein that is ubiquitously expressed and binds multiple actin-regulatory proteins directly, including N-WASP[263]. Tuba plays crucial roles in signalling and trafficking pathways with cytoskeletal rearrangement events, promotes accumulation of F-actin around mitochondria and regulates actin

assembly (via N-WASP dependent process)[263][264]. NPC1 mouse brain showed hyper phosphorylated cytoskeletal proteins in axon spheroids in presymptomatic stage (5 weeks), followed by spread of pathology to other regions, namely brainstem, basal ganglia, cerebellum, and eventually, the cortex, which might contribute to neurodegeneration [265].

When 1mg lysate was used with D-Biotin elution, the highest affinity interaction was to low density lipoprotein receptor-related protein 1 (LRP1/CD91), which is a ubiquitously expressed type 1 transmembrane receptor[266]. It takes part in lipid metabolism, cell growth and migration by two main physiological functions: endocytosis and regulation of signalling pathways. It is able to mediate endocytosis of lipoproteins, extracellular matrix proteins, viruses, to cytokines and growth factors[267]. LRP1 has also been shown to have a major pathophysiological role in the central nervous system (CNS); it is able to interact with N- methyl-D-aspartate (NMDA) receptor subunits, and glutamate system (via its effect on calcium signalling)[267]. Mice lacking LRP in neurons displayed severe behavioural and motor abnormalities with tremor, ataxia, hyperactivity and finally cachexia and premature death[268]. On the other hand, LRP1 gene, linked to Alzheimer's disease, was found to be up regulated in NPC cells, which might potentially be contributing factor to the NPC pathology by interacting with APP and mediating APP accumulation in cells[269].

Experiments using 1mg of lysate but with different elution methods gave two common protein interactors: Isoform 2 of elongator complex protein (ELP2), which binds to microtubules and mediates histone acetylation and cytoskeleton association[270] cell motility, neuronal growth and migration[271], and Inositol 1,4,5-trisphosphate receptor type 3 (ITPR3), which is intracellular Ca^{2+} release

channel on the endoplasmic reticulum membrane, receptive to the binding of a second messenger inositol 1,4,5-triphosphate (IP3)[272]. The expression level of ITPR3 is associated with growth and aggressiveness of different types of tumors, susceptibility to various immune-mediated diseases, such as type 1 diabetes rheumatoid arthritis, and Graves' disease[272].

Experiment 0.6 mg used displayed Phosphoglycerate mutase 1 (PGAM1) protein as the strongest interactor. PGAM1 is an important enzyme in the glycolytic pathway that catalyses the conversion of 3-phosphoglycerate into 2-phosphoglycerate, it can also upregulate pyruvate production directly[273]. It has been reported that PGAM1 is promotes cancer cell glycolysis and growth with increased enzyme levels in number of cancers (colorectal, liver, lung and breast cancer), and its enzymatic activity is also higher than normal tissues, which makes PGAM1 as a very attractive therapeutic target[274]. Stable knockdown of PGAM1 present lower glycolytic rates, low lactate production, RNA biosynthesis, lipogenesis, and relatively low cell proliferation rates[275].

Shared interactor between 1mg and 0.6mg protein lysate with D-biotin elution is D-Coiled-coil domain-containing protein 22 (CCD22), whose function is unknown. However, it was found that this protein was significantly down regulated in X-linked intellectual disability disorder[276].

The forth experiment, in which we used 0.4mg cell homogenates for drug-protein interaction study presented Beta Hexosaminidase A (HEXA) as the strongest interactor. HEXA is a crucial enzyme that locates in the lysosomes, responsible for GM2 breakdown and metabolism. Its loss results in another lysosomal storage disorder: Tay-Sachs disease, which manifests abnormal accumulation of GM2 gangliosides in especially CNS, sharing many biochemical

pathologies with NPC[277]. Affected individuals with disease exhibit a virtually identical clinical course of neurodegeneration leading to death in early stage[277]. Shared interactor between 0.6mg and 0.4mg experiments is peroxisomal multifunctional enzyme type 2 (HSD17B4). HSD17B4, also known as D-bifunctional protein (DBP), is a peroxisomal enzyme involved in fatty acid β -oxidation and steroid metabolism. Mutations in HSD17B4 cause DBP deficiency (MIM 261515), which is generally fatal within the first two years of life. Patients with DBP deficiency present hypotonia and seizures by one month of age[278]. DBP deficiency results in the accumulation of β -oxidation substrates (very long chain branched chain fatty acids, and bile acid intermediates) that are all detectable in serum and cultured patient fibroblasts[278]. Remarkably, defective peroxisomal fatty acid oxidation[279] and bile acid accumulation[280] in NPC have been reported.

Finally, the common interactor between 2nd (1mg, D-Biotin eluted) and the 4th (0.4mg, D-Biotin Eluted) experiments was Nucleoside diphosphate kinase 7(NME7). NDP kinases, which NME7 belongs to, are responsible for numerous regulatory processes associated with proliferation, development, and differentiation[281]. It was shown to interact with gamma-tubulin complex; its absence impairs centrosome-based microtubule nucleation.

Nine proteins that have been found to interact with ALL highlight the importance of cytoskeletal dynamics, lipid and glucose metabolism, which ultimately serves to the cell viability and growth in NPC. Interestingly, leucine's known interactor mTORC1[282] was not detected with biotin-conjugated ALL. This might be due to the inhibition of mTOR interaction due to N-acetylation [170]. Further studies to confirm and validate these data and show which player

has the highest impact on reducing mitochondrial and lysosomal NPC1 pathologies are needed. This study highlights the challenges of protein interaction studies when no positive control interactors are known and so optimising conditions in an evidence-based way is difficult.

Chapter 6: Concluding remarks and future directions

In this thesis, we have explored the efficacy of leucine amino acid treatments in neurodegenerative lysosomal storage diseases. The lead compound we explored was Acetyl-DL-Leucine, which was shown to reduce ataxia and balance problems, and improve motor function in observational studies in NPC patients [131]. However, the mechanism of action of ADLL in NPC remains unknown. We therefore utilised *in vitro* and *in vivo* models of NPC to better understand how this modified amino acid mediates its clinical benefit.

In **Chapter 2**, we used the *Npc1^{-/-nih}* mouse model to explore the pharmacology of the lead compound Acetyl-DL-Leucine (ADLL) on behavioural, biochemical and hispathologic parameters, and also investigated how individual enantiomers (ALL and ADL) behave, as ALL was shown to be the active enantiomer for vestibular compensation in the literature[147].

In agreement with the clinical data[131], we confirmed that ADLL administered acutely reduces ataxia in symptomatic *Npc1*^{-/-} mice treated for a week; however, interestingly, we also saw beneficial effects with ADL and ALL. The same anti-ataxic effect was also observed with ALs when the treatments were initiated from weaning (3 weeks of age) when the mice are pre-symptomatic. Normalisation of ataxia with either symptomatic or pre-symptomatic treatments was comparable; suggesting the anti-ataxic effect of ALs is through different mechanism of action and requires further investigation.

The early initiation of treatment with AL drugs had differential effects on *Npc1*^{-/-} mice, with ADLL and ALL, but not ADL, improving motor function, gait parameters and modestly increasing life span. ADLL and ALL were found to attenuate neuropathology. For example, ADLL increased Purkinje cell survival in the cerebellum, while ALL provided a significant reduction in neuroinflammation. It was rather surprising to observe only ADLL was able to provide neuroprotection, but not ALL; which conflicts with ALL being the active enantiomer that enables the beneficial effect in ADLL formulation (50%ALL, 50%ADL) as stated in the literature[164]. When we measured lipid storage in the brain, we found that ADL-only treatment was able to reduce sphinganine levels, while ALL-only treatment reduced GSL levels. ADLL the racemic mixture had intermediate effect on these parameters.

When we investigated the possible mechanistic targets of ALs we observed that ADLL activated a key metabolic enzyme, pyruvate dehydrogenase, an enzyme that is known to have reduced activity in NPC[93]. This enzyme is inactivated by phosphorylation and we observed reduced phosphorylation of PDH in ADLL treated mice. This correlated with reduced pyruvate

dehydrogenase kinase 2 levels. ALL-only treatment reduced SOD1 levels and shifted the PDH and LDH ratio, the indication of aerobic/anaerobic glucose metabolism ratio, towards PDH the most. ADL-only treatment increased SOD2 levels, normalising to WT level. These data strongly implies the notion of ALL and ADL synergy and possible consequent neuroprotective effect of ADLL

In vivo, ADLL and ALL treatment was associated with a significantly greater benefit (relative to ADL) in terms of neuropathology, motor function and lifespan, suggesting new approach to pharmacological therapy for this and other related neurodegenerative diseases could target PDH activation or LDH reduction in NPC. Many neurodegenerative diseases are associated with defects in underlying metabolism, a common theme being a shift to anaerobic respiration with less energy (ATP) produced as a consequence. A major finding of this thesis is that shifts in metabolic pathway utilisation are a viable therapeutic target.

We tested the synergy of ADLL with the approved therapy miglustat in NPC and observed a substantial life span increase, normalisation of low-weight pattern seen with miglustat treatment. These results clearly showed that leucine based treatments were effecting the cells' nutrient utilisation overall energy metabolism and ADLL's target on metabolic enzymes may create a synergy with miglustat as ADLL and miglustat have different mechanisms of action. Most patients treated with ADLL in observational studies are also on standard of care miglustat so clinically we are observing the complementary effects of both treatments.

In **Chapter 3**, we investigated whether the beneficial effect of ADLL could be translated to another LSD; Sandhoff Disease (GM2 gangliosidosis). When we

initiated ADLL treatment from weaning in Sandhoff (*Hexb*^{-/-}) mice we found that ADLL treatment extended life span, improved motor function and gait, and reduced lipid storage in the brain. However, the mechanism of action was different from NPC. ADLL was found to reduce the phosphorylation (inactivation) of hyperactive Sandhoff pyruvate dehydrogenase enzyme in the brain by increasing pyruvate dehydrogenase kinase 4 levels, and increase very low LDH enzyme levels and SOD1 protein expression. In addition, ADLL treatment increased autophagic flux. Finally, we found that ADLL and miglustat combination did not increase the life span more so than miglustat monotherapy suggesting that ADLL's effects on metabolic enzymes are disease specific and depend on the metabolic changes present in each specific disease.

ADLL reduces PDK2 (regulated by NADH and Acetyl CoA) levels further, activates PDH in NPC1 cerebellum, and it increases PDK4 (regulated by starvation and nutrient deprivation) levels and inactivates PDH in Sandhoff cerebellum (**Table 6.1**). These results suggest that ADLL might be causing glucose utilisation in NPC1 disease that is of low insulin and glucose consumption, and triggering glucose deprivation in Sandhoff disease that displays hyperinsulinism profile. This could be due to leucine's paradoxal effect on insulin signalling.[136]. BCAAs have been proposed to elicit different or even opposite effects on metabolism, depending on the prevalence of catabolic and anabolic states; leucine supplementation led to abnormal catabolism of BCAA and the incompletely oxidized lipid species that contributed to mitochondrial dysfunction HFD-fed rats in the early stage of insulin resistance, but it induced no remarkable alternations in BCAA catabolism and enhanced mitochondrial

biogenesis with a concomitant improvement in lipid oxidation and mitochondrial function in the diabetic state [283].

Cerebellum	Niemann Pick Type C (Lipid/Lys trafficking jam)	Sandhoff (Enzyme deficiency-GM2)
Autophagy	Jammed (increased autophagic vacuoles-increased p62)	Increased-normal p62 
Branched Chain AA catabolism	Increased	Normal
PDH	Very inactive 	Very active 
PDK2	Low 	Low
PDK4	Normal	Normal 
LDH	High	Normal 
SOD1	Normal	High 
SOD2	Low	Normal
GDH	Normal	High 

Table 6.1. Summary of NPC1 and Sandhoff mice cerebellum pathologies investigated and their response to ADLL treatment. Green arrows indicate the effect of ADLL treatment.

In **Chapter 4**, we investigated the efficacy of non-acetylated leucine amino acids in NPC1 mouse and cell. We observed the same synergistic trend between LL and DL when combined as the racemic mixture (DLL); it was able to increase life span, and reduce sphinganine levels in the brain in mice (**Table 6.2**). Individually, LL treatment improved motor function and reduced inflammation in the cerebellum, while DL improved one of the gait parameters in *Npc1*^{-/-} mice. N-Acetylation of the leucine racemic mixture (ADLL) was more effective than the non-acetylated form (DLL), attenuating neurodegeneration in cerebellum and lipid accumulation in liver and CNS. Similarly, the acetylated form of L-Leucine (ALL) performed significantly better than LL treatment, with additional life span extension and lowering of lipid accumulation in *Npc1*^{-/-} mice (**Table 6.2**). When L-Leucine's ethyl ester form was trialled in *Npc1*^{-/-} mice, it provided

neuroprotection on top of LL's effect and showed better efficacy. Intriguingly, when L-Leucine is N-acetylated (ALL), it lost the neuroprotective effect, showing that there is scope for further medicinal chemistry around these branch chain amino acids to better understand structure:function and to potentially generate more potent compounds. In parallel, N-Acetylation of D-Leucine also resulted in increased efficacy (**Table 6.2**).

	<i>In vivo</i> Parameters	ADLL	DLL	ALL	LL	ETLEU	ADL	DL
1	Survival	+	+	+	0	0	0	0
2	Gait Duty cycle	+	0	+	0	0	0	+
3	Average Speed	0	0	0	0	0	0	0
4	Rotarod	+	0	+	+	+	0	0
5	Liver Sphingosine	+	0	+	0	0	+	0
6	Liver Sphinganine	+	0	+	0	0	+	0
7	Liver GSL	+	0	+	0	0	+	0
8	Liver Cholesterol	+	0	+	0	0	+	0
9	Brain Sphingosine	0	+	0	0	0	0	0
10	Brain Sphinganine	+	+	0	0	0	+	0
11	Brain GSL (Total)	0	0	0	0	0	0	0
12	Cerebellum Sphinganine	0	0	+	+	0	0	0
13	Cerebellum Sphinganine	0	0	0	0	0	+	0
14	Cerebellum GSL	0	0	0	0	0	0	0
15	Cerebellum CD68	0	0	+	+	+	0	0
16	Cerebellum Purkinje cells	+	0	0	0	+	0	0

Table 6.2. Summary of leucine amino acid effects in NPC mouse. (+) indicates a significant improvement, (0) indicates no difference was found compared to NPC1 untreated group.

In vitro studies in **Chapter 4** (DLL-LL-DL-Etleu) and **Chapter 5** (ADLL-ALL-ADL) for all leucine treatments showed that the most profound effects with 24h treatments were on high mitochondrial ROS levels and elevated mitochondrial volume of NPC1 cells, whereas elevated lysosomal volume was very mildly reduced (**Figure 6.3**). The main pathologic aspects of NPC1, which are lipid accumulation and overall lysosomal volume was significantly reduced after 72h treatment, proposing that the mitochondrial pathology is the main

target of these drugs. This finding also speculates the impact of mitochondria on lysosomal function. Biotin conjugation to ALL and ADL resulted in differentiated activities *in vitro* (**Figure 6.3**). Biotin addition to amide tail showed no effect with ADL on cells while Biotin-ALL was active similar to ALL and LL. However, *tert*-butyl addition to carboxylic acid tail of ALL led to activity loss (**Figure 6.3**). Same biotinylation site of Acetyl-leucine's individual enantiomers showed potency in L form, but not in D form, which might be a strong hint of the existence of different active sites of isoforms of acetyl-leucine.

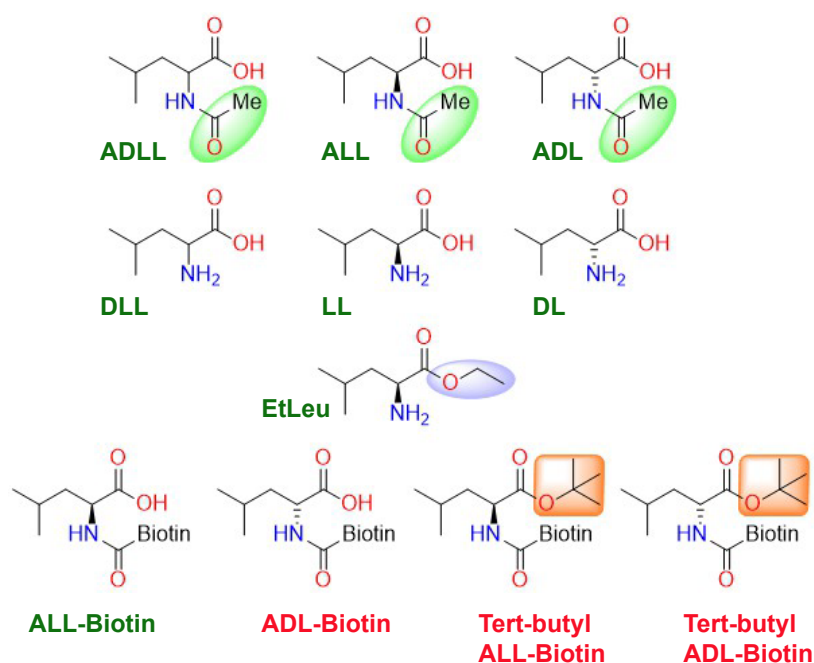


Figure 6.3. Summary of leucine amino acid effects in NPC1 cells. Green-coloured compounds were pharmacologically active, red-coloured compounds were found to be inactive. N-acetylation is highlighted in green, ethyl esterification in purple, *tert*-butyl addition in orange.

Chapter 5 investigated the interacting proteins of Acetyl-L-Leucine, using avidin-biotin interaction. From this preliminary study we identified possible acetyl-leucine interactors, which affect β -oxidation, cytoskeletal dynamics and glucose metabolism. The same approach should be used for Acetyl-D-Leucine interacting proteins to confirm these findings and better optimisation of this

method would also be needed. However, the studies to date show that combining affinity column and proteomic approaches potential interacting proteins can be identified and this could be developed further in the future.

In conclusion, this thesis has identified unanticipated neuroprotective benefits of acetyl-leucine treatments in NPC and Sandhoff disease. To our knowledge, this is the first study that investigates energy metabolism as a therapeutic target, and highlights the importance of pyruvate dehydrogenase and potentially insulin signalling for disease modification in LSDs. We have found unanticipated benefits of ADLL and ALL as neuroprotective drugs that slow disease progression suggesting that they may have broader therapeutic utility in other neurodegenerative diseases. The striking synergy of ADLL with miglustat in *Npc1*^{-/-} mice suggests that combining these two drugs will lead to improved short- and longer-term clinical benefit and merits placebo controlled clinical trials.

If time had allowed, I would have liked to pursue further the actual target of ADLL in NPC1 and Sandhoff mice that results in differential effect on the metabolic response and glucose metabolism, as it is still unknown. I was only able to assess the sphingosine, sphinganine, GSL and cholesterol changes with the therapies *in vitro* and *in vivo*; I believe measuring ceramide levels and investigating insulin-signalling pathways would uncover a very important aspect of ADLL's pharmacology. Conducting gene expression profiling for both NPC and Sandhoff disease with and without ADLL treatment also has the potential to reveal the protein targets that explain ADLL's effects.

In addition, I would have pursued the how mitochondrial enzyme PDH manipulation by pharmacological tools would effect the pathologies in NPC1

non-neuronal cells. For example looking at lysosomal functions (volume and cumulative lipids) and mitochondrial volume and ROS could be assessed with PDK inhibitors.

Furthermore, having shown the availability of applying chemical modifications to leucine amino acid to increase its efficacy, I believe it is also a very promising part to follow further. I would be very interested in trialling different leucine compounds and different branched chain amino acids that are metabolised in a same way as leucine *in vivo* and *in vitro* LSD models. Very exciting finding that we were able to present and this thesis which should also be investigated further is the low levels of NAD and NADH cofactors in the *Npc1* mouse brain. Agents that would increase these cofactors could display a promising therapy option for NPC disease.

Our results show that a new pharmacologic approach for LSDs can be manipulating glucose metabolism. For example, Metformin (N, N-dimethyl biguanide) is widely used first-line anti-hyperglycaemic drug for treatment of type 2 diabetes (T2D), which can reverse fatty liver, improve insulin sensitivity, and ameliorate cardiovascular dysfunctions associated with T2D [284]. Moreover, cancer incidence was found to be decreased in patients treated with metformin [284]. Based on the encouraging research results, a clinical trial will test whether metformin can delay the onset of age-related diseases, including cancer and Alzheimer's disease[285]. Metformin was found to acidify the lysosomal/endosomal compartment and increase AMPK activity in microglial cells[284]. It would be interesting to trial Metformin, or AMPK-related compounds in NPC1 and Sandhoff disease models.

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