

Luis Carlos Serrachino Granadeiro

Understanding how ACBD5 mediates lipid dysregulation and neuropathology

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Orientador – Doutor Pedro Miguel Brites
Categoria – Investigador Principal
Afiliação – Instituto de Biologia Molecular e
Celular, Instituto de Investigação e Inovação em
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À minha família.

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Summary

The nervous system is one of the most complex tissues and consists of a variety of tissue-specific cell types: neurons, oligodendrocytes, Schwann cells, astrocytes, and microglia. The nervous tissue is divided into the central nervous system (CNS), comprising the brain, spinal cord, optic nerve and retina, and the peripheral nervous system (PNS). The complexities of the nervous system and the close interaction of all these cells render this system susceptible to disturbance. Metabolic dysfunction associated with impairment of peroxisomal functions is often associated with the perturbation of brain formation, function, or maintenance; thus, pathological nervous system abnormalities are prominent features in most peroxisomal disorders. Peroxisomes are multifunctional organelles present in all eukaryotic cells and are crucial in the metabolism of lipids. They play unique roles in cellular metabolism being involved in key processes that include beta-oxidation of very long-chain fatty acids (VLCFA; >C22:0), pristanic acid, bile acid intermediates, and polyunsaturated fatty acids (PUFA); biosynthesis of ether-phospholipids including plasmalogens and alpha-oxidation of phytanic acid. Their importance is marked by the existence of a large number of inborn errors in which an impairment in one or more of their metabolic functions can occur. Patients present different clinical signs and symptoms depending on the enzyme that is affected and the extent of the deficiency; however, the disorders are usually severe, with neurological dysfunction and early death in some cases. Peroxisomal disorders can be divided into a group called Multiple Peroxisomal Function Defects (MPFD) or a group with distinct deficiencies called Single Peroxisomal Function Defects (SPFD). The MPFD disorders are typified by the Zellweger spectrum disorder (ZSD) in which the complete loss of peroxisomes and their functions causes severe abnormalities, such as neurological malformations, hypotonia, seizures, hepatomegaly, bone abnormalities, and leukodystrophy; but also biochemical abnormalities, including VLCFA accumulation, marked depletion of ether phospholipids (plasmalogens), and reduced levels of docosahexaenoic acid (DHA). Moreover, different failures in the functioning of peroxisomes can lead to a single function defect within the peroxisome. This defect can be attributed to either matrix enzymes or membrane proteins, which are primarily involved in transporting metabolites. X-linked adrenoleukodystrophy is the most well-known single peroxisome enzyme deficiency and is caused by mutations in the peroxisomal ACBD1 membrane transporter, essential for the translocation of CoA-activated VLCFAs across the peroxisomal membrane. ALD patients present severe defects in the CNS with progressive demyelination (leukodystrophy), motor dysfunction, and developmental delay, being these disorders first diagnosed by an elevated plasma VLCFA ratio, such as C26:0/C22:0 and C26:1/C22:0.

Recently, a new disorder was identified with a nosologically distinct presentation. Mutations in the gene acyl-CoA binding domain-containing protein 5 (ACBD5) were identified in patients presenting cone-rod dystrophy, spastic paraparesis, psychomotor delay, and leukodystrophy. Previous proteomic studies have identified ACBD5 as a peroxisomal protein, bringing the newly found ACBD5 deficiency to be considered a novel peroxisomal disease. More recently, ACBD5 was identified as another critical factor in VLCFA catabolism. ACBD5 is a peroxisomal protein containing an acyl-CoA binding domain (ACBD) and a FFAT motif that can mediate interaction with ER-resident proteins VAPA and VAPB. The ACBD and the observation of VLCFA accumulation in ACBD5 deficient patients consolidated a central role of ACBD5 as a possible handler of VLCFA on route for ABCD1-mediated transport and peroxisomal β -oxidation. ACBD5 deficiency was initially discovered in a subgroup of patients presenting rod-cone dystrophy. Notwithstanding the retina defects, psychomotor delay, spasticity, ataxia, and involvement of white matter are also prominent features of the disorder.

Histological studies on ALD tissues significantly contributed to the characterization of the neuropathology caused by the accumulation of VLCFA. Active myelin destruction with infiltrating monocytes and lipid-laden macrophages are key signatures of the cerebral inflammatory presentation. In contrast, spinal cord axonopathy and peripheral neuropathy are the main presentations of adult AMN disorder. The lack of correlative features between VLCFA accumulation and disease onset or pathology highlights a complex system through which VLCFA mediates cell and tissue pathology. The limited availability of mouse models with partial phenocopying of the corresponding disease also hindered this understanding. The *Abcd1* knockout (KO) mouse has a very late onset of axonal degeneration. In support of this, it has been observed that the double *Abcd1:Abcd2* KO mutant mouse presents a worsened pathology and disease presentation, allowing significant progress in understanding mechanisms and evaluating candidate therapies.

On the other hand, ACBD5 seems to have a more centralized role as it may be critical for handling VLCFA and necessary for the correct presentation of these fatty acids to ABCD1. In support of this, an *Acdb5tm1a* mutant mouse with blocked transcription has the characteristic accumulation of VLCFA and displays cerebellar and hepatic changes. To investigate the consequences of abnormal VLCFA metabolism within the CNS, we developed, using CRISPR/Cas9, an *Acdb5* Gly357* mutant mouse model. Here, we show that Gly357* mice faithfully mimic key aspects of the human disorder. Gly357* mutant mice developed progressive ataxia and severe neuropathology affecting the retina, cerebellum, and spinal cord, with neurodegeneration, gliosis, and myelin loss. Targeted lipidomic analysis revealed that *Acdb5* deficiency leads to generalized lipid dysregulation. Proteomic analysis unveiled the cellular defects in the degenerating spinal cord and uncovered a

disruption in actin dynamics. Despite the multitude of cellular defects, the ataxia and neurodegeneration can be prevented through adeno-associated virus (AAV)-mediated expression of *Acbd5*. Our results provide mechanistic insights into how impaired VLCFA oxidation induces severe neuropathology. Additionally, we demonstrate that AAV-mediated gene therapy is capable of preventing the escalating degeneration observed within the CNS.

Sumário

O sistema nervoso é um dos tecidos mais complexos e compreende uma variedade de tipos celulares específicos: neurónios, oligodendrócitos, células de Schwann, astrócitos e micróglia. O tecido nervoso é dividido em sistema nervoso central (SNC), que compreende o cérebro, a medula espinhal, o nervo ótico e a retina, e o sistema nervoso periférico (SNP). As complexidades do sistema nervoso e a interação próxima de todas essas células tornam este sistema suscetível a distúrbios. A disfunção metabólica associada ao comprometimento das funções peroxissomais é frequentemente associada à perturbação da formação, função ou manutenção do cérebro; portanto, anormalidades patológicas do sistema nervoso são características proeminentes na maioria dos distúrbios peroxissomais. Os peroxissomas são organelos multifuncionais presentes em todas as células eucarióticas e são cruciais no metabolismo de lipídios. Eles desempenham papéis únicos no metabolismo celular, estando envolvidos em processos-chave que incluem a beta-oxidação de ácidos gordos muito longos (AGMLs; >C22:0), ácido pristânico, intermediários de ácidos biliares e ácidos gordos poli-insaturados (AGPIs); biossíntese de éter-fosfolipídios, incluindo plasmalógenos e alfa-oxidação do ácido fitânico. A sua importância é marcada pela existência de um grande número de erros inatos nos quais o comprometimento em uma ou mais funções metabólicas pode ocorrer. Os pacientes apresentam diferentes sinais e sintomas clínicos, dependendo da enzima que está afetada e da extensão da deficiência; no entanto, os distúrbios geralmente são graves, com disfunção neurológica e morte precoce em alguns casos. Os distúrbios peroxissomais podem ser divididos em um grupo chamado Defeitos de Múltiplas Funções Peroxissomais (DMFP) ou grupo com deficiências distintas chamado Defeitos de Função Peroxissomal Única (DFPU). Os distúrbios MPFD são tipificados pelo espectro de desordem de Zellweger (ZSD), no qual a perda completa de peroxissomas e das suas funções causa anormalidades graves, como malformações neurológicas, hipotonia, convulsões, hepatomegalia, anormalidades ósseas e leucodistrofia; mas também anormalidades bioquímicas, incluindo acumulação de AGMLs, depleção acentuada de éter-fosfolipídios (plasmalógenos) e níveis reduzidos de ácido docosahexaenoico (DHA). Além disso, diferentes falhas no funcionamento dos peroxissomas podem levar a um defeito de função único dentro do peroxissomo. Este defeito pode ser atribuído a enzimas de matriz ou proteínas de membrana, que estão principalmente envolvidas no transporte de metabólitos. A adrenoleucodistrofia ligada ao cromossomo X é a deficiência enzimática peroxissomal única mais conhecida e é causada por mutações no transportador de membrana peroxissomal ACBD1, essencial para a translocação de AGMLs ativados por CoA através da membrana peroxissomal. Os pacientes com ALD apresentam defeitos graves no SNC

com desmielinização progressiva (leucodistrofia), disfunção motora e atraso no desenvolvimento, sendo esses distúrbios diagnosticados inicialmente por uma razão elevada de AGMLs plasmáticos, como C26:0/C22:0 e C26:1/C22:0.

Recentemente, uma nova patologia foi identificada com uma apresentação nosologicamente distinta. Mutações no gene da Proteína de ligação à acil-CoA contendo o domínio 5 (ACBD5) foram identificadas em pacientes apresentando distrofia dos cones e bastonetes, paraparesia espástica, atraso psicomotor e leucodistrofia. Estudos proteômicos anteriores identificaram o ACBD5 como uma proteína peroxissomal, fazendo com que a deficiência recém-descoberta seja considerada uma nova doença peroxissomal. Mais recentemente, o ACBD5 foi identificado como outro fator crítico no catabolismo de AGMLs. O ACBD5 é uma proteína peroxissomal contendo um domínio de ligação à acil-CoA (ACBD) e um motivo FFAT que pode mediar a interação com proteínas residentes no RE, como VAPA e VAPB. O ACBD e a observação da acumulação de AGMLs em pacientes deficientes em ACBD5 consolidaram um papel central do ACBD5 como um possível manipulador de AGMLs na rota para o transporte mediado por ABCD1 e beta-oxidação peroxissomal. A deficiência de ACBD5 foi inicialmente descoberta num subgrupo de pacientes apresentando distrofia dos cones e bastonetes. Apesar dos defeitos na retina, atraso psicomotor, espasticidade, ataxia e envolvimento da substância branca também são características proeminentes da patologia.

Estudos histológicos em tecidos de ALD contribuíram significativamente para a caracterização da neuropatologia causada pela acumulação de AGMLs. Destruição ativa da mielina com monócitos infiltrantes e macrófagos carregados de lipídios são assinaturas-chave da apresentação inflamatória cerebral. Em contraste, a axonopatia na medula espinhal e a neuropatia periférica são as principais apresentações da AMN adulta. A falta de características correlacionadas entre a acumulação de AGMLs e o início da doença destaca um sistema complexo através do qual AGMLs medeiam a patologia celular. A disponibilidade limitada de modelos de ratinhos com fenótipo parcial da doença correspondente também dificultou essa compreensão. O ratinho knockout (KO) de *Abcd1* tem um início muito tardio de degeneração axonal. Em apoio a isso, foi observado que o ratinho mutante duplo *Abcd1:Abcd2* KO apresenta uma patologia e apresentação de doença mais agressivas, permitindo progressos significativos na compreensão dos mecanismos e na avaliação de terapias.

Por outro lado, o ACBD5 parece ter um papel mais centralizado, pois demonstra ser crítico na translocação de AGMLs e necessário para a apresentação correta desses ácidos graxos para o transportador ABCD1. Em apoio a isso, um modelo de ratinho mutante *Acbd5^{tm1a}* com transcrição bloqueada apresenta a característica acumulação de AGMLs e exibe alterações cerebelares e hepáticas. Para investigar as consequências do

metabolismo anormal de AGMLs no SNC, desenvolvemos, usando CRISPR/Cas9, um modelo de murganho mutante Gly357* de Acbd5. Aqui, mostramos que os murganhos Gly357* mimetizam os aspectos-chave da patologia humana. Os murganhos mutantes Gly357* desenvolveram ataxia progressiva e neuropatologia grave afetando a retina, cerebelo e medula espinhal, com neurodegeneração, gliose e perda de mielina. A análise lipidômica revelou que a deficiência de Acbd5 leva à desregulação lipídica generalizada. A análise proteômica revelou os defeitos celulares na medula espinhal em degeneração e mostrou uma alteração na dinâmica da actina. Apesar da multiplicidade de defeitos celulares, a ataxia e a neurodegeneração podem ser prevenidas por meio da expressão mediada pelo vírus adeno-associado (AAV) de Acbd5. Os nossos resultados permitiram dar uma visão mais aprofundada sobre os mecanismos pelos quais a oxidação de AGMLs comprometida induz neuropatologia grave. Além disso, demonstramos que a terapia genética mediada por AAV é capaz de prevenir a degeneração progressiva observada no SNC.

Abbreviations List

ABC - ATP-binding cassette

ABCD1 - ATP Binding Cassette Subfamily D Member 1

ABCD2 - ATP-binding cassette sub-family D member 2

ABCD3 - ATP-binding cassette sub-family D member 3

ABCD4 - ATP Binding Cassette Subfamily D Member 4

ABP - Actin-binding proteins

ACAA1 - 3-ketoacyl-CoA-thiolase

ACBD - Acyl-CoA binding domain

ACBD5 - Acyl-CoA Binding Containing Domain Protein 5

ACBP - Acyl-CoA Binding Proteins

ACOX1 - Acyl-CoA oxidase 1

ACOX2 - Acyl-CoA oxidase 2

AGPS - Alkylglycerone Phosphate Synthase

ALD - Adrenoleukodystrophy

ALDH1L1 - Aldehyde Dehydrogenase 1 Family Member L1

ALDP - Adrenoleukodystrophy protein

AMN – Adrenomyeloneuropathy

AT - Acid track

ATP - Adenosine triphosphate

BBB - Blood-brain barrier

C – Carbon

cALD - Cerebral ALD

CAMK II - Calcium/calmodulin-dependent protein kinase type II

Cas9 – CRISPR-associated protein 9

CD51- Cluster of differentiation 51

CD63 - Cluster of differentiation 63

CD71 - Cluster of differentiation 71

CNPase - 2',3'-Cyclic-nucleotide 3'-phosphodiesterase

Co-A – Coenzyme A

CRISPR - Clustered regularly interspaced palindromic repeats

DBP - D-bifunctional protein

ELOVL - Elongase of very long-chain fatty acid

ELOVL4 - Elongase of very long-chain fatty acid 4

ELOVL5 - Elongase of very long-chain fatty acid 5

ER - Endoplasmic Reticulum

FA - Fatty acids

F-actin – Filamentous actin

FAR1 - Fatty Acyl-CoA Reductase 1

FF - Two-phenylalanines

GABA - Gamma-aminobutyric acid

G-actin - Globular actin

GalC - Galactosylceramidase

GDP - Guanosine diphosphate

GFAP - Glial fibrillary acidic protein

GL - Glycerolipids

Gly - Glycine

GNPAT - Glyceronephosphate O-Acyltransferase

GPL - Glycerophospholipids

kDa - Kilodalton

LCFA - Long-chain Fatty Acids

LTD - long-term depression

LTP - Long-term potentiation

MAG - Myelin associated glycoprotein

MAP-1 - Microtubule-associated protein 1

MAP-2 - Microtubule-associated protein 2

MAPs - Microtubules associated proteins

MBP - Myelin basic protein

MCFA - Medium-chain Fatty Acids

MFP-2 – Multifunctional Protein 2

MOG - Myelin-oligodendrocyte glycoprotein

MT - Microtubules

MUFAs - Monounsaturated Fatty Acids

NF155 - 155-kDa neurofascin

NMDA - N-methyl-D-aspartate

OPC - Oligodendrocyte progenitor cells

PC - Phosphatidylcholine

PE – Phosphatidylethanolamine

PEX7 - Peroxin 7

PhyH - Phytanoyl-CoA hydroxylase

PKA - Protein kinase A

PKC - Protein kinase C

PLP - Myelin proteolipid protein

PTS - Peroxisomal targeting sequences

PUFAs - Polyunsaturated Fatty Acids

RCDP - Rhizomelic chondrodysplasia Punctata

RNA - Ribonucleic acid

SCPx - Sterol carrier protein X

SP -Sphingolipids

ST - Sterol lipids

TM - Transmembrane domain

VAPA - vesicle-associated membrane protein–associated proteins A

VAPB - vesicle-associated membrane protein–associated proteins B

VLCFA - Very-long chain fatty acids

ZSD - Zellweger spectrum disorder

α/β -spectrin – Alpha/Beta spectrin

α -oxidation – Alpha-oxidation

β -oxidation – Beta-oxidation

μm – Micrometer

Chapter 1: Introduction

Introduction

1. Nervous tissue

The nervous tissue is one of the most complex tissues of the body. It is divided into two different parts, namely the central and peripheral nervous systems. The central nervous system (CNS) comprises the brain, spinal cord, optic nerve and retina and is responsible for processing and coordinating information throughout the body ^{(1); (2)}. The brain is responsible for a wide range of functions, including sensory perception, motor control, cognition, and emotions ^{(1); (2)}. It is divided into several regions, including the cerebrum, cerebellum, and brainstem ^{(1); (2)}. The spinal cord is a long structure that extends from the brainstem down through the vertebral column and is responsible for transmitting sensory and motor information between the brain and the rest of the body ^{(1); (2)}. The peripheral nervous system (PNS) comprises the dorsal root ganglia and nerves and can be divided into two main systems: sensory and motor ^{(1); (2)}. While the sensory system is responsible for transmitting sensory information from different parts of the body to the CNS, including temperature, pain, and proprioception, the motor system is responsible for transmitting motor commands from the CNS to the muscles and glands of the body ^{(1); (2)}. The motor system can be further divided into the somatic and autonomic nervous systems, which control voluntary movements of skeletal muscles and involuntary movements of smooth muscles and glands, respectively ^{(1); (2)}. Lastly, the autonomic nervous system is divided into the sympathetic and parasympathetic systems, which have opposing effects on the body's functions. The sympathetic nervous system prepares the body to respond to stress or danger ^{(1); (2)}. This response includes an increase in heart rate, blood pressure, and breathing rate, as well as a decrease in digestive and immune system activity ^{(1); (2)}. On the other hand, the parasympathetic nervous system promotes relaxation and conserves energy by decreasing heart rate, blood pressure, and breathing rate, as well as increasing digestive and immune system activity ^{(1); (2)}.

The central nervous tissue has a high cellular density with limited extracellular space ⁽³⁾. The two central cells in the CNS are neurons and glia, which include astrocytes, microglia, oligodendrocytes.

1.1 Neurons

Neurons are the principal cells of the nervous tissue. They are polarized and highly specialized cells able to form circuits/networks crucial for tissue function. Morphologically,

they are divided into cell body, dendrites, and axons ^{(1); (2); (4)}. The cell body is the compartment that contains the nucleus and the most organelles and is responsible for processing the information received; dendrites are specialized extensions that arise from the cell body, which receive information from other neurons or sensory cells ^{(1); (2); (4)}. Typically, dendrites are branched and have a tree-like appearance, with the number and shape varying depending on the neuron's type and its location in the nervous tissue ^{(1); (2); (4)}. Dendrites are covered by small protrusions called dendritic spines, which increase their surface area and allow them to efficiently receive more signals ^{(1); (2); (4)}. Dendrites are highly dynamic and can be modified by experience, learning, and memory formation ^{(5); (6)}. This process is designated as dendritic plasticity and involves changes in the number and shape of the dendritic spines ^{(5); (6)}. Lastly, the axon is a long process that usually stems from the cell body. There are types of neurons where the axon can also arise from dendrites, which is called axodendritic. The axon is responsible for generating and propagating the electrical signal and transmitting it to other neurons or effector cells such as muscles or glands ^{(1); (2); (4); (7)}. This process starts in the axon initial segment (AIS) ^{(7); (8)}. The AIS is a specialized region located at the proximal part of the axon, which, due to a high density of voltage-gated channels, is responsible for generating the action potential ^{(7); (8)}. The AIS also contains a specific cytoskeletal organization essential for maintaining the integrity and function of the axon. Moreover, it exhibits selective properties that regulate the localization, distribution, and function of proteins, lipids, vesicles, and organelles within the axon, ultimately contributing to its integrity and proper function in neuronal signaling ^{(7); (8)}.

1.1.1 Neuronal communication

Due to the cell's importance for a multitude of body's actions and functions, proper communication is crucial. Neurons can be classified based on their functional effects on other neurons or cells as excitatory or inhibitory ^{(1); (2); (4)}. The excitatory neurons are responsible for the release of neurotransmitters that stimulate other neurons or cells, making them more likely to fire action potentials and transmit signals; contrarily, inhibitory neurons release neurotransmitters that inhibit or decrease the possibility of other neurons or cells firing action potentials and transmit signals ^{(1); (2); (4)}. Depending on the neurotransmitter released, neurons can be further categorized as cholinergic, adrenergic, dopaminergic, serotonergic, GABAergic, glutamatergic, or histaminergic ^{(1); (2); (9); (10)}. Chemical signals are transmitted across synapses, specialized junctions at the interface between the axon and its target (e.g., dendritic spines, muscle cells) ^{(1); (2); (11)}. When an action potential reaches the end of the axon of the presynaptic neuron, it triggers the release of neurotransmitters that bind to receptors on the postsynaptic cell (that can be another

neuron or a target cell) ^{(1); (2)}. When a neuron receives a stimulus, sodium ion channels on the membrane open, allowing sodium ions to flow into the cell, which makes the cell more positively charged and the outside more negatively charged ^{(1); (2)}. This process is called depolarization, and if it reaches a certain threshold, it can trigger an action potential ^{(1); (2)}. Once the action potential is triggered, voltage-gated potassium channels open, allowing potassium ions to flow out of the cell, repolarizing the neuron by making the inside of the cell more negative again ^{(1); (2)}. At the same time, the sodium ion channels close, preventing further influx of sodium ions, which lead neurons to restore the resting membrane potential ^{(1); (2)}.

1.1.2 Cytoskeleton and axonal transport

The neuronal cytoskeleton, composed of neurofilaments, actin, and microtubules, is an intricate network of proteins that is essential for maintaining the structure and function of neurons (Figure 1) ^{(8); (12)}. Most of these cytoskeletal proteins are synthesized in the soma and transported along the axon, where they are critical for maintaining its integrity and morphology ^{(8); (12)}. In the early stages, the cytoskeleton is important for neurons to establish neuronal polarity and maintain cell shape ⁽¹³⁾. Additionally, it is responsible for the transport of organelles and proteins along the axon ^{(8); (12); (14)}. In this sense, a unique cytoskeleton organization is necessary to ensure the proper transmission of information along the axons.

Neurofilaments (NF) belong to the class IV of the intermediate filaments ^{(8); (15)}. In the CNS, NF are composed of four subunits including the NF-Light, NF-Medium, NF-Heavy and alpha-internexin ^{(8); (15)}. While relatively sparse in dendrites and cell body, NF are particularly abundant in the axon and are essential for controlling the diameter and thereby the axonal conductance ^{(8); (15); (16)}. As NF establish extensive cross-bridges with each other and with actin filaments and microtubules through several types of cross-bridging proteins, including spectrin ⁽¹⁷⁾, plectin ⁽¹⁸⁾, and microtubules-associated proteins (MAPs) such as MAP-1 and MAP-2 ^{(17); (19); (20)}, these cross-bridges are proposed to help maintaining the integrity and density of neurofilaments but also to keep the neurofilament network anchored to plasma membrane and the other cytoskeletal components ^{(8); (12)}. NF are among the most highly phosphorylated proteins in nervous tissue, and their phosphorylation depends on a complex regulation between kinases and phosphatases, which is critical to impede pathological alterations ⁽²¹⁾. The phosphorylation mediated by protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase type II (CAMK II) play a key role in regulating the assembly, stability, transport, and interaction with other cytoskeletal components ⁽⁸⁾. Moreover, phosphorylation of the NF-Heavy and NF-Medium sidearms

promotes their cross-linking and the formation of a highly stable axonal neurofilament cytoskeleton ^{(15); (22)}. On the other hand, dephosphorylation is performed by phosphatase proteins. The protein serine/threonine phosphatases comprise a multigene family highly conserved in evolution ⁽²³⁾. Considering sequence homologies and biochemical properties, the known phosphatases can be divided into four interrelated families, including the protein phosphatase types 1, 2A, 2B, and 2C ⁽²³⁾. Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) have been identified as key players in catalyzing the dephosphorylation of neurofilament proteins ^{(23); (24); (25)}. PP2A is recognized as the primary phosphatase responsible for dephosphorylating all three neurofilament subunits ⁽²³⁾. PP1 is also involved in neurofilament regulation, although to a lesser extent, playing a minor role in their dephosphorylation ⁽²³⁾.

Actin filaments, known as F-actin, are polymers built up from globular actin (G-actin) ^{(8); (26)}. These filaments are abundantly present in all cells being important for cell division, adhesion, and motility ⁽²⁷⁾. In mammals, there are three actin isoforms: α , β , and γ , of which α and mostly β are expressed in neurons ⁽²⁷⁾. Due to the arrangement of actin monomers within the filament, F-actin exhibits polarity. This polarity allows for the formation of distinct functional domains within the actin filament, leading actin to undergo a process called treadmilling ^{(26); (27)}. At the growing end, also known as the barbed end, ATP-bound actin monomers are added to the filament, and at the opposite end, the pointed end, GDP-bound monomers dissociate from the filament ^{(8); (26); (27)}. Actin polymerization is believed to push the membrane forward, leading to the formation of membrane protrusions ⁽²⁸⁾. Actin filaments are also involved in the short-distance transport of various cargoes, with myosin Va serving as a motor protein that binds to actin filaments to transport these cargoes ^{(27); (29); (30)}. The movement of myosin Va along the actin filament is driven by ATP hydrolysis, which induces conformational changes in the protein and results in its movement along the filament, transporting the associated cargo locally to its destination ^{(27); (29); (30)}. During development actin waves lead actin and actin-binding proteins (ABP) to be delivered to the putative axon to promote its elongation ^{(31); (32)}. At later stages, during axonal outgrowth, microtubules and actin dynamics are critical for growth cone motility and axon guidance ^{(33); (34)}. Additionally, in mature axons, actin filaments play a crucial role in creating a sub-plasmalemmal scaffolding providing support to the plasma membrane and docking sites for various membrane protein complexes ⁽²⁷⁾. The actin cytoskeleton also exists underneath the plasma membrane as periodically spaced ring-like structures organized by spectrin and adducin proteins ^{(8); (12)}. This unique organization is predicted to provide the axon with elastic and stable support and to provide molecular organization to the axonal membrane ^{(8); (12)}. Besides this organization, actin is also present along the axon as bundles. These actin bundles, defined as actin trails, represent a more dynamic F-actin as they extend in both

anterograde and retrograde directions ^{(8); (12)}. This suggests a dual population of axonal actin with the more stable actin rings providing mechanical support, and the more dynamic actin filaments providing flexibility needed for maintaining the axonal and synaptic plasticity ^{(8); (35)}. Actin filaments are dynamic structures which continuously grow and shrink facilitating the continuous remodeling of the actin cytoskeleton ⁽⁸⁾. Actin dynamics are regulated by several actin binding proteins, and their actions include sequestering of G-actin (Profilin), nucleation of filaments (Formins, Arp2/3 complex proteins), capping or bidding the barbed or the pointed end (Tropomodulin, Tensin), severing actin filaments (Gelsolin, Cofilin), bundling (Fascin, Alpha-actinin), crosslinking (Filamin, Transgelin), stabilizing and anchoring the actin to other cellular components (Vinculin, Annexin) ^{(8); (12); (36), (37)}.

Microtubules (MT) are cylindrical polymers built up from alpha- and beta-tubulin heterodimers that have essential roles in cell division, directional transport of proteins and vesicles, cytoplasmic organization, cell shape, polarity, and motility ⁽³⁸⁾. They have a characteristic polarity, with a faster-growing plus-end and a more stable minus-end. The plus end is usually in a state of dynamic instability, alternating from growth phases (polymerization), pauses at a constant length, and catastrophe (fast depolymerization) ^{(12); (39)}. While in the axon, microtubules are orientated in a unique direction, with the plus-end toward the axon tip, in dendrites, there is a mixed orientation. This is important for axons to keep their functions since it affects the specific sorting of cargos to be transported ^{(12); (40); (41)}. MT dynamics are regulated by MAPs, motor proteins, post-translational modifications of tubulin and plus-end tracking proteins ^{(8); (42); (43)}. Post-translational modifications of tubulin are important for regulating microtubule's structure and function and include detirosination, polyglutamylation, polyglycylation, palmitoylation, phosphorylation, and acetylation ^{(44); (45); (46)}. Together with MAPs, these modifications can alter the number, stability, and affinity of motors to MT, thus modulating the transport ^{(12); (47); (48)}.

Microtubules, actin, and neurofilaments are crucial for neuronal morphology and functions. However, axonal transport is almost exclusively based on microtubules. The axonal transport is a highly complex process that requires neurons to sustain proper function and clear misfolded proteins or damaged organelles for degradation and recycling ⁽⁴⁸⁾. It can be classified based on the speed at which cargoes move, with vesicles, organelles, and RNA being transported by fast axonal transport, while cytoskeletal components and some soluble proteins are transported by slow axonal transport ^{(14); (29); (48); (49)}. Furthermore, the slow anterograde transport can still be subdivided into slow component a (mainly tubulin and neurofilaments) and slow component b (cytosolic proteins) ⁽⁴⁸⁾. Besides this, transport can occur towards the presynaptic tip, i.e., the anterograde transport, or back to the cell body, i.e., retrograde transport ^{(14); (50)}. In the axons, synaptic vesicles, cytoskeletal components, and cytosolic proteins are cargoes transported in the anterograde

manner ⁽⁵¹⁾. On the other hand, signaling endosomes, autophagosomes, and proteins involved in injury signaling are transported in the retrograde transport ^{(48); (51)}. Mitochondria, a variety of endosomes, lysosomes, and mRNA are transported in both manners ^{(48); (51)}. Axonal transport is fundamental for neuronal function, as it allows the transport of essential molecules within the neurons, including the transport of organelles, proteins, lipids, and RNA ^{(14); (52); (53)}. It is mediated by two ATP-dependent motor proteins, kinesin, and dynein, which carry their cargoes along microtubule tracks ^{(14); (29); (52)}. The various cargoes that are transported along the axons move in a saltatory manner, exhibiting periods of rapid movements, pauses and directional switches ⁽²⁹⁾. Kinesin is the motor driving anterograde transport, while dynein is the major motor driving retrograde transport ^{(48); (52)}. Regarding kinesin, its superfamily comprises about 45 proteins, with kinesin-1, kinesin-2, and kinesin-3 contributing for axonal transport ^{(14); (54); (55)}. Kinesin-1 is a heterotetramer that is composed of two kinesin heavy chains and two light chains and is responsible for the fast anterograde transport of organelles, most vesicles, proteins, and RNA, and the slow transport of cytoskeletal proteins ^{(48); (52); (54); (56)}. Kinesin-2 and Kinesin-3 are also important for axonal transport, being responsible for the transport of late endosomes and lysosomes and synaptic vesicle precursors ^{(52); (57); (58)}. On the other hand, a unique Dynein protein is responsible for retrograde transport. Dynein is a complex containing two catalytic heavy chains, two intermediate chains, four light intermediate chains and several light chains ^{(48); (59)}, being the binding of the cargo to the intermediate and light chains modulated by dynactin ^{(60); (61); (62)}. Dynein is responsible for the retrograde transport of essential cargoes from the nerve terminals towards the cell body located in the central nervous system, and this process is critical for the replenishment of cellular components/recycling protein and organelles and for retrograde signaling ^{(60); (61); (62)}

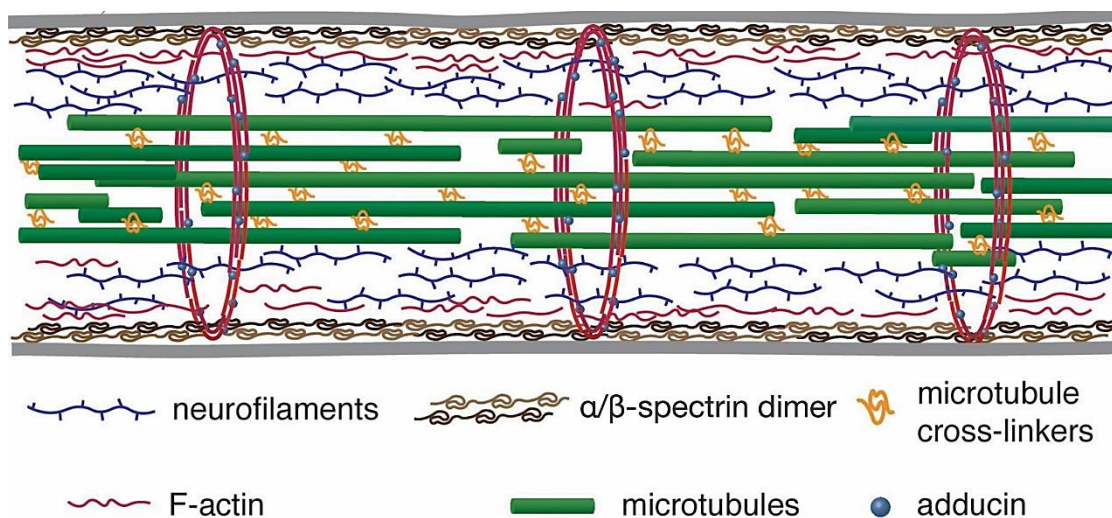


Figure 1. Schematic representation of the axonal cytoskeleton. Cytoskeleton is composed of different components, including F-actin, microtubules, and neurofilaments. The microtubules form the core of the axon and are cross-linked by microtubule-associated proteins. The neurofilaments surround the core and provide additional support. The outermost layer of the axonal cytoskeleton comprises regularly spaced rings composed of F-actin filaments. These actin rings are connected by α/β -spectrin tetramers and adducin proteins, which align these structures along the axonal axis. Other cortical F-actin filaments also exist orientated along the axon. Several proteins, including motor proteins (not shown), can interconnect the various components and the membrane (grey lines) to the inner skeleton. Adapted from (63).

1.2 Astrocytes

Astrocytes are the most abundant glial cell in the CNS. They are involved in a multitude of processes, performing a variety of complex functions in health and disease, and are able to trigger responses that can contribute to tissue repair or promote CNS pathology ⁽⁶⁴⁾. Astrocytes are highly specialized cells involved in ion and water homeostasis, neurotransmitter recycling, blood-brain barrier (BBB) formation and maintenance, immune signaling, and regulation of neuronal synaptogenesis ^{(64); (65); (66); (67)}. Due to the expression of transporters for glutamate, gamma-aminobutyric acid (GABA), and glycine, astrocytes are critical for neuronal excitability ⁽⁶⁸⁾. Besides these functions, astrocytes are also the primary cells responsible for cholesterol synthesis in the brain ⁽⁶⁹⁾ and are the major provider of glutamine to neurons ⁽⁶⁸⁾. So far, five populations of astrocytes have been defined based on the expression of different markers, such as Aldh1l1, CD51, CD63, and CD71 ⁽⁶⁴⁾. These populations may be present in different regions of CNS and may display differences in volume, orientation, and arborization ^{(70); (71)}. Morphologically, astrocytes are highly branched star-shaped cells. Stemmed from the cell body, primary branches that gradually divide into thinner processes to generate a dense network of terminal processes ⁽⁷²⁾. Depending on the region, one single astrocyte can cover a huge spatial domain, wrapping multiple neuron processes, and contacting with several individual synapses ^{(72); (73); (74); (75); (76)}. In mice, astrocytes have been characterized based on their reactivity status as A1 or A2 ⁽⁶⁹⁾. A1 astrocytes display neurotoxic activity and have been proposed to participate in several neurological disorders and be pro-inflammatory; on the other hand, A2 is associated with neuroprotective activity and is proposed to create anti-inflammatory conditions ⁽⁶⁴⁾. However, despite their transcriptional signature that is correlated to A1/A2 status, astrocytes can present an intermediate state, and all these astrocyte types can co-exist within the same tissue ⁽⁶⁹⁾. They can be further classified into two main types based on their morphology and location. Protoplasmic astrocytes are found in the gray matter and have several fine processes that extend in all directions. Fibrous astrocytes are found in the white matter and have few unbranched processes that extend along the length of axons ⁽⁷⁷⁾. In

the protoplasmic astrocytes, these processes are closely associated with neurons and synapses ⁽⁷²⁾. The close interaction between astrocytes and the pre- and post-synaptic structures forms what is called “tripartite synapse” and makes astrocytes capable of monitoring and altering synaptic function ^{(78); (79)}. Here, they can modulate the synaptic plasticity by releasing molecules such as glutamate and D-serine that can modulate the activity of N-methyl-D-aspartate (NMDA) receptors, which are important for long-term potentiation (LTP) and long-term depression (LTD) ^{(73); (74)}, and control synapse elimination during development, by secreting molecules such as complement proteins that tag synapses for elimination by microglia ^{(79); (80)}.

Under conditions promoted by injury, astrocytes can undergo morphological and functional changes, becoming reactive and starting a process of neuroinflammation ⁽⁶⁸⁾. This change is called astrogliosis, and one common feature in is the up-regulation of the glial fibrillary acidic protein (GFAP) and the hypertrophy of the main processes ^{(64); (68)}. GFAP is an intermediate neurofilament found primarily in astrocytes and plays a crucial role in maintaining the cytoskeleton of these cells ⁽⁸¹⁾. It is described as one of the best hallmarks of reactive astrocytes. However, its expression can still vary depending on the astrocyte's location, proximity to the injury site, and injury type ^{(81); (82)}. The biochemical and structural changes during neuroinflammation are triggered to minimize and repair the initial damage. However, sustained inflammatory responses under conditions of severe or prolonged insults can be driven by positive feedback loops between astrocytes and microglia, leading to chronic neuroinflammation and, consequently neurodegeneration ^{(83); (84); (85)}.

1.3 Microglia

Microglia are considered the immune cells of the CNS, being around 10% of the total cells in this tissue ^{(69); (86)}. Microglia play important roles in promoting neuronal survival by releasing neurotrophic factors, and participating in synaptic elimination, by phagocytosing dendritic spines that are not receiving inputs to ensure proper neuronal communication ^{(69); (80); (87); (88)}. During development, several neurons fail to differentiate, migrate, and establish proper neuronal circuits ⁽⁸⁹⁾. These neurons undergo programmed cell death, which can be initiated by cell-intrinsic factors or induced by other cells such as microglia ^{(89); (90)}. Here, microglia play important roles in inducing programmed cell death and cleaning up the resultant cell debris ^{(89); (91); (92)}. Throughout adulthood, they continue to play critical roles in CNS, being involved in synaptic remodeling, tissue homeostasis by phagocytosing dead cells, maintaining myelin homeostasis, and host defense against pathogens ^{(69); (93); (94)}. Besides this, microglia also establish important interactions with astrocytes, making this interaction

important in homeostasis, inflammation, and neurodegeneration ⁽⁹⁵⁾. Microglia have been categorized into two different states of activity: resting and activated ^{(69); (93)}. Resting microglia are described as highly ramified and dynamic cells ^{(69); (93)}. Their thin processes are responsible for scanning the microenvironment for pathogens and injuries to keep tissue homeostasis ^{(69); (93)}. On the other hand, activated microglia are characterized by enlarged cell bodies with thicker and shorter processes ^{(69); (93)}. Changes in tissue homeostasis associated with infection, damage, or disease induce cells to alter their morphology to amoeboid-like and to become reactive ^{(69); (93); (96)}. Additionally to morphologic alterations, microglia also alter their genetic profile and start expressing pro- or anti-inflammatory molecules ^{(69); (97)}. Activated microglia can be further divided into two types: M1 and M2. M1 microglia are characterized by expressing pro-inflammatory cytokines, exhibiting antigen presentation markers, and increased phagocytic activity ^{(69); (98)}. M2 microglia are cells associated with a healing process and are responsible for promoting changes in the extracellular matrix as a restorative process and for expressing anti-inflammatory cytokines ^{(69); (99)}. Microglia are closely associated with neurons. Due to this, defective or dysfunctional neurons can trigger microglia to become reactive and display a neuroprotective response. However, this process can turn into chronic inflammation and exacerbate neurodegeneration, as observed in diseases such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis ⁽⁸⁹⁾. Here, microglia become activated and release pro-inflammatory cytokines and other factors that can cause neuronal damage and cell death. Moreover, with age, microglia's transcriptomic profile change, and this is seen as a major contributor to disease in neurodegenerative conditions ^{(69); (100)}.

1.4 Oligodendrocytes

Oligodendrocytes are the CNS cells that produce myelin, which insulates neurons and facilitates efficient electrical signal transmission. Morphologically, oligodendrocytes are highly branched small cells with unique characteristics, such as the absence of intermediate filaments and glycogen in the cytoplasm and the presence of a large number of microtubules in their many thin processes that may be involved in their stability ⁽¹⁰¹⁾. Due to their extensive branching, one single oligodendrocyte is able to myelinate multiple axons ⁽¹⁰²⁾. Besides their involvement in myelination, oligodendrocytes, in coordination with astrocytes, are also crucial for giving metabolic and trophic support to neurons, supplying them with essential nutrients and growth factors to maintain the neuronal health and functionality ^{(103); (104); (105)}.

Oligodendrocytes derive from the oligodendrocyte progenitor cells (OPC) lineage through a process known as oligodendrogenesis, which follows a well-defined sequence of events including proliferation, migration, and differentiation ^{(106); (101)}. During the

differentiation, OPC give rise to pre-myelinating oligodendrocytes that extend short processes and start expressing specific markers such as the sulfatide recognized by the O4 antibody and the GPR17 protein, as well as OPC markers (e.g., platelet-derived growth factor receptor α (PDGF-R α), ganglioside A2B5, proteoglycan NG2, polysialic acid-neural cell adhesion molecule and fatty-acid-binding protein (FABP)), which persist until the immature oligodendrocyte stage ⁽¹⁰⁷⁾. As immature oligodendrocytes stop expressing A2B5 and NG2 and start expressing galactocerebroside C (GalC), they become post-mitotic cells with extensive branched processes ⁽¹⁰⁷⁾. In the final developmental stage of oligodendrocytes, mature oligodendrocytes extend membranes that form compact enwrapping sheaths around the axons and start expressing myelin proteins ⁽¹⁰⁷⁾.

During development, for myelination to occur, migration of OPC into the developing white matter tracts is crucial ⁽¹⁰⁸⁾. OPC derives from neural progenitor cells in specific parts within the tissue (ventricular-subventricular zone), and to overcome the spatial distance, OPC can migrate along blood vessels within the CNS to populate the tissue ⁽¹⁰⁹⁾. Under appropriate conditions, immature oligodendrocytes start engaging with a target axon and enwrap it with their membrane ⁽¹⁰⁸⁾. After maturing and become myelinating oligodendrocytes, they produce myelin and specific types of proteins essential for myelin maintenance, including myelin basic protein (Mbp), 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (Cnpase), myelin proteolipid protein (Plp), myelin-associated glycoprotein (Mag), membrane marker galactocerebroside and the surface marker myelin-oligodendrocyte glycoprotein (Mog) ^{(101); (108)}. A large fraction of OPCs do not differentiate and remain in a cycling state throughout adulthood ⁽¹¹⁰⁾. Actually, OPCs represent the largest dividing population among neural cells and are evenly distributed, making up to 8% of total CNS cells ⁽¹¹¹⁾. This is extremely important due to their capability to detect perturbations within the CNS, and when myelin repair is needed, local OPC proliferation occurs, followed by OPC differentiation in oligodendrocytes ^{(112); (113); (114)}.

As for the other cells present in the CNS, oligodendrocytes can also play a role in disease. Dysfunction of oligodendrocytes and demyelination have been implicated in several neurological disorders, including leukodystrophies such as Niemann-Pick disease, Metachromatic leukodystrophy or Adrenoleukodystrophy (ALD) ^{(115); (116)}.

1.4.1 Myelin

Myelin is a multilamellar membrane consisting of several wrapped lipid bilayers. The formation of myelin is one of the most complex processes of transformation of a plasma membrane, involving timely coordinated cell-cell interactions, intercellular signaling,

wrapping, and extension of membranes around the axon ⁽¹⁰⁸⁾. Myelination follows a relatively consistent chronological and topographical pattern, starting in areas dedicated to basic homeostasis, proceeding to regions controlling more complex tasks, and ending in areas required for the highest intellectual functions ^{(117); (118) (119)}. Within a neuronal pathway, myelination often progresses in a proximal to distal direction; within the brain, it generally extends from central zones toward the poles ⁽¹¹⁹⁾. The role of myelin in facilitating the rapid transmission of electrical signals along the axon and providing metabolic support to the axonal region that is ensheathed makes it unique, and thus, any damage or loss of myelin, as seen in leukodystrophies, can lead to impaired nerve conduction and neurological symptoms ⁽¹⁰⁸⁾.

Myelin's presentation along the axons is distinctive. Myelinated segments, also called internodes, are interchanged with areas devoid of myelin, called nodes of Ranvier ⁽¹²⁰⁾. At nodes of Ranvier, the presence of a high number of sodium channels facilitates rapid membrane depolarization, driving the electric signal along the axon in a saltatory manner ⁽¹²⁰⁾. Structurally, myelin also presents different compaction regions with different lipid and protein compositions. While within the internodes, myelin is compacted and enriched in glycosphingolipid GalC, Plp, and Mbp, the areas at their edges, called paranodes, consist of non-compact myelin enriched in glycosphingolipid sulfatide and the protein 155-kDa neurofascin (NF155) ⁽¹²⁰⁾.

In contrast with most biological membranes, myelin is primarily composed of a high proportion of lipids (70-85%) and a low proportion of proteins (15-30%) ⁽¹²¹⁾. Important for conferring viscosity, stabilization, and compaction to myelin, the major lipid constituents are cholesterol, phospholipids, and glycolipids, with a ratio of 40%:40%:20%, respectively ^{(121); (122)}. Along with the unique lipid composition, myelin contains a specific set of proteins, including Plp, Mbp, NF155, Cnpase, Mag, and Mog ⁽¹⁰¹⁾. Among these, Plp and Mbp are the most abundant and are indispensable for the proper assembly, compaction, and physical stability of myelin in the CNS ⁽¹⁰¹⁾. This distinctive composition (i.e., ratio lipids/proteins and lipids/proteins constituents) gives myelin single properties, contributing to the close packing and tight organization of the membrane, which are of extreme importance for myelin formation, maintenance and dynamics ^{(123); (124); (125)}.

2. Lipids

Lipids are a diverse group of biomolecules that play several essential roles in biological systems, including energy storage, metabolic substrates, membrane structure, and cell signaling ^{(126); (127)}. They can be broadly categorized into two main types based on their polarity: non-polar (neutral) lipids and polar lipids ^{(128); (129)}. This distinction is crucial for

understanding their functions in biological systems. Non-polar lipids are often involved in energy storage, while polar lipids are integral components of cell membranes and participate in cell signaling and recognition processes ^{(128); (129)}. Lipids can be grouped in four main classes, namely glycerophospholipids, sphingolipids, glycolipids and sterols ⁽¹³⁰⁾. An additional class of biological lipids that do not constitute the lipid bilayers, the glycerolipids, can also be identified ⁽¹³⁰⁾.

The nervous tissue is highly enriched in lipids ⁽¹³¹⁾. In this tissue, they are a key constituent of the plasma membrane, having an important role in a vast number of functions, including synaptogenesis, neuritogenesis, and myelin formation and maintenance ^{(127); (130); (131); (132)}.

2.1 Lipids in the plasma membrane

The plasma membrane is a highly dynamic and semi-permeable structure comprising amphipathic lipids and proteins (Figure 2). Its primary function is to separate the cell interior from the extracellular environment, enabling at the same time cells to communicate with their surroundings and access the necessary resources ^{(127); (133); (134)}. The central properties of the plasma membrane are conferred by its lipid composition, which plays a critical role in various functions such as signaling pathways, modulation of protein-protein interactions, vesicle fusion, and regulation of membrane organization, structure, and dynamics ^{(134); (135)}. Lipids can provide versatile anchoring platforms that modulate protein interactions and affect how proteins associate with the membrane ⁽¹³⁴⁾. Membranes are not homogenous structures, and depending on their biological role, the lipid composition is also different ⁽¹³⁵⁾. In mammals, the plasma membrane has a unique lipid composition that falls into three distinct classes: glycerophospholipids, sphingolipids, and sterols ^{(127); (132)}. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant glycerophospholipids, while sphingomyelin (SM) is the most abundant sphingolipid ^{(132); (136)}. Cholesterol is the predominant sterol found in this structure, making up to 50% of the lipid content, and its planar and rigid structure enables it to affect membrane fluidity and rigidity ⁽¹²⁷⁾. The compact arrangement along with the preference for cholesterol to intercalate with the acyl chains of sphingomyelin, creates liquid-order domains called lipid rafts ⁽¹³⁷⁾. Lipid rafts are heterogeneous structures that can vary in size and composition and are thought to be involved in membrane organization and function ⁽¹³⁷⁾. They have the capability to decrease membrane fluidity locally, allowing for the clustering and organization of specific membrane-associated proteins, including signaling molecules, receptors, and enzymes, in a spatially restricted manner ^{(137); (138)}. The clustering of these proteins in the lipid rafts can then

enhance signal transduction efficiency and specificity and may also facilitate the formation of signaling complexes ^{(139); (140)}. Moreover, lipids also interact with cytoskeletal proteins, in particular with F-actin, being able to regulate cell shape and movement ^{(138); (141); (142)}. Furthermore, interactions between lipid rafts and cytoskeletal components can contribute to the assembly of these membrane domains and be involved in the regulation of the cytoskeleton dynamics ^{(133); (143); (144); (145)}.

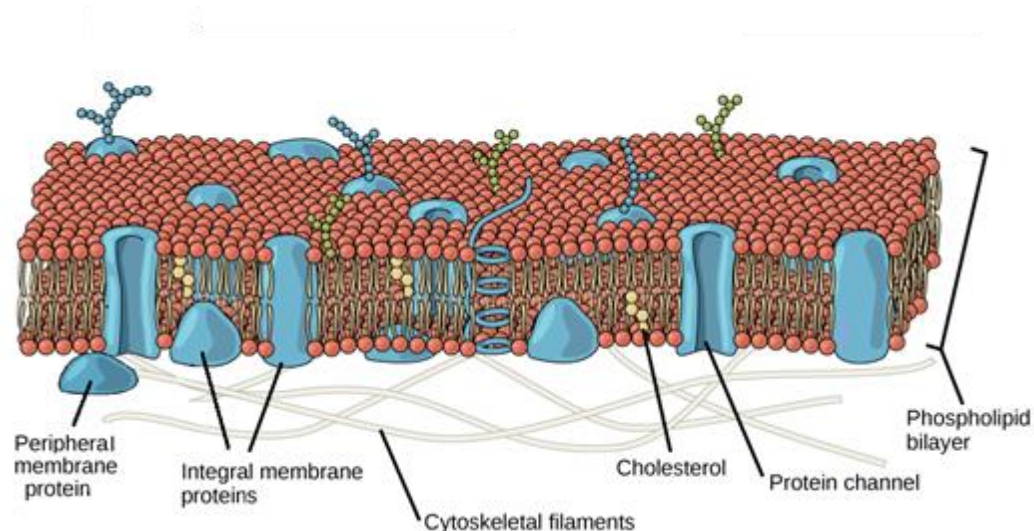


Figure 2. Representative scheme of Plasma membrane. The plasma membrane is a thin and flexible barrier that encloses the cell and separates its internal environment from the external environment. It is made up of two layers of phospholipids arranged with their hydrophobic tails facing each other and their hydrophilic heads facing outward. One important lipid constituent is cholesterol, which helps to stabilize the phospholipid bilayer and regulate its fluidity. Inserted into the plasma membrane are two types of proteins: integral membrane proteins, proteins that are embedded within the phospholipid bilayer, and peripheral membrane proteins, proteins that are located on the outer or inner surface of the membrane and interact with either the phospholipids or integral membrane proteins. Moreover, this structure makes important interactions with the cytoskeleton, providing structural support and helping maintain the cell's shape. Adapted from Concepts of Biology, 2013.

2.2 Fatty acids

Fatty acids are the major building blocks for lipids, consisting of a carbon chain with a methyl and a carboxyl group ^{(130); (146)}. In CNS and PNS, FA are important substrates for the synthesis of a wide array of more complex lipids, including glycerophospholipids, sphingolipids, and cholesterol esters ^{(131); (147)}. They are crucial biomolecules, either in their free form or as part of complex lipids, playing important functions in cells such as in metabolism, cell membrane composition, and gene regulation ⁽¹⁴⁶⁾. FA can be divided into saturated and unsaturated fatty acids. Saturated FA are those in which all carbon bonds

are linked to hydrogen ⁽¹⁴⁶⁾. In contrast, unsaturated fatty acids contain at least one carbon-carbon double bond, with monounsaturated FA (MUFAs) having one double bond and polyunsaturated FA (PUFAs) containing two or more ⁽¹⁴⁶⁾. FA can further exist in various forms such as short-chain FA, which have carbon (C) chains consisting of 1-6 carbons, medium-chain FA (MCFA), consisting of C6-12, long-chain FA (LCFA), consisting of C12-20 and very-long-chain FA (VLCFA), consisting of C $\geq$ 22. Although various forms of FA exist, most species are MCFA, and only a small fraction of FA are present in the other forms ^{(147); (148); (149); (150)}. LCFA are essential for a wide range of cell functions, including regulation of intercellular signaling, ion channels, or metabolic enzymes ⁽¹⁵¹⁾. For this reason, the intracellular concentration of FA is tightly controlled either by processes of lipid biosynthesis or degradation ⁽¹⁵¹⁾. Moreover, FA can be converted intracellularly to other FA by elongation, desaturation, or β -oxidation ⁽¹⁴⁷⁾. Actually, a significant amount of the fatty acids *de novo* synthesized or taken up through the diet are elongated into VLCFA ⁽¹⁵¹⁾. This occurs in a four-step reaction cycle performed by endoplasmic reticulum membrane enzymes, which include condensation, reduction, dehydration and reduction ^{(147); (152)}. The first step is performed by a FA elongase (ELOVL), where an acyl-CoA is condensed with malonyl-CoA to produce 3-ketoacyl-CoA; in the second step, 3-ketoacyl-CoA is reduced to 3-hydroxyacyl-CoA by a 3-ketoacyl-CoA reductase; 3-hydroxyacyl-CoA is transformed into 2,3-trans-enoyl-CoA by the 3-hydroxyacyl-CoA dehydratase; lastly, this product is reduced to an acyl-CoA with two more carbons than the original ^{(147); (153)}. In mammals, there are seven ELOVL responsible for the elongation of FA, and each exhibits distinct preferences for acyl chain length and the degree of unsaturation ^{(147); (154)}. Of all the enzymes, ELOVL4 and ELOVL5 are the most expressed in the CNS, being their expression especially pronounced in the olfactory bulb, hippocampus, cerebral cortex, thalamus, and cerebellum ⁽¹⁵⁴⁾.

VLCFA are present in all lipid classes, however, in mammals, most saturated and monounsaturated VLCFA are found as constituents of sphingolipids ⁽¹⁴⁷⁾. In these lipids, chain-lengths vary from C16 to C24, with the most FA being C16:0 (palmitic acid) and the VLCFA C24:0 (lignoceric acid) and C24:1 (nervonic acid) ⁽¹⁵⁵⁾. Although present in organisms as a minor fraction, VLCFA have key functions such as skin barrier formation, retinal functions, mediation of inflammation, formation, and maintenance of myelin, sperm development and maturation, and liver homeostasis ^{(147); (151)}. Moreover, and specifically in the nervous tissue, they have also been described as modulators of synaptic function and membrane fusion ⁽¹⁵⁴⁾. This goes in accordance with the fact that the brain is the richest source of VLCFA in most higher organisms ⁽¹⁵⁶⁾. These FA have been linked to the regulation of various cellular functions, and this is due to their ability to change membrane properties, including fluidity, permeability, curvature, and microdomain formation ⁽¹⁵⁵⁾. Similar to LCFA, the amount of VLCFA needs to be tightly controlled to avoid their

accumulation in the cell and consequently disturbing organelle structure and function, leading to diseases such as ALD and Zellweger syndrome ^{(155); (157); (158)}.

To control the abundance of VLCFA, peroxisomal β -oxidation is critical (Figure 3). In mammals, FA catabolism occurs in both mitochondria and peroxisomes ^{(159); (160)}. Although initially it was thought that peroxisomes were only involved in the β -oxidation when mitochondrial oxidative capacity was exceeded, today it is clear that peroxisomes have a critical role in FA degradation, specifically VLCFA, and impairment of their function is related to certain peroxisomal disorders ^{(157); (159)}. In this metabolic process, most fatty acids undergo degradation by the removal of two carbon units from the carboxyl end of the molecule and are able to re-initiate a new cycle of oxidation ⁽¹⁵⁹⁾. Both mitochondrial and peroxisomal β -oxidation share a similar mechanism involving four consecutive steps: dehydrogenation that removes two hydrogen molecules and introduces a *trans* double bond; hydration across the double bond, forming a 3-L-hydroxyacyl-CoA; dehydrogenation of the 3-L-hydroxyacyl-CoA forming a 3-ketoacyl CoA; and thiolitic cleavage of the terminal acetyl-CoA group forming a new acyl-CoA molecule shorter by two carbons ^{(159); (161)}. Although the process of β -oxidation is essentially the same in both organelles, there are notable differences in the enzymes involved (Figure 3) ⁽¹⁶¹⁾. In the peroxisome, the degradation of VLCFA is initiated by the entrance of VLCFA in the lumen via ATP Binding Cassette Subfamily D Member 1 (ABCD1), a member of the ATP Binding Cassette family of transporters, which is followed by sequential reactions performed by specific enzymes ⁽¹⁶²⁾. Acyl-CoA oxidase 1 (ACOX1) is the primary enzyme involved in the first step, which is the oxidation of VLCFA; the second and third steps are mainly performed by D-bifunctional protein (DBP), while the fourth step is carried out by 3-ketoacyl-CoA-thiolase (ACAA1) or sterol carrier protein X (SCPx) ⁽¹⁵⁷⁾. Depending on the VLCFA, several cycles of β -oxidation are necessary to fully process VLCFA. The processed VLCFA, now LCFA, can then be shuttled to mitochondria for further catabolism or can be used to acylate DHAP, a step critical for the biosynthesis of ether-phospholipids ⁽¹⁶³⁾.

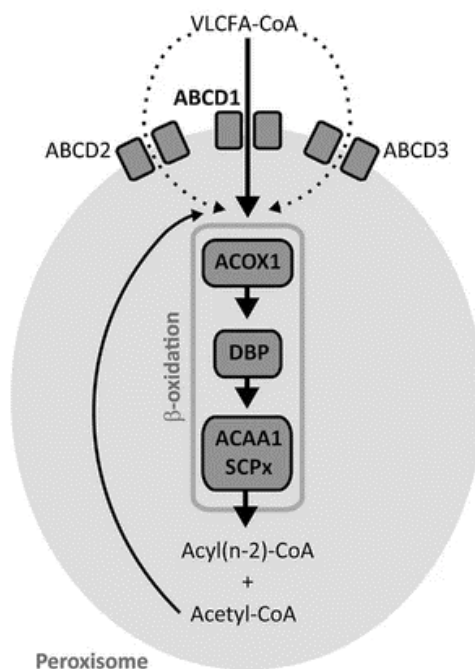


Figure 3. Overview of peroxisomal β -oxidation mechanisms. Activated VLCFAs (VLCFA-CoA) enter into peroxisomes by ABCD 1, 2 and 3 transporters. VLCFA-CoA is then shortened by β -oxidation, resulting in an acyl-CoA shortened in two carbon molecules and one acetyl-CoA. This process begins with the VLCFA-CoA desaturation catalyzed by acyl-CoA oxidase (ACOX), and requires molecular oxygen. The next steps of hydration and dehydrogenation, are catalyzed by enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, D-bifunctional proteins (DBP), respectively. The final step is catalyzed by 3-oxo-acyl thiolase (ACAA1) and sterol carrier protein X (SCPx). Adapted from (164).

3. Peroxisomes

Peroxisomes are highly versatile organelles found in all eukaryotic cells, which play a crucial role in various cellular processes. Initially observed by Johannes Rhodin in the mouse kidney and named as “microbodies” ⁽¹⁶⁵⁾, these organelles were later identified and named peroxisomes by Christian de Duve when observing that enzymes, including oxidases and catalase, which are involved in hydrogen peroxide metabolism, were found here in equilibrium density gradients ⁽¹⁶⁶⁾. Actually, due to de Duve’s experiments, it was thought that their primary function was related to the metabolism of hydrogen peroxide ⁽¹⁵⁹⁾. Among their many functions, peroxisomes are particularly important in the metabolism and synthesis of lipids. These functions were demonstrated by showing that enzymes involved in fatty acid oxidation were colocalized in plant peroxisome-like organelles called glyoxysomes (capable of degrading fatty acids to metabolic intermediates of carbohydrate synthesis) ^{(165); (167)}. Moreover, in 1976, Paul Lazarow provided evidence supporting the role of peroxisomes in lipid metabolism. His research demonstrated that β -oxidation enzymes

were present in peroxisomes located in the liver of rats, and their activity was significantly increased following treatment with clofibrate (a drug used to lower blood fats and found to be involved in the proliferation of peroxisomes) ⁽¹⁶⁵⁾. Regarding their structure, peroxisomes can vary in shape and size, ranging from 0.1-0.5µm in diameter ⁽¹⁵⁹⁾. The importance of these organelles is marked by the fact that they are very plastic, responding rapidly to cellular or environmental changes by modifying their size, number, morphology, and function ⁽¹⁶⁸⁾. Two models have been proposed for peroxisome biogenesis. Peroxisomes can be generated by *de novo* synthesis, where pre-peroxisomal vesicles bud from the endoplasmic reticulum and fuse with each other to form a mature peroxisome, and/or pre-existing peroxisomes which growth, and by fission give rise to new peroxisomes ^{(169); (170); (171)}. Regardless of their origin, peroxisomes rely on a specific set of proteins called peroxins to facilitate their assembly and protein import ⁽¹⁵⁹⁾. Peroxins are involved in the regulation of several aspects of biogenesis, including factors that control the assembly of peroxisomal membrane, interaction of peroxisomal targeting sequences (PTS), allowing proteins to be shuttled to the peroxisome and docking receptors for peroxisomal proteins ⁽¹⁵⁹⁾.

Peroxisomes are described as key metabolic organelles, and in accordance with this, they are assigned several metabolic functions, including β-oxidation of VLCFA, α-oxidation of branched-chain FA, synthesis of ether lipids, bile acids and docosahexaenoic acid (C22:6 n-3) and production and degradation of hydrogen peroxide (H₂O₂), glyoxylate detoxification and amino acid metabolism ^{(157); (159)}. Additionally, due to their pivotal metabolic role, they also interact and cooperate with many organelles, including the endoplasmic reticulum, mitochondria, lipid droplets, lysosomes, and phagosomes, making peroxisomes a central organelle in organisms ⁽¹⁷²⁾. In this way, the established repertoire of peroxisome functions and their need to interact and cooperate with a wide range of other organelles make clear, from the clinical standpoint, that peroxisomal functions are highly relevant in health ^{(172); (173); (174)}.

3.1 Peroxisomal disorders

Peroxisomal functions are extremely important, as evidenced by the numerous genetic disorders linked to this organelle. These disorders are clinically heterogeneous but are consistently associated with impaired lipid metabolism, resulting in the accumulation of VLCFA or phytanic acid or abnormal synthesis of ether lipids or bile acids ⁽¹⁵⁹⁾. Peroxisomal disorders can be divided into a group called Multiple Peroxisomal Function Defects (MPFD) or a group with distinct deficiencies called Single Peroxisomal Function Defects (SPFD) ⁽¹⁷³⁾.

^{(175); (176); (177)}. The MPFD disorders are typified by the Zellweger spectrum disorder (ZSD) ^{(176); (178)}. In this disorder, the complete loss of peroxisomes or their functions causes severe abnormalities, such as neurological malformations, hypotonia, seizures, hepatomegaly, bone abnormalities, and leukodystrophy, but also biochemical abnormalities, including VLCFA accumulation, marked depletion of ether phospholipids (plasmalogens), and reduced levels of docosahexaenoic acid (DHA) ^{(175); (178); (179); (180)}. Another disorder associated with Multiple Peroxisomal Function Defects is Rhizomelic Chondrodysplasia Punctata (RCDP) type 1, which is caused by mutations in *PEX7* and is characterized by distinctive facial features, including prominent forehead and hypertelorism, growth failure, proximal shortening of long bones, developmental delay, seizures, congenital cataracts but also a severe deficiency in the biosynthesis of plasmalogens, accumulation of phytanic acid and VLCFA (notably, increased levels were so far only detected in the mouse model for *PEX7* deficiency) ^{(159); (181)}. Moreover, different failures in the functioning of peroxisomes can lead to a single function defect within the peroxisome. This defect can be attributed to either matrix enzymes or membrane proteins, which are primarily involved in transporting metabolites ^{(178); (182)}. Here, several peroxisomal diseases have been identified as being responsible for impacting single metabolic pathways. ALD (ABCD1 deficiency), Acyl-CoA oxidase 1 deficiency (ACOX1 deficiency), D-bifunctional protein deficiency (DBP/MFP-2 deficiency), Sterol carrier protein X deficiency (SCPx deficiency), and the recently discovered ABCD5 deficiency are described as affecting β -oxidation; Acyl-CoA oxidase 2 deficiency (ACOX2 deficiency) and Peroxisomal ABCD3 deficiency are linked to bile acid metabolism; RCDP type 2 (GNPAT deficiency), RCDP type 3 (AGPS deficiency), and RCDP type 4 (FAR1 deficiency) are associated with plasmalogen synthesis; Refsum disease (PhyH deficiency) is known to affect α -oxidation, while Acatalsaeemia (Catalase deficiency) impacts hydrogen peroxide metabolism ⁽¹⁷⁸⁾.

3.1.1 Beta-oxidation related disorders

The various enzymes and membrane transporters known to play a role in β -oxidation cause significant peroxisomal malfunctioning and lead to human disorders. For instance, patients carrying mutations on ACOX1 or MFP-2 reveal an altered VLCFA profile and severe neurological conditions. ACOX1 deficiency is an autosomal recessive disorder that compromises VLCFA catabolism ⁽¹⁸³⁾. Due to this, patients present symptoms such as hypotonia, seizures, developmental delay, and loss of vision and hearing, but also present white matter abnormalities ^{(184); (185)}. MFP-2 deficiency is an autosomal recessive inborn error that, as occurs in ACOX1 deficiency, leads to impaired peroxisomal degradation of

VLCFA^{(186), (187)}. The disorder presents a severe phenotype and is characterized by neonatal hypotonia, craniofacial dysmorphic features, seizures, psychomotor delays, ataxia, visual and hearing impairment, and most of them die before the age of 2 years^{(186), (187); (188)}.

ALD, caused by mutations in the peroxisomal ABCD1 membrane transporter, is the most well-known and frequent single peroxisomal function defect disorder⁽¹⁸⁹⁾. ABCD1 is also known as ALDP, a protein that belongs to the family of the ATP-binding cassette (ABC) transporters and is responsible for the translocation of CoA-activated VLCFA across the peroxisomal membrane for β -oxidation^{(190); (191)}. This family consists of three other transporters, namely ABCD2, ABCD3, and ABCD4, which exhibit some degree of similarity with ABCD1. Notably, the resemblance observed in ABCD2 has prompted investigators to suggest potential overlapping functions among these transporters^{(191); (192); (193)}.

ALD is initially diagnosed by elevated levels of VLCFA in the plasma, as evidenced by increased ratios of C26:0/C22:0 and C26:1/C22:0, and is characterized by defects in the CNS with progressive demyelination (leukodystrophy), motor dysfunction and adrenal insufficiency⁽¹⁹⁴⁾. Most patients receive the defective ABCD1 allele from one parent. However, a significant number of cases report *de novo* mutations (up to 19%)^{(195); (196); (197)}. Around 2700 mutations have been described to date, yet there is no relation between genotype/phenotype^{(191); (198)}. Both males and females born with mutations in ABCD1 develop with normal brain function and normal myelination^{(191); (199)}. However, depending on the presented phenotype, they may develop the disease at certain point of life. The most frequent phenotypes are the cerebral ALD (cALD) and the adrenomyeloneuropathy (AMN)⁽¹⁷³⁾. cALD is the most severe form of the disease, which primarily affects males during childhood, typically between 3 and 10 years of age⁽¹⁹¹⁾. Individuals with cALD experience rapid and progressive inflammatory demyelination, leading to deterioration in behavior, cognition, and neurological function, often leading to total disability within three years⁽¹⁷³⁾. There is a 20% chance of AMN adult males developing cALD, which rapidly progresses to disability and eventually to death⁽²⁰⁰⁾. AMN, unlike cALD, typically affects males between the ages of 20-30 and females between 40-50 and presents a lower rate of progression⁽²⁰¹⁾. Here, patients may exhibit slowly progressive demyelinating axonopathy and a mild or absent inflammatory response primarily affecting the spinal cord, that result in the development of progressive spastic paraplegia, characterized by weakness and stiffness in the legs^{(173); (196); (202)}.

To date, there are few treatments available for the disease. Those include Lorenzo's oil to reduce the levels of C26:0 in plasma, metabolic modulators, or the hematopoietic stem cell transplantation, which may prevent or revert clinical deterioration; however, this treatment is only applicable for cALD at the very early stage of the disease^{(191); (203); (204); (205);}

(206); (207). Considering the insights gained from allogeneic transplantation experiences, there was a natural interest in exploring the possibility of employing a gene therapy approach for ALD. Various pre-clinical studies have been conducted using in vivo gene delivery approaches to treat ALD. For instance, in vivo, gene editing via homology-independent targeted integration showed *ABCD1* mRNA level significantly increased, and the plasma levels of C24:0-LysoPC (lysophosphatidylcholine) and C26:0-LysoPC, sensitive diagnostic markers for ALD, reduced considerably in mice (208). Another study also showed positive results with reduced VCFA content in treated mice (209). AAV gene therapy has also been used as an approach to prevent/minimize the effects of other peroxisomal disorders. For instance, AAV-mediated PEX1 gene delivery improved visual function in the PEX1-Gly844Asp mouse model for mild Zellweger spectrum disorder (210).

Cartier et al. pioneered the initial human trials of gene therapy for individuals with cerebral disease (211); (212). Carter and her team documented the utilization of lentiviral-mediated gene transfer into mobilized peripheral blood stem cells (specifically CD34+ selected cells) induced by the administration of granulocyte-colony stimulating factor (G-CSF) in patients diagnosed with cerebral ALD (211); (212). In those patients, cerebral demyelination was halted with no additional advancement noted until the latest follow-up, mirroring a clinical outcome similar to what was observed following allogeneic hematopoietic stem cell transplantation (211); (212). Bluebird Bio has sponsored two other trials focusing on lentiviral-mediated gene therapy for cerebral ALD. In these trials, isolated haematopoietic stem cells from the patients were transfected with a lentiviral vector containing a functional cDNA copy of *ABCD1* (213); (214); (215). Most patients met the primary study end point of survival with no major functional disabilities at 24 months; in addition, patients showed no or minimal clinical symptoms and exhibited stabilization of lesion progression on MRI (213); (214); (215). Two patients died of disease-related causes that were unrelated to the treatment (213); (214); (215). Preliminary from these studies showed that lentivirus gene therapy was well tolerated and provided clinical benefit to subjects with cALD (213); (214); (215). More recently, Lauer et al., showed in a phase 2-3 clinical study that treatment with autologous hematopoietic stem cells transduced with the Lenti-D lentiviral vector containing *ABCD1* cDNA halts CALD lesion progression and improves microvascular circulation of the entire cerebral white matter (216).

So far, the clinical studies have achieved promising results, however, the authors also highlighted the necessity for an extended follow-up duration and larger sample size are needed to verify the efficacy and safety of this approach.

3.1.1.1 VLCFA in ALD

Due to the impaired transport of VLCFA into peroxisomes for β -oxidation, the primary defect in ALD is the accumulation of those FA in different tissues. VLCFA are found mainly esterified with cholesterol, glycerophospholipids, lysophospholipids, and sphingolipids in the brain, spinal cord, adrenal cortex and testis, causing the pathology in individuals ^{(217); (218); (219)}. Postmortem analysis of white matter made it possible to identify different lesion regions, including active, gliotic, and inactive, which presented different profiles of lipids accumulating VLCFA ⁽²²⁰⁾. In the active zone of the pathology, VLCFA accumulate significantly in cholesterol, while gangliosides containing C24:0 are increased in the active and gliotic zones ⁽²²⁰⁾. Moreover, the most striking observation was the significant increase of phosphatidylcholine containing C26:0 in the active and inactive zones when compared to control samples ⁽²²⁰⁾. Additionally, data obtained from studies in animal models and *in vitro* showed that VLCFA are also directly involved in inducing endoplasmic reticulum stress response, modulation of mitochondrial function by impairing electron transport, and generation ROS ^{(198); (221); (222); (223)}. The changes in organelle homeostasis and membrane lipid composition associated with ALD have been shown to contribute to both cell death and neurodegeneration. Additionally, these alterations can affect the structural integrity of myelin, leading to inflammation and demyelination, which are characteristic features of the disease.

3.1.1.2 Animal models in ALD

Animal models play a significant role in biomedical research, assisting in the study of disease mechanisms and the development of potential treatments. They are used to assess/test the effectiveness and safety of various interventions, such as gene therapy and pharmacological treatments. Following this, different models were developed to study disorders with impaired VLCFA metabolism. In *Drosophila*, the gene *CG2316* was identified as the sole homolog of human *ABCD1* ⁽²²⁴⁾. The disruption of this transporter in *Drosophila* resulted in an age-dependent neurodegeneration specified by retinal degeneration and pigment cell loss, suggesting that the functioning of this protein in flies does not impact the nervous system similarly to humans ⁽²²⁴⁾. Zebrafish with mutations in have also been used to study ALD, and compared to *Drosophila*, it better mimicked human disease. Increased levels of VLCFA were found in the mutants when compared to the wild type, suggesting that fish phenocopied the key biochemical marker of human ALD ⁽²²⁵⁾. Moreover,

hypomyelination in the spinal cord was observed in mutant zebrafish, and decreased numbers of oligodendrocytes were also detected ⁽²²⁵⁾. Along with those defects, zebrafish also showed reduced survival and impaired motor function ⁽²²⁵⁾.

Mouse models are the most used in vivo system, as they present high phylogenetic similarities to humans and share more than 90% of the genome ⁽²²⁶⁾. Due to this, different models were created to study ALD. However, they were not able to develop the most severe form of the disease, i.e., cALD, being only able to develop a milder phenotype similar to patients with AMN. In the *Abcd1* mutant mouse model for ALD, mice do not present a detectable phenotype despite the increased VLCFA from an early age ⁽²²⁷⁾. Even in later stages, mice only showed focal abnormalities in axons and myelin, both in spinal cord and sciatic nerve ⁽²²⁷⁾. Knockout for *Abcd2* showed a similar presentation without significant clinical phenotypic alterations. Similar to the *Abcd1* mutant, only significant axonal defects were found in older animals ⁽²²⁸⁾. Trying to achieve more severe neurological manifestations of ALD, *Abcd1* null mice have been crossed with other mice carrying mutations that are considered possible modifier genes for ALD. Interestingly, the double knockout for *Abcd1* and *Abcd2* presented a more severe phenotype, with an earlier onset of the disease, high levels of oxidative damage, and more severe locomotor impairment ^{(229); (230)}.

4. Acyl-CoA Binding Containing Domain Protein 5: a novel peroxisomal protein

Acyl-CoA Binding Containing Domain Protein 5 (ACBD5) is a novel protein that was recently identified in peroxisomes ^{(231); (232)}. Its peroxisomal location was confirmed by proteomic analysis from mouse kidney, rat, and human liver ^{(231); (232); (233); (234)}. ACBD5 belongs to the Acyl-CoA binding protein (ACBP) family, a group of intracellular lipid-binding proteins consisting of seven members in mammals ^{(151), (235)}. The protein is characterized by an N-terminal acyl-CoA binding domain (ACBD) and a two-phenylalanines (FF) in an acid track (AT)-like motif (FFAT-like; Figure 4A) ^{(236); (237)}. The ACBD is thought to mediate the binding of acyl-CoA esters, and the FFAT-like domain is thought to allow the interaction with the endoplasmic reticulum (ER) vesicle-associated membrane protein-associated proteins A and B (VAPA and VAPB) bringing these organelles closer (Figure 4B) ^{(232); (236); (238)}. ACBD5 is predicted to be inserted into the peroxisomal membrane with its C-terminal region while the acyl-CoA binding domain faces the cytosol, allowing the protein to interact with acyl-CoA molecules and thus facilitating its function in lipid metabolism ^{(232); (239)}.

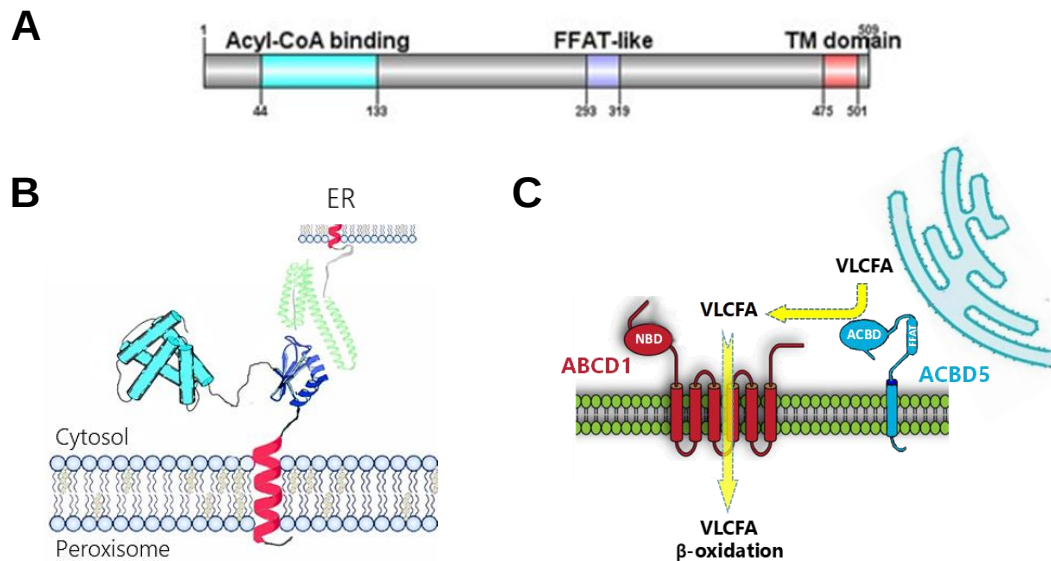


Figure 4. ACBD5 protein. Schematic representations of ACBD5 and its proposed functions. A – Representation of ACBD5 domains and motifs. ACBD5 is a peroxisomal membrane protein containing a single transmembrane domain (TM domain) in its C-terminal region. The N-terminal regions contain the acyl-CoA binding domain and the two phenylalanines (FF) in an acidic tract (FFAT)-like motif. B – Schematic drawing of ACBD5 proposed topology in peroxisomes showing the interaction of ACBD5 with the ER proteins vesicle-associated membrane protein A and B (VAMP-A/B) to mediate a closer interaction between peroxisomes and the ER. C - Schematic drawing of the proposed mechanism by which ACBD5 is thought to mediate the transport of VLCFA by facilitating their entrance via ABCD1 inside peroxisomes to be β -oxidized.

The discovery of patients with mutations in *ACBD5* led to the identification of a novel peroxisomal disorder included in the SPFD category. This disorder was identified with a nosologically distinct presentation and is characterized by retinal dystrophy, ataxia, and progressive leukodystrophy^{(240); (241)}. The biochemical characterization of *ACBD5*-deficient patients revealed the accumulation of VLCFA in plasma and fibroblasts, with increased levels of C26:0 and C26:0/C22:0 ratio, unraveling the role of *ACBD5* in VLCFA metabolism and β -oxidation^{(237); (240); (241)}. Cytosolic and/or ER-derived VLCFA are required to be translocated across the peroxisomal membrane to undergo degradation inside peroxisomes. This accumulation of VLCFA may have various repercussions on neurons and glia, which may cause neurodegeneration⁽²⁴¹⁾. Current models predict that ABCD1 and ABCD2, two ATP-binding cassette peroxisomal transporters, are involved in the transport of VLCFA across the peroxisome membrane. However, the metabolic findings in *ACBD5*-deficiency support the role of *ACBD5* as an initial carrier of VLCFA on-route for beta-oxidation in peroxisomes (Figure 4C). Studies *in vitro* reinforce this function since the biochemical phenotype of the patient's cells after transfection with wild-type *ACBD5* was reverted, confirming that the impaired VLCFA metabolism is caused by the deficiency in

ACBD5⁽²⁴¹⁾. Despite the available literature on the roles of ACBD5 in key peroxisomal functions, further research is needed to determine the mechanisms behind these processes and how their impairments cause tissue pathology and a disease state. The limited number of patients diagnosed with ACBD5 deficiency, combined with limited access to relevant patient-derived target tissues, has been partially overcome by the generation of mouse models. Recently, Darwisch and colleagues showed in their mouse model, alterations in lipid homeostasis, with neuronal degeneration, astrogliosis/microgliosis, and demyelination in the cerebellum and an ataxic phenotype in mice lacking *Acbd5*, resembling the pathology described in humans⁽²⁴²⁾.

In this work, we generated, using CRISPR-Cas9 technology, a novel mouse model for ACBD5 deficiency. The *Acbd5* Gly357* mutant mouse was instrumental in investigating how *Acbd5* deficiency contributes to lipid dysregulation, axonal degeneration, and white matter disease in CNS. Our work also impacted the field of neuroscience by establishing how the burden of lipid dysregulation affects neuronal and glial function. Determining the consequences and mechanisms behind ACBD5 deficiency will be a major contribution to understanding the neuropathology of the human disorder, as well as other neurodegenerative peroxisomal diseases that share common metabolic defects, and it will also be fundamental for the development of therapeutic strategies.

Aims of the work

Following the discovery of a novel peroxisomal disorder associated with mutations in the *ACBD5* gene and the accumulation of VLCFA, it became of added value to study the pathophysiology of the disease. Since VLCFA accumulation is also present in other human disorders, studying neuropathology, disease mechanisms, and potential therapies is of heightened interest and demand. Using a novel *Acbd5* mouse model, this work aimed to characterize the impact of *Acbd5* deficiency in the nervous tissue and determine the mechanisms behind the neuropathology. Moreover, we aimed to develop an *in vivo* gene delivery therapy to rescue the defects observed in the CNS.

Chapter 2: Material and Methods

Material and Methods

Generation of *Acbd5* mutant mouse, animal breeding, and genotyping

For the generation of the *Acbd5* mutant mouse, the Geneious software was used to analyze potential gRNAs targeting exon 9 of the *Acbd5* gene using the MEGAscript™ T7 Transcription Kit (Invitrogen). The target sequence 5'-AAAGGCGAAGTGAAACACGG-3' displayed high on-target activity and low off-target scores and was used to synthesize gRNAs. Next, the ribonucleoprotein consisting of Cas9 protein (IDT) plus the gRNA were mixed with a ultramer 5'-gatgtagcagtcaaaggaaaaggcgaagtgaacaCTGAGTGAGTGAGAATTCGGGcggaggagaagatggcagaagtagcagtggagcaccgcaccgtgagacgagaggtggagagagcgaggacttctccagtgtcaggagagg-3' modified with phosphorothioate bonds and were co-injected into fertilized eggs of F1 C57BL/6J:129S2/SvPasCrl mice. F0 founders were screening for the insertion of 21bp using PCR followed by sequencing. Founder #9 contained the desired mutation and was used to generate a congenic strain by backcrossing to C57BL/6J. At generation N7, we started breeding heterozygous mice to characterize the novel strain – B6.B6/129-*Acbd5*^{em1#9PB}/PB. PCR genotyping with specific primers (see below) yielded amplicons of 180bp for the WT allele and 201bp for the mutant *Acbd5* Gly357* allele.

All animals were maintained with ad libitum access to rodent food and water and were kept in a 12:12h light and dark cycle facility. Experiments and mouse manipulations were performed in compliance with the institutional guidelines and recommendations of the Federation for Laboratory Animal Science Association (FELASA) and were approved by the National Authority for Animal Health (DGAV; Lisbon, Portugal). Genotyping for wild-type (WT), *Acbd5*^{-/-} (Gly357*) and heterozygous (Htz) animals was performed using a thermocycler (VWR). All reactions were performed on PCR tubes containing 6.25µl of Supreme NZYTaQ II 2x Green Master Mix, 1µl of specific primers at 10µM for the exon 9 of *Acbd5* gene, Fwd-5'-TCAAACAATGGACACTTTCAGT-3' and Rev-5'-GCTACTTCTGCCATCTTCTCC-3', 3.25µl of MiliQ water and 1µl of genomic DNA. Amplification parameters were as follows: heat lid at 110°C, followed by 30 cycles of 95°C for 30 seconds (sec), 60°C for 30sec and 72°C for 30sec. A final elongation step of 5 minutes (min) at 72°C was performed. Amplicons were run on agarose gel with GreenSafe Premium and visualized on Molecular Image® Gel Doc™ XR+ Imaging System (Bio-Rad).

Behavioral assessments

Mouse behavior was evaluated using a set of qualitative scoring tests to assess motor and coordination function. Limb clasping, horizontal ladder, and paw print analysis tests were conducted at different ages in a blinded manner in different WT and Gly357* animal groups. For limb clasping analysis, animals were followed over several months (3, 4, 5, 6, 9, and 12 months) and scored for the response of the hind paws when gently lifted by the tail. A score of 0 was given for no clasping, 0.5 for partial clasping, and 1 for full clasping ^{(243); (244)}. For the horizontal ladder test, animal performance was assessed at 6, 9, and 12 months. The test was conducted by placing the mice at the beginning of the ladder with evenly spaced rungs, allowing them to cross the ladder (3 runs per animal), and recording the number of slips ⁽²⁴⁵⁾. For paw print analysis, the walking pattern was evaluated at 6 and 12 months by applying black ink to the hind paws of the mice and allowing them to walk on a white sheet of paper (four to five sheets per animal). The resulting paw prints were analyzed for stride and step length, toe spreading, base of support, and paw area ^{(246); (247)}.

Histological and morphological analysis

Mice were anesthetized and euthanized with a lethal mixture of ketamine/xylazine (125 and 12.5mg/kg body weight, respectively), and tissues were isolated and fixed by immersion. For ultrastructural analysis, tissues were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 5 days at 4°C. After postfixation with 1% OsO₄ in 0.1M cacodylate buffer (pH 7.4) for 2 hours (h), tissues were dehydrated and embedded in Epon (Electron Microscopy Sciences). Semi-sections (1µm) prepared on a Leica ultramicrotome were stained for 30sec with Richardson's or 10min with 1% p-phenylenediamine (PPD) in absolute methanol, dried, mounted with DPX (Merk) and used for tissue evaluation. In the spinal cord, the ventrolateral funiculus was chosen as the region of interest as it was the region where we observed most of the dystrophic axons. For axonal diameter analysis, five to eight images per animal at 40x magnification were acquired on an Olympus optical microscope equipped with an Olympus DP 25 camera and Cell B software and imported into Photoshop (Adobe) for quantification. Ultrathin sections (60nm) prepared on a Leica ultramicrotome were placed on 200-mesh copper grids (Electron Microscopy Sciences) and were counterstained, firstly with alcoholic uranyl acetate solution (2% w/v; 10 minutes), then by aqueous uranyl acetate solution (2% w/v; 10 min) and lead citrate (4% w/v; 10 minutes). Grids were observed on a JEOL JEM-1400 transmission electron microscope (JEOL, Japan) equipped with an Orious Sc1000 digital camera. Nonoverlapping images were acquired and used for tissue evaluation of g-ratio and myelin thickness analysis at 6 and 12

months. For g-ratio and myelin thickness, five to eight images per animal at 12000x magnification were analyzed. The Photoshop Quick Selection and the Recording Measurement tools were used to determine the ratio of the diameter of the axon to the diameter of the myelinated fiber (axon plus myelin).

For immunohistochemistry, tissues were fixed either in modified Carnoy's solution (absolute ethanol: methanol: glacial acetic acid - 6:3:1) for 2h at room temperature (RT), processed for paraffin embedding, sectioned at 4µm, cleared and rehydrated; or 4% Paraformaldehyde overnight (O/N) at 4°C, immersed in 30% sucrose O/N at 4°C, covered and kept in Cryomatrix (Thermo Scientific) at -80°C, sectioned at 25µm and stored in PBS 1x. Sections (two to three sections per animal) were permeabilized in 100% methanol at RT for paraffin or -20°C for cryosections, treated with 0.1% NaBH₄ in 10 mM Tris, 1mM EDTA at pH 9.0, and blocked with 5% normal donkey serum (NDS) for paraffin or 1% fish gelatin for cryosections in PBS. Primary antibodies were diluted in blocking buffer and incubated for 1h30min at RT or O/N at 4°C (Supplementary Table 1). Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488 and 594; Jackson ImmunoResearch Europe) were diluted in blocking buffer and incubated at RT for 1h. All tissue sections were mounted with Ibbidi mounting medium (Ibbidi) with 1x DAPI in microscope slides and visualized by epifluorescence in a Zeiss Axio Imager Z1 microscope equipped with an AxioCam MR3.0 camera and Axiovision 4.7 software. All quantifications, including cell counting and signal intensity measurement, were performed using the imaging software Fiji Image J.

Plasmids and AAV therapy

Two different plasmids either expressing the full-length mouse *Acbd5* gene (WT m*Acbd5*) linked by the 2A self-cleaving small peptide (P2A) to the reporter gene Turbo green fluorescent protein (tGFP, *Acbd5* plasmid) or the reporter gene Enhanced green fluorescent protein (EGFP, GFP plasmid), under the control of the cytomegalovirus promoter (CMV) were designed (VectorBuilder).

For the *in vivo* therapy, plasmids were packaged into an AAV-PHP.eB virus to obtain the pAAV[Exp]-CMV>TurboGFP(ns):P2A:m*Acbd5* (AAV-*Acbd5*) and pAAV[Exp]-CMV>EGFP (AAV-GFP, Control) vectors (VectorBuilder). At 3 months of age, a single tail intravenous injection (i.v.) of the viral solution containing 1.06×10^{12} gene copies for AAV-*Acbd5* or 1.48×10^{12} for AAV-GFP was delivered to Gly357* mice. WT-untransduced mice served as controls. Mouse behavioral was assessed at 3 months and at the end of the experimental setup, 6 months of age. The histological analysis was performed at 6 months of age, as described above.

Mouse fibroblast culture and transfection

Mice at 6 months of age were euthanized as described above, and lungs were isolated to a falcon containing Dulbecco's modified eagle medium with ultraglutamine 1 and 4.5g/L glucose (DMEM, Lonza). After being cut into small pieces in a petri dish, tissue was collected into a tube and digested with 0.05% trypsin-1x EDTA (Alfagene) for 30min at 37°C. Digestion was stopped by adding fibroblast medium (DMEM containing 10% Fetal Bovine Serum and 1% penicillin and streptomycin (P/S), and tissues were further disrupted by mechanical dissociation with a P1000 micropipette tip. The resulting suspension was plated on a petri dish and cultured at 37°C, 5% CO₂. Upon confluency, cells were split using 0.05% trypsin-1x EDTA and plated at 50% confluency.

For transfection, cells were cultured on a 6-well plate at 50-70% confluency O/N at 37°C, 5% CO₂. After removing the medium, cells were maintained in 1ml of Reduced Serum Media (Opti-Mem, Gibco) and transfected using Lipofectamine™ 3000 Transfection Reagent kit for 5h at 37°C, 5% CO₂. Transfection was performed by adding to cells 250µl of Opti-Mem containing 1.5µg of GFP plasmid DNA with 2.5µl of Lipofectamine P3000 Reagent and 3µl of Lipofectamine or 10µg of *Acbd5* plasmid DNA with 7.5µl of Lipofectamine P3000 Reagent and 5µl of Lipofectamine. After transfection, Opti-Mem was replaced by a fibroblast medium, and cells were maintained in culture at 37°C, with 5% CO₂ for 24h.

Mouse cortical neuron culture and very long chain fatty acids treatment

Cortical neuron culture was performed as described in Mar et al., 2016, with minor alterations. Cortical neurons were isolated from individual WT and Gly357* embryos brains at embryonic day 17.5 (E17.5). Cortices were digested at 37°C in 0.05% Trypsin + 1x EDTA for 8min. Digestion was stopped with DMEM/F12 medium (Pan-Biotech) containing 10% FBS. After dissociated, cells were filtered and resuspended in culture medium containing Neurobasal medium (Invitrogen) supplemented with 2x B-27 (Invitrogen), 1% P/S, and 2mM L-glutamine (Invitrogen). Cells were plated onto 24-well (Costar®) or 15µ-slide 8-well (Ibidi®) plates coated with 20µg/ml poly-L-lysine (Sigma) and 0.4µg/ml of laminin (Sigma). Cortical neurons were maintained in culture and used for experiments at Div7, Div8, and Div11.

For treatment with VLCFA, tetracosanoic acid methyl ester (meC24:0; Cymit Quimica) was used. A meC24:0 work solution at 5mM was prepared in 50% DMSO/ 50%

H₂O. WT and Gly357* cortical neurons were treated with 60μM or the vehicle, which were added to the culture medium at Div1, Div4, and Div7 for the longer experience period.

Mitochondria morphology and dynamics evaluation

For mitochondria morphological analysis, 12500 cells/well of cortical neurons were plated on a 24-well plate and were treated at Div7 with 100nM of the mitochondrial fluorescent dye MitoTracker™ Orange CMTMRos (M7510, Invitrogen, Thermo Fisher Scientific) for 45min at 37°C. After the incubation, the culture medium was removed, and the cells were fixed with PFA 4% for 10 minutes at RT. Fixation was followed by permeabilization with 0.1% triton X-100 in PBS for 10min and autofluorescence quenching with 0.2M ammonium chloride (Merck) for 10min at RT. Non-specific labeling was blocked by incubation with blocking buffer (5% NDS in PBS 1x) for 1h at 37°C. The primary antibody, mouse anti-tubulin βIII (Promega), was diluted in blocking buffer and incubated for 1h at RT. After two 5min washes in PBS, incubation with secondary antibody, donkey anti-mouse IgG antibody Alexa Fluor® 488 (Jackson ImmunoResearch), was performed for 1hr at RT. For analysis of mitochondria morphology images acquired in Zeiss Axio Imager Z1 (Carl Zeiss, Germany) were analyzed using Fiji software. After the application of the threshold function, so that all the mitochondria were highlighted, area and circularity parameters were determined.

For mitochondria live imaging, 12500 cells/well of cortical neurons were plated on a 15μ-slide 8-well plate and were treated at Div11 with 50nM of the mitochondrial fluorescent dye MitoTracker™ Orange CMTMRos (M7510, Invitrogen, Thermo Fisher Scientific). Half of the culture medium was removed and kept at 37°C, while the other half was completed with fresh medium. The cells were then treated with Mitotracker and incubated at 37°C for 45min. After incubation, the medium was substituted with the original culture medium supplemented with 50% fresh solution to achieve the total volume, and cells were kept in culture for 1h to recover. For live cell imaging at the axon shaft, excitation was carried out using 561nm laser lines, and time-lapse recordings every 2sec for 2min on a Leica TCS SP5 (Leica Microsystems, Germany) at 37°C and 5% CO₂. Images were analyzed and processed using Fiji software, and mitochondria dynamics were assessed using the plug-in MTrackJ. The velocity of the particles was manually tracked, and all organelles were tracked to determine the percentage of motile and stationary mitochondria. The percentage of motile mitochondria corresponds to the number of moving mitochondria divided by the total number of mitochondria (motile and stationary). The percentage of mitochondria moving

either in anterograde or retrograde direction is the number of moving mitochondria in the specific direction divided by the total number of mitochondria.

Actin and microtubule dynamics evaluation

For actin and microtubule dynamics analysis, 50000 cells/well of cortical neurons were plated on an 8-well plate were transfected at Div5 using Lipofectamine™ 3000 (Invitrogen). The culture medium was removed and stored at 37°C, and transfection was performed by adding to the neurons for 35min 150µl of Opti-Mem containing 0.4µg of Lifeact-GFP plasmid⁽²⁴⁸⁾ or 0.4µg of EB3-GFP plasmid⁽²⁴⁹⁾, 0.3µl of Lipofectamine and 0.5µl of Lipofectamine P3000 Reagent. After the incubation, the cortical neuron transfection medium was replaced by the original medium, and cells were maintained in the culture for 24-48h until live cell imaging was performed.

The analysis of actin and microtubule dynamics was performed as described in⁽²⁵⁰⁾. 24h or 48h after transfection, i.e., Div7 or Div8, time-lapse recordings every 2sec for 3min in the growth cone or axon shaft, respectively, were performed at 37°C and 5% CO₂, on a Spinning Disk Confocal System Andor Revolution XD with an iXonEM+ DU-897 camera and an IQ 1.10.1 software (ANDOR Technology). Transfected GFP⁺ neurons were considered for analysis. For the quantification, kymographs were obtained using the Fiji KymoResliceWide plug-in (distance, x axis; time, y axis), and initial and end positions of the traces were defined using the Fiji Cell Counter plug-in.

Immunolabeling and super-resolution imaging were performed as described in⁽²⁵¹⁾, with minor alterations. Cortical neurons were fixed at Div8 with 4% PFA for 10min at RT, followed by permeabilization with 0.1% triton X-100 in PBS for 5min and autofluorescence quenching with 0.2M ammonium chloride (Merck) for 3min at RT. Non-specific labeling was blocked by incubation with blocking buffer (5% NDS in PBS) for 1h at RT. The primary antibody, mouse anti-tubulin βIII (Promega), was diluted in blocking buffer and incubated for 1h at RT. After two 5min washes in PBS, incubation with the secondary antibody, goat anti-mouse STAR 580 (Abberior GmbH), was performed for 1hr at RT. Actin staining was performed with 0.3mM phalloidin 635P (Abberior GmbH) diluted in PBS, which was incubated for 30min at 37°C following the secondary antibody incubation.

STED imaging was performed as described in⁽²⁵¹⁾. Images were acquired with a single point scanning confocal Stellaris 8, equipped with a fully motorized inverted Leica DMI8 microscope, a HC PL APO 93x/1.3STED WHITE glycerol immersion objective equipped with a correction collar (Leica Microsystems), a WLL, and Falcon and STED modalities. The STED images were recorded sequentially by frame scanning

unidirectionally at 400Hz using the galvanometer-based imaging mode, with a line accumulation of 16, and a pixel size of 0.02 μ m for an area size of 20,83 μ m x 20,83 μ m (1024x1024, zoom factor of 6, pixel dwell time of 1.575 μ s) in Leica LasX software (version 5.1.0). Phalloidin (Abberior STAR 635P) was excited with a 633nm laser line with a laser power of 2%, and emission was collected on a Leica HyDX4 (counting mode) detector with a collection window of 643-720nm. Tubulin β III, labeled with Abberior STAR 580 was excited with a 587nm laser line with a laser power of 1%, and emission was collected on a Leica HyDX2 (counting mode) detector with a collection window of 597-623nm. The STED depletion was performed with a synchronized pulsed 775nm (set at 100 %) depletion laser at 40 or 80%, respectively. For all images, the pinhole size was 151.9 μ m, calculated at 1 AU for 580nm emission, and TauSTED was applied with the following parameters: Tau background: ON; Tau strength: 100; Denoise: 50.

Western Blotting

For Western blot, lysates of the spinal cord and mouse fibroblasts (MF) were prepared by sonication in lysis buffer containing 1% Triton, 0.1% SDS, 140mM NaCl, and 1x Halt Protease and phosphatase inhibitor (Thermo Scientific) in 20mM Tris- 1mM EDTA pH 8.0. Total protein levels were measured using DC™ Protein Assay (Bio-Rad). Protein samples (5 μ g total protein for myelin marker analysis, 10 μ g of total protein for MF, and 20 μ g total protein for the remaining applications) were prepared on 1x sample loading buffer (SLB; 31.25mM Tris.Cl pH 6.8, 1.5% SDS, 12.5% Glycerol, 358mM β -mercaptoethanol and 0.05% Orange G) denatured at 100°C and separated by SDS-Page in Criterion™ TGX Precast (Bio-Rad) or Tris-Glycine NG (NuSep) 4-20% gels and transferred to nitrocellulose blotting membranes (0.45 μ m; Amersham) using a semidry transfer blotting system. Membranes were blocked with 5% skim milk (Millipore) in Tris-buffered saline (TBS) for 1h at RT, washed and incubated for 1h30min at RT with primary antibody (Supplementary Table 1) diluted in 5% BSA in TBS with 0.1% Triton (TBS-T). Horseradish peroxidase-conjugated (HRP, Jackson ImmunoResearch Laboratories) or IRDye (LI-COR) secondary antibodies were diluted 1:10000 in 5% skim milk in TBS-T and incubated for 1h at RT. HRP-labeled membranes were developed using ECL (Luminata Crescendo Western HRP Substrate, Millipore) and exposed to X-ray film (Fujifilm). Films were scanned on a Molecular Imager GS800 and quantified using Quantity One software. 14-3-3 (pan) or p44/42 MAPK (T-erk1/2) were used as housekeeping proteins ⁽⁷⁾. Li-cor IRDye-labeled membranes were scanned on an Odyssey CLx Infrared Imaging System, and images were

quantified using Image Studio 5.2 software. Total Protein Staining (TPS; LI-COR) was used as a loading control.

RT-qPCR

Brains from WT, Gly357*-AAV-GFP, and Gly357*-AAV-*Acbd5* mice at 6 months of age were isolated, frozen on dry ice, and kept at -80°C. Total RNA was prepared using NZY Total RNA Isolation Kit and following the manufacturer's protocol (Nzytech). RNA integrity and concentration were assessed by checking the absorbance ratio at 260nm and 280nm (A260/A280) using NanoDrop 1000 Spectrophotometer (Thermo Scientific) and the ND-1000 3.5.2 software. cDNA was prepared from 0.9µg of RNA using NZY First-Strand cDNA Synthesis Kit (Nzytech) as recommended by the manufacturer.

SYBR-green quantitative PCR was performed using a CFX384 Touch™ Real-Time PCR Detection System (Bio-rad) with primers for *Acbd5* (Fwd- 5'-GCCGTGAAGGTGATCCAGAG -3' and Rev- 5'- CAGAATCCAGGCCGTGAAAG -3') and for *β-actin* (Fwd- 5'-GGGCTGTATTCCTCCATC -3' and Rev- 5'-TCTCCATGTCGTCCCAGTTG -3'). All reactions were performed in 96-well plates and contained: 5µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.5µl of 10µM forward and reverse primers, 3µl of molecular biology grade water and 1µl of a 1:5 dilution of the cDNA solution. Amplification parameters were as follows: 95°C for 3min, followed by 40 amplification cycles at 95°C for 10s and 66°C for 30s. A final dissociation reaction (melting curve) was performed with the following steps: 65°C for 5s and incremental temperatures of 0.5°C until 95°C. The fold change in gene expression was calculated using the $\Delta\Delta C_t$ relative expression method (Livak method) and normalized for *β-actin*.

LC-MS Proteomics

Lysates of the spinal cord (cervical region) were prepared by sonication in lysis buffer (as above). Total protein levels were measured using DC™ Protein Assay (Bio-Rad). Proteomics analysis was performed by the i3S Proteomics service. Briefly, 100µg of each sample (6M, WT n=3, Gly357* n=3; 12M, WT n=2, Gly357* n=3) were processed in duplicate for proteomic analysis following the solid-phase-enhanced sample-preparation (SP3) protocol and enzymatically digested with Trypsin/LysC (2µg) overnight at 37 °C at 1000 rpm. The peptide concentration was measured by fluorescence.

Protein identification and label-free quantitation were performed by nanoLC-MS/MS using an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) as

described in ⁽²⁵²⁾. For protein identification and quantification, the UniProt *Mus Musculus* Proteome database was considered (2019_09). The proteomics data analysis was performed with the Proteome Discoverer 2.4.0.305 software (Thermo Scientific, San Jose, CA, USA). For determination of differentially expressed proteins between the Gly357* and WT animals, the following filters were considered: all samples must have at least confidence peak found, at least two unique peptides identified, the p-value adjusted using Benjamini–Hochberg correction for the FDR set to ≤ 0.05 and the Gly357*/WT considered ratio was set as follows: 6M, ≥ 1.20 and ≤ 0.85 and 12M, ≥ 1.25 and ≤ 0.75 , for the selection of upregulated proteins and downregulated proteins, respectively. The threshold values considered for up-regulation were obtained based on the fold change number of the first protein, which was identified as statistically altered. The considered threshold values for down-regulation were obtained by making the inverse of the fold change value of the first value of the first protein identified as statistically up-regulated. Volcano plot analysis (Proteome Discoverer 2.4 software), Protein functional enrichment analysis (ShinyGO 0.77 software), Venn diagram, and Heatmap analysis (Excel software) were performed after applying the above-described filters.

Lipid extraction and LC-MS Lipidomics

Spinal cord was collected from WT and Gly357* and immediately frozen and stored at -80°C . Lipid extraction was performed by Metabolomics Core Facility (EMBL, Heidelberg, Germany) as briefly described: frozen tissue was grounded to a powder, added 200 μL of cold methanol and vortex for protein precipitation, added 500 μL of chloroform and vortex and kept at cold for 10min, added 200 μL of water for phase separation, centrifuge at 600rpm for 5min and carefully remove the bottom chloroform layer and transfer to new amber color glass vial. After the initial extraction, a second extraction was performed with 2:1 chloroform: methanol and the lower organic phase was collected and dried under a stream of nitrogen gas.

LC-MS/MS analysis was performed by Metabolomics Core Facility (EMBL, Heidelberg, Germany) using a Vanquish UHPLC system coupled to a Q-Exactive plus HRMS (Thermo Scientific, MA, USA) in ESI positive mode as follows: the separation of lipids and fatty acids was carried out on Kinetex C18 (100x 2.1; 2.6 μM) column at a flow rate of 0.26 ml/min and maintained at 30°C . The mobile phase consisted of solvent A (0.1% formic acid in isopropyl alcohol:acetonitrile (9:1) and solvent B (0.1% formic acid water:acetonitrile (6:4), were buffered with 10mM ammonium formate for positive mode

analysis. Lipid detection and data acquisition were performed as described in ⁽²⁵³⁾. Lipid annotation was performed using BasicLipids and Waters Library hits from Progenesis.

Statistics

Results were expressed as the mean $\pm$ SEM, and data were analyzed with Prism software (GraphPad 8.1.1 Software). A parametric unpaired student's T-test was used to compare the two groups. For comparison of more than two groups, One-way ANOVA or Two-way ANOVA tests were used, followed by Sidak's or Tukey's post hoc tests. For statistical significance, $P < 0.05$ was considered a significant difference.

Chapter 3: Results

Results

Gly357* mice recapitulate Acbd5 deficiency in human

To understand and unravel the mechanisms behind the Acbd5 deficiency, a CRISPR- Cas9 gene-targeting approach was used to generate a mutant mice model. A specific gRNA for exon 9 was used to introduce 21 nucleotides in the genome through homologous directed repair (Figure 1A). The knock-in sequence introduces a premature stop codon, causing the mutation p.Gly357* (Figure 1B). Sequencing of the WT and mutant amplicons through a primer-based approach using primers flanking the knock-in sequence confirmed the presence of GGA to TGA mutation, and the same primer set was used to differentiate between wild-type (WT), heterozygous (Htz), and homozygous mutants (Gly357*; Figure 1C). To eliminate possible off-target mutations, a founder carrying the mutation was selected, and a congenic strain was generated by backcrossing to C57BL/6J. At generation N7, with an estimated 99% of the donor genome eliminated, we started breeding Htz mice to generate and characterize the Gly357* mutants. The offspring from heterozygous intercrosses were born at the expected Mendelian ratio.

To confirm the absence of Acbd5 in Gly357* mice, as observed in most of the described patients with Acbd5 deficiency, western blot analysis was performed in liver and spinal cord lysates from WT and Gly357* at 6 months of age. The expression of Acbd5 was not detected in either tissue of Gly357* mutants, indicating that mice carry a null allele (Figure 1D).

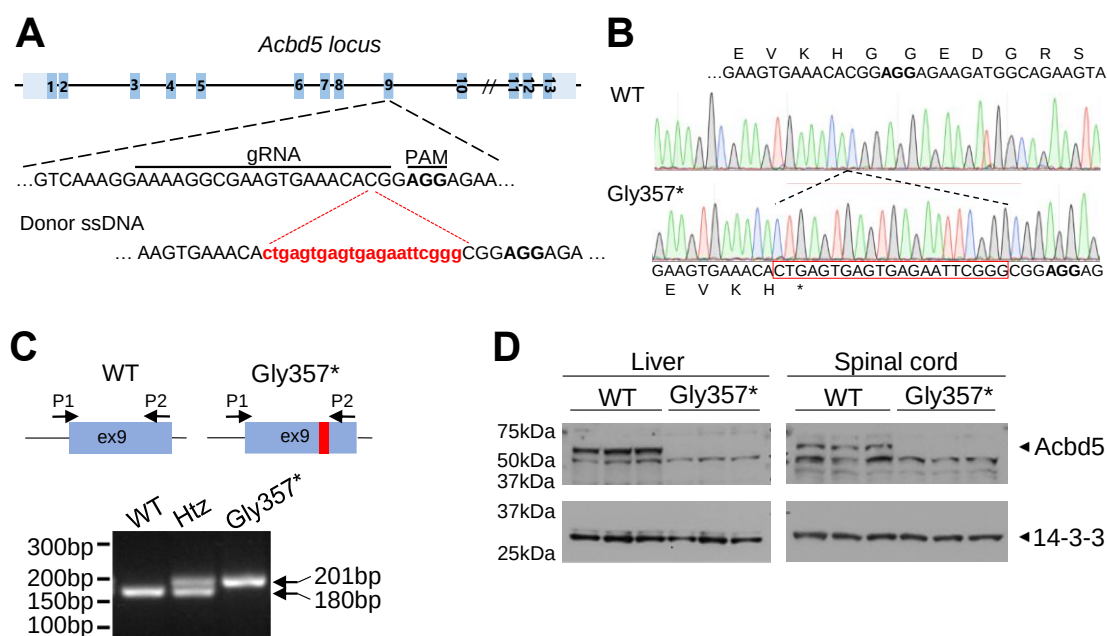


Figure 1. *Acbd5* mutant mouse generated by Crispr. (A) Schematic representation of *Acbd5* gene (upper panel). Representation of exon 9 highlighting the gRNA site and PAM (middle panel). Donor ssDNA sequence used as a template for homologous directed repair (HDR), containing the 21 nucleotides (in red) introduced on the genome (bottom panel). (B) Sanger sequencing confirmed the GGA to TGA mutation corresponding to Gly357* in homozygous mutant mice (bottom electropherogram). The 21bp mutant sequence introduced by HDR is boxed in red. (C) Primers (P1 e P2) flanking the exon 9 for genotyping WT and Gly357* mice (upper panel). Agarose gel showing amplicons for WT (180bp), Gly357* homozygous (201bp) and heterozygous mice (bottom panel). (D) Representative blot in the liver (left panel) and spinal cord (right panel) showing the expression in WT and the absence of *Acbd5* in Gly357* at 6 months of age. Blot for 14-3-3 used as loading control (WT n=3, Gly357* n=3).

***Acbd5* deficiency leads to CNS lipid homeostasis disruption in Gly357* mice**

Acbd5 deficiency pathology in humans has been associated with impaired β -oxidation of VLCFA and its subsequent accumulation in tissues ^{(237); (240); (241)}. To assess lipidomic changes caused by the mutant allele, spinal cords from WT and Gly357* mice were analyzed by LC-MS. Lipidomics revealed a total of 139 lipids that were dysregulated, including increased levels of C26:0 in the spinal cord from Gly357* mice (Supplementary Table 1). Within the dysregulated lipids, our analysis showed that the main lipid classes altered were phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingolipids (SL). Accumulation of lipids in Gly357* mice containing VLCFA was found to be distinctly different with high fold changes when compared to WT mice (Figure 2A). PC containing C32:0 and C24:1, and lactosylceramide (LacCer) and ceramide (Cer) containing C26:0

were the most increased lipids in Gly357* spinal cords (Figure 2A). Besides the accumulation of VLCFA, lipidomics also found several lipid species containing LCFA altered in Gly357*. Alkyl- and alkenyl-glycerophospholipids were among the lipids with the highest accumulation in Gly357* spinal cords (Figure 2B). Notably, our results also revealed that lipids did not only accumulate in Gly357* mice. Lipids containing VLCFA, particularly polyunsaturated C22 fatty acids, were found decreased in Gly357* (Figure 2C). Additionally, lipids containing LCFA, mainly lyso-phospholipids, were also found to be significantly decreased in the mutant mice compared to WT (Figure 2D). Overall, the altered lipid composition demonstrates that *Acbd5* is important for lipid metabolism and maintenance of lipid homeostasis, and its loss causes a complex metabolic outcome with the dysregulation of major lipid classes in CNS.

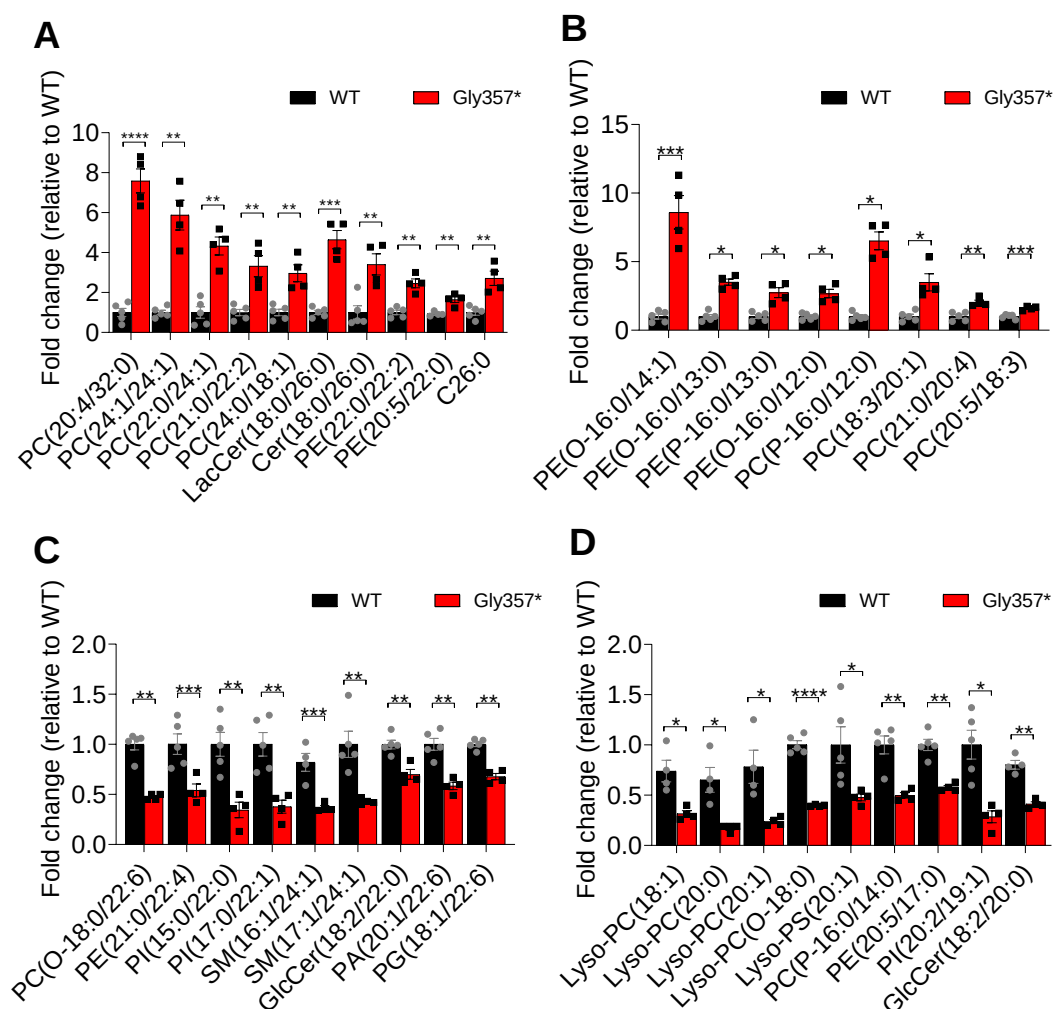


Figure 2. Lipidomic profiling in Gly357* mutant mice. Relative abundances of lipids containing VLCFA and LCFA in spinal cords from WT and Gly357* mice at 6 months of age (WT n=5, Gly357* n=4). The Y axis illustrates the fold change relative to the mean of WT for each lipid. Lipid classes accumulated in Gly357* mice containing VLCFA (**A**) or LCFA (**B**). Lipid species containing VLCFA that are reduced in Gly357* mice (**C**) and general lipid

species that are reduced in Gly357* mice (**D**). Data are represented as mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; unpaired parametric student's t-test.

Loss of ACBD5 causes retinal defects and ataxia in mutant mice

Retinal dystrophy is described as one of the main clinical symptoms present in *Acbd5* deficiency in humans ^{(237); (240); (241)}. To address the retinal pathology, an analysis of Richardson's and PPD stained eye sections from WT and Gly357* mice at 12 months of age was performed (Supplementary Figure 1A). Measurement of retinal layers revealed a decreased length of the photoreceptor outer segment (POS; Supplementary Figure 1B) and outer nuclear layer (ONL; Figure Supplementary 1C), which contains the cell bodies of photoreceptors. In addition, the normal length of the inner nuclear layer (Supplementary Figure 1D) in Gly357* highlights a targeted degeneration of the synaptic terminals of rods and cones with concomitant loss of neurons (Supplementary Figure 1E).

Notably, the most striking phenotypic presentation of Gly357* mice and an early indicator of motor and coordination defects was the presence of limb claspings (Figure 3A). A follow-up analysis showed a progressive phenotype up to 6 months of age when all mutants displayed full limb claspings (Figure 3B). Given the ataxic presentation, the locomotor performance was assessed. The horizontal ladder test (Figure 3C) showed that mutant mice performed poorly (with a higher percentage of paw slips) when compared to WT mice, and this phenotype tended to be more severe with aging (Figure 3D). Paw print analysis also indicated motor and coordination deficiencies in Gly357* (Figure 3E). At 6 months of age, Gly357* mice had decreases in stride and step lengths (Figure 3F and 3G). Worsening was observed at 1 year of age, and at this stage, Gly357* also had decreases in toe spreading (Figure 3H), base of support (Figure 3I), and paw area (Figure 3J). Next, we investigated the potential involvement of the cerebellum in the presented ataxic phenotype. Analysis of Purkinje cells by histology showed focal loss in 6-month-old Gly357* mice (Supplementary Figure 2A-C). The pathology was progressive, and by 1 year of age, Gly357* mice showed generalized loss of Purkinje cells in the anterior lobules (Supplementary Figure 2A-C). Multiple axonal swellings were also found in axons of Gly357* Purkinje cells (Supplementary Figure 2B), which is indicative of cell damage and can be a contributing factor to the progressive loss of these neurons. Our results indicate that *Acbd5* has an important role in the cerebellum, and the progressive loss of Purkinje cells contributes to the ataxic phenotype in Gly357* mice.

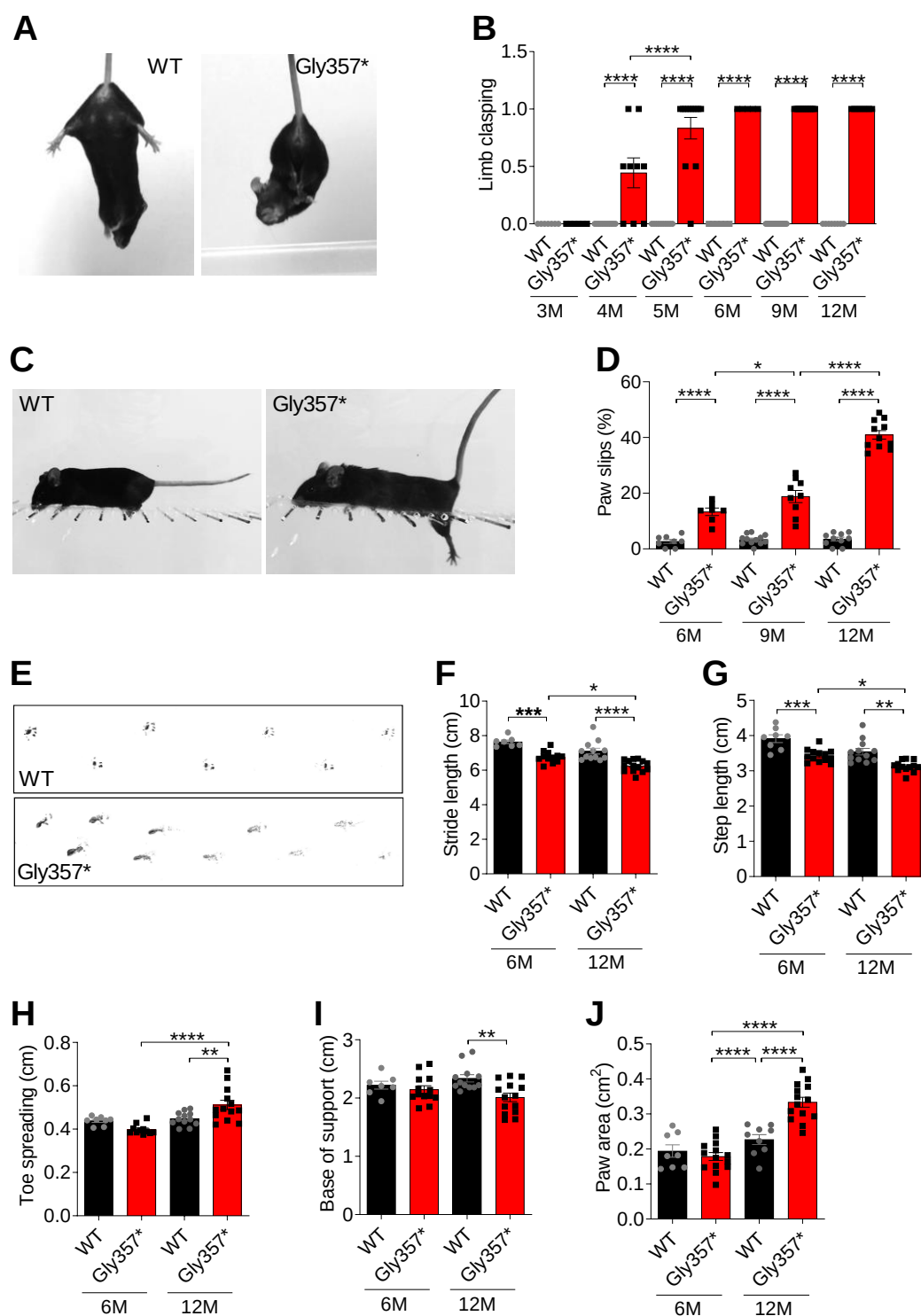


Figure 3. Gly357* mutant mice develop a severe ataxia. (A) Representative image of WT and Gly357* mice behavior when picked by the tail (limb clasping) at 12 months of age. (B) Limb clasping quantification at 3, 4, 5, 6, 9 and 12 months of age revealed phenotype starting at 4 months of age in Gly357* (3M, WT n=7, Gly357* n=6; 4M, WT n=12, Gly357* n=9; 5M, WT n=10, Gly357* n=12; 6M, WT n=6, Gly357* n=5; 9M, WT n=17, Gly357* n=13; 12M, WT n=8, Gly357* n=8). (C) Representative image of WT and Gly357* mice on horizontal ladder test. (D) Quantification of WT and Gly357* paw slips at 6, 9 and 12 months of age showed an increased percentage of missed steps of mutant mice (6M, WT n=8, Gly357* n=7; 9M, WT n=12, Gly357* n=9; 12M, WT n=10, Gly357* n=11). (E)

Representative paw print images of WT and Gly357* mice. **(F-J)** Quantification at 6 and 12 months of age of WT and Gly357* stride length **(F)**, step length **(G)**, toe spreading **(H)**, base of support **(I)** and paw area **(J)**. (6M, WT n=7-8, Gly357* n=11-14; 12M, WT n=9-12, Gly357* n=13-14). Data are represented as mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; One-way ANOVA followed by Sidak's post hoc test **(B, D)**; One-way ANOVA followed by Tukey's post hoc test **(F- J)**.

Extensive gliosis highlights CNS dysfunction in *Acdb5* deficient mice

Alterations in astrocyte and microglia reactivity status are often associated with many CNS injuries and pathologies ^{(68); (69); (81); (85); (254)}. To address the participation of these glial cells upon *Acdb5* deficiency, we analyzed the gliotic state on spinal cords from 6 and 12 months old mice using immunofluorescence with antibodies against the glial fibrillary acidic protein (Gfap) and ionized calcium-binding adapter molecule 1 (Iba1; Figure 4A and 4C). At 6 months, increased Gfap reactivity was detected throughout the grey matter (Figure 4B). At 1 year old, increased astrocytosis was observed in both grey and white matter (Figure 4A and 4B). A similar pattern was observed for microglia, where cells were found to be more reactive with increased expression of Iba1 in Gly357*. Similarly to astrocytes, microglia were found to be more reactive at 1 year of age (Figure 4C and 4D). The initial prevalence of grey matter vs. white matter gliosis may demark differential neuronal and myelin defects. Also, the progressive regional expansion of the gliosis from 6 to 12 months indicates the development of a generalized neuropathological condition.

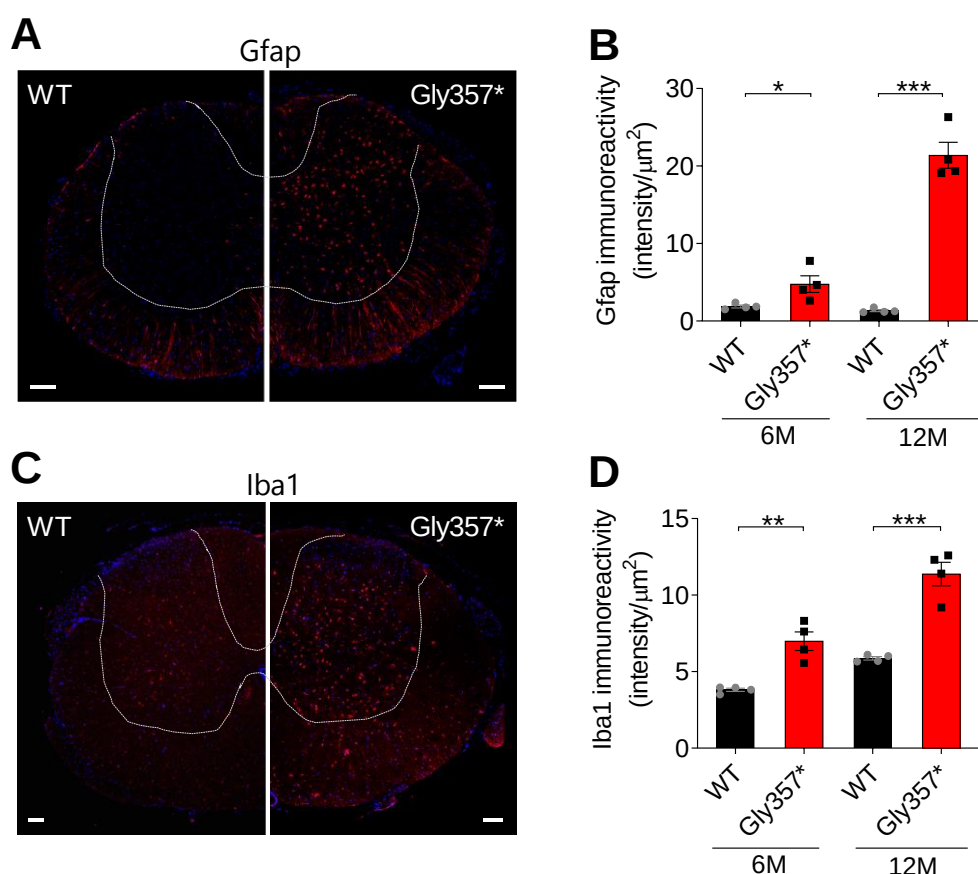


Figure 4. *Acbd5* deficiency leads to extensive glial response in CNS. (A) Spinal cord paraffin sections of WT and Gly357* mice at 12 months of age stained with anti-Gfap (in red) and DAPI (in blue) showed increased reactivity response of astrocytes in mutant mice (scale bar: 100 μm). **(B)** Quantification of the Gfap immunoreactivity in the spinal cord of WT and Gly357* at 6 and 12 months of age (6M, WT n=4, Gly357* n=4; 12M, WT n=4, Gly357* n=4). **(C)** Paraffin sections of WT and Gly357* spinal cord at 12 months of age stained with anti-Iba1 (in red) and DAPI (in blue) showed increased reactivity response of microglia in mutant mice (scale bar: 100 μm). **(D)** Quantification of the Iba1 immunoreactivity in the spinal cord of WT and Gly357* at 6 and 12 months of age (6M, WT n=4, Gly357* n=4; 12M, WT n=4, Gly357* n=4). Data are represented as mean ± SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; unpaired parametric student's t-test.

Loss of *Acbd5* causes demyelination in Gly357* mutant mice

Considering the white matter involvement observed in *Acbd5* patients and the gliotic condition of Gly357* mice, we next evaluated the impact of a deficiency in *Acbd5* in myelination. Ultrastructural analysis of the spinal cord revealed that Gly357* fibers displayed thinner myelin sheets (Figure 5A). Additionally, analysis of g-ratio (Figure 5B) and myelin thickness (Figure 5C and 5D) confirmed the defective myelination in mutant mice. The loss of myelin was not restricted to the spinal cord, as we also observed demyelination in the cerebellar white matter (Figure 5E). To further characterize the myelin defects, we

quantified the expression levels of different myelin markers in WT and Gly357* mice. One-year-old Gly357* mice revealed reduced levels of myelin basic protein (Mbp), 2',3'-cyclic nucleotide 3'-phosphodiesterase (Cnpase), and tubulin β -IV (Figure 5F-J). Altogether, these results demonstrate a demyelination condition in the CNS of Gly357* mice, highlighting *Acbd5* as a crucial player in the proper maintenance of myelin.

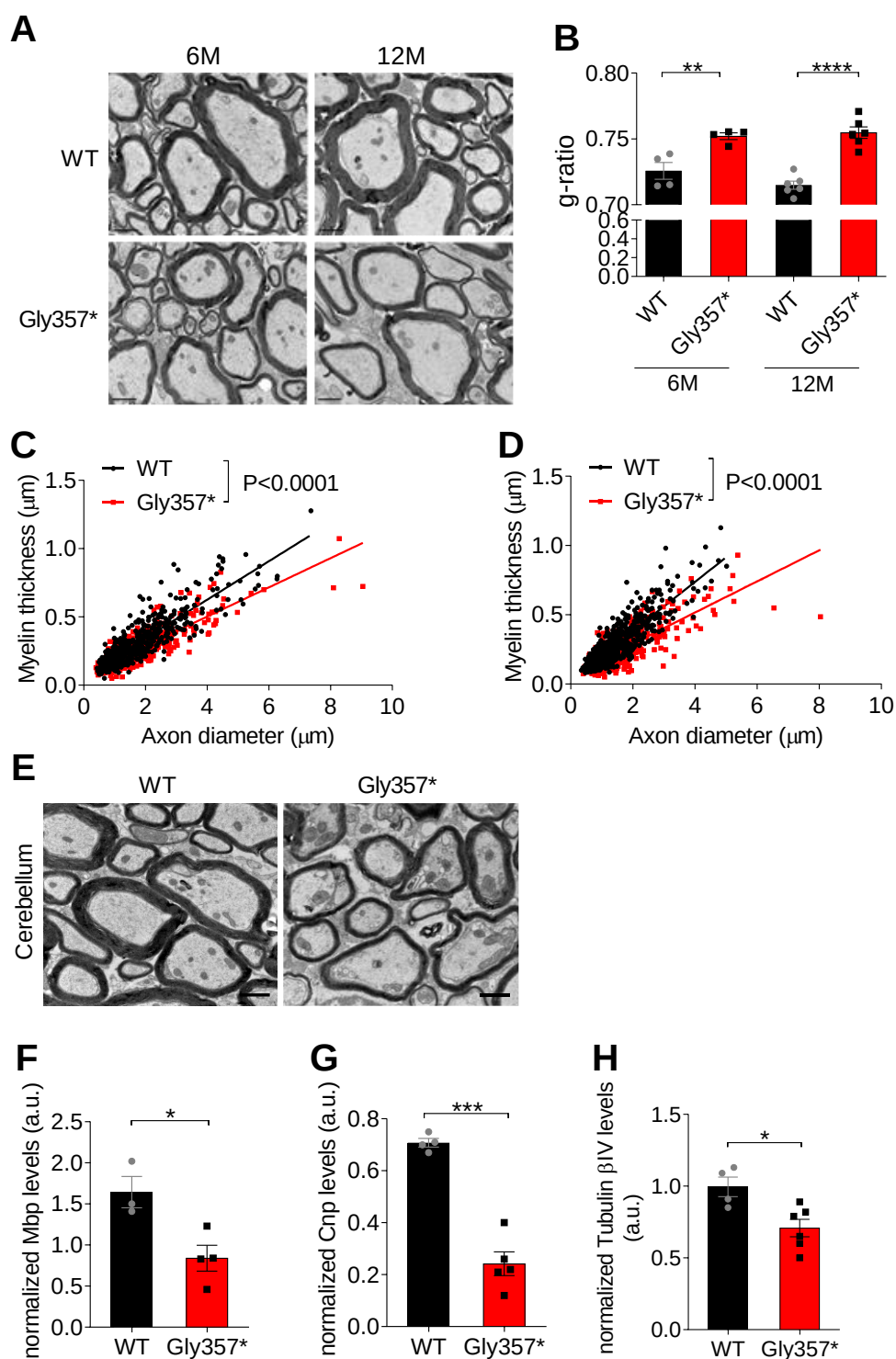


Figure 5. *Acdb5* deficiency causes demyelination in CNS. (A) Electron microscopic images of WT and Gly357* spinal cords at 6 and 12 months of age revealed a loss of myelin in mutant mice (bottom panel; scale bars: 1 μ m). (B) Quantification of myelin in WT and Gly357* spinal cord at 6 and 12 months of age by determination of g-ratio (6M, WT n=4, Gly357* n=4; 12M, WT n=6, Gly357* n=6). (C-D) Linear regression analysis of myelin thickness in the spinal cord at 6 (C) and 12 months of age (D) in WT and Gly357* (6M, WT=4, Gly357*=4; 12M, WT=6, Gly357*=6). (E) Representative electron microscopic images of WT and Gly357* cerebellum at 12 months of age revealed a loss of myelin in mutant mice (scale bars: 1 μ m). (F-H) Western Blot quantification of myelin proteins present in WT and Gly357* spinal cords at 12 months of age showed decreased levels of Mbp (F), Cnpase (G), and Tubulin β -IV (H) in mutant mice (WT n=3-4, Gly357* n=4-6). Data are represented as mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; unpaired parametric student's t-test.

Gly357* mice display axonopathy with giant axon swellings

The structural analysis of the spinal cord at 6 and 12 months revealed clear signs of axonal damage and the presence of giant axons in Gly357* mice (Figure 6A). Analysis of axon diameter highlighted the magnitude of the increased axon size, which in some cases almost reached 50 μ m in diameter (Supplementary Figure 3A). The analysis also revealed that spinal cords from Gly357* mice had an increased frequency of axons with large diameters (Figure 6B), and with age, this feature was more evident, suggesting a worsening of the pathology. To better understand the structure of these axons, we analyzed longitudinal sections of the spinal cord. The analysis of near consecutive sections confirmed that the giant structures were swellings and that individual axons could present several of these giant swellings separated by stretches with an apparent normal diameter (Figure 6C).

The giant axonal swellings were a generalized pathological feature observed in the spinal cord, brain, optic nerve, and cerebellum (Figure 6D and Supplementary Figure 3B). The ultrastructural analysis has also revealed that swellings accumulate organelles and present a disorganized cytoskeleton (Figure 6D–F). Another striking feature observed in axons from Gly357* mice was the presence of alterations in the structure and distribution of the endoplasmic reticulum (ER) in stretches of axons just preceding or just following the giant swelling (Supplementary Figure 3C). This prompted us to investigate if ER stress was a component of the cellular changes caused by *Acdb5* deficiency. Using western blot and immunohistochemistry, we observed increased levels of protein disulfide isomerase (Pdi) and C/EBP homologous protein (Chop; Supplementary Figure 3D and 3E), two well-established markers of ER stress ⁽²⁵⁵⁾.

The giant axonal swellings presented a severe disruption of the cytoskeleton organization with a mesh network of neurofilaments and microtubules. Given that alterations and damaged axons are known to display increased levels of non-phosphorylated

neurofilaments, we assessed the levels of non-phosphorylated and phosphorylated neurofilaments (not showed) by immunohistochemistry in spinal cords from WT and Gly357* mice. Analysis at 6 and 12 months of age revealed increased SMI32 reactivity in axons of Gly357* spinal cords (Figure 6G and Supplementary Figure 3F). We also observed increased SMI32 reactivity in axons in the cerebellar white matter of Gly357* mice (Supplementary Figure 3G). Combined, our results indicate that *Acbd5* is important for neuronal homeostasis, and its loss is associated with axonal defects in CNS, resulting in a severe axonopathy with increased axon diameter and the formation of giant swellings. The disruption of the axonal cytoskeleton architecture is likely a contributing factor leading to defects in organelle transport and their regional accumulation.

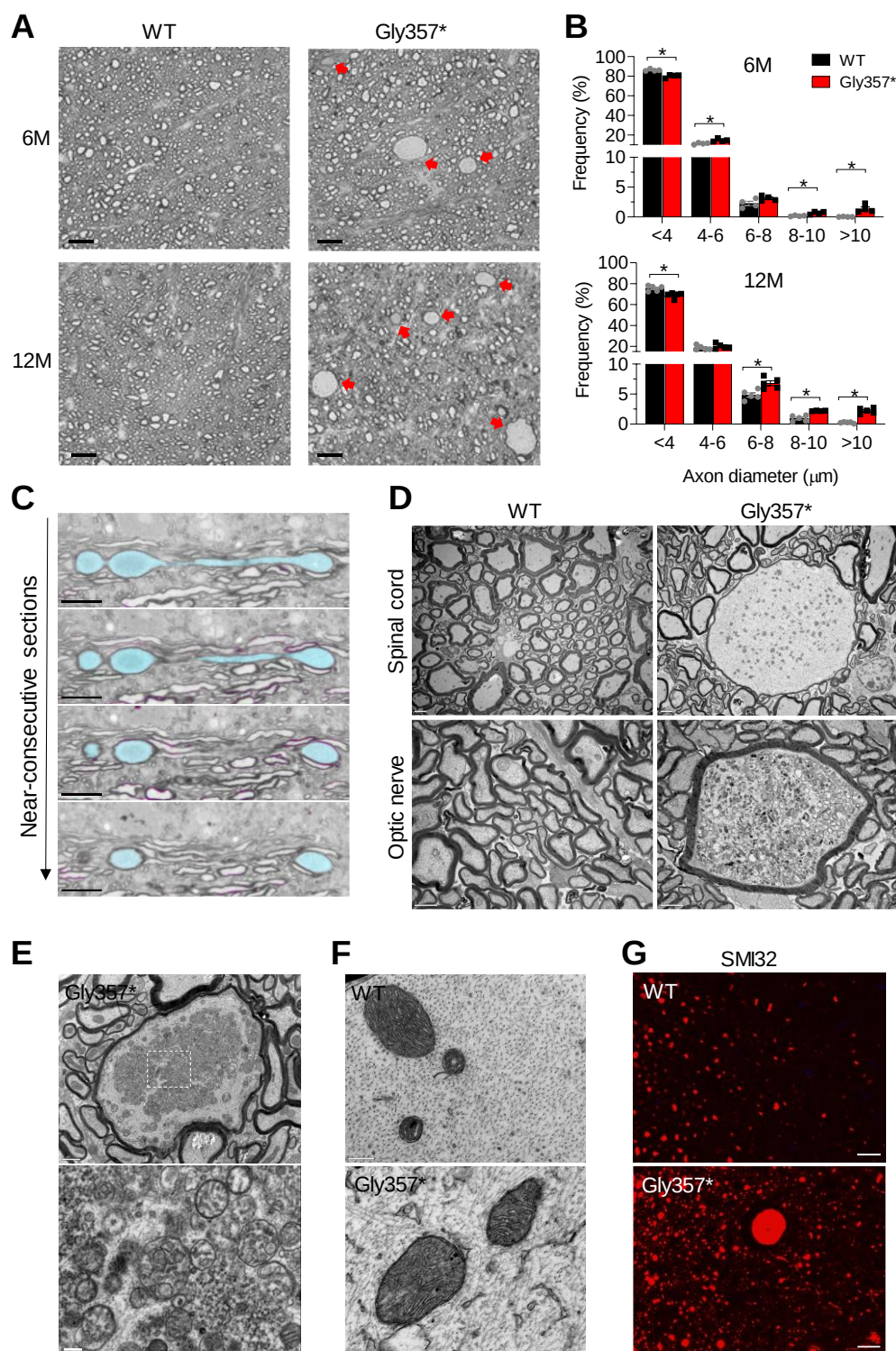


Figure 6. Axonal pathology in *Acbd5* deficiency mice. (A) PPD stained sections of WT and Gly357* spinal cord at 6 months (upper panels) and 12 months (bottom panels) revealed the presence of axonal swellings (arrows) in Gly357* mice (6M, WT n=4, Gly357*=4; 12M, WT n=5, Gly357* n=5; scale bars: 20 μm). (B) Frequency of axon diameters in spinal cords from WT and Gly357* mice at 6 and 12 months. (C) PPD stained near consecutive semi-sections of Gly357* spinal cord highlighted the presence of multiple focal axonal swellings in one axon pseudocolored blue (scale bars: 20 μm). (D) Electron microscopic analysis of WT and Gly357* spinal cord and optic nerve at 12 months of age

showing axonal swellings with an accumulation of organelles in mutant (scale bars: 2 μ m). **(E-F)** Electron microscopic analysis of spinal cord in WT and Gly357* at 12 months showing axonal defects marked by dense organelle accumulation in the swelling **(E)** and disorganized cytoskeleton **(F)** in mutant mice. Bottom panel in **(E)** is a zoom-in of dotted box in the upper panel. **(G)** Paraffin sections of WT and Gly357* spinal cord stained with anti-SMI32 (in red) at 12 months of age showed an increase in non-phosphorylated neurofilaments in mutant mice axons (12M, WT n=5, Gly357* n=5; scale bars: 20 μ m). Data are represented as mean $\pm$ SEM. * $P < 0.05$; Multiple student's t-test followed by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

Spinal cord proteomic signature mirrors cellular pathology and unravels actin defects in Gly357* mice neurons

To assist in the characterization of the pathogenesis of *Acbd5* deficiency and to identify proteins associated with the phenotypic alterations observed in our mouse model, we performed a proteomic analysis in spinal cords from WT and Gly357* mice at an early and a later stage of the disease progression. A total of 2108 and 1904 proteins were found in the proteomic analysis for 6 and 12 months of age, respectively. From those, using the p-value and fold change criteria, 52 proteins were found down-regulated and 31 up-regulated at 6 months of age; at 12 months of age 29 proteins were found down-regulated and 73 up-regulated in Gly357* mice (Figure 7A; Supplementary Table 2). Among the proteins differentially expressed, 58 proteins were found altered only at 6 months, 77 proteins only at 12 months, and 25 proteins at both ages (Figure 7B, upper panel). Using data from single-cell RNAseq databases ^{(256); (257)}, analysis of the 160 dysregulated proteins revealed that 9 were mainly expressed in neurons, 9 in astrocytes, 10 in microglia, and 14 in oligodendrocytes (Figure 7B, bottom panel), suggesting that the main cell types of the CNS are contributing for the CNS pathology in Gly357* mice. Heatmap clustering of proteomics expression data revealed that proteins commonly found at both ages displayed a higher dysregulation at 1 year of age, mirroring the progressive worsening of the pathology (Figure 7C). Independent validation using western blot of the expression levels of aldehyde dehydrogenase 1 family member L1 (*Aldh1l1*), galectin-3 (*Lgals3*), cannabinoid receptor 1 (*Cnr1*), catalase (*Cat*), dehydrogenase/Reductase 1 (*Dhrs1*), filamin2 (*Fln*), *Iba1*, *Gfap*, vimentin (*Vim*), fibromodulin (*Fmod*), crystallin alpha b (*Cryab*), and ubiquilin 2 (*Ubqln2*), confirmed the dysregulation observed on the proteomic analysis (Figure 7D and Supplementary Figure 4B). The potential altered pathways were identified by Gene Ontology analysis. Here, we observed an enrichment for different molecular functions, including extracellular matrix and actin/cytoskeleton organization (Supplementary Figure 4A). Notably, the cluster related to actin binding contained 18 dysregulated proteins, which prompted further analysis. Independent validation of the dysregulation using western blot

revealed the upregulation of alpha-actinin (Actn1), transgelin 2 (Tgln2) and transgelin 3 (Tgln3), calponin 3 (Cnn3), heat shock protein beta-1 (Hsp β 1), ezrin (Ezr) and moesin (Msn; Figure 7E). Combined, our data identified a signature of protein dysregulations that seem to delineate the pathology and highlight neurons, oligodendrocytes, astrocytes, and microglia as targets of *Acbd5* deficiency.

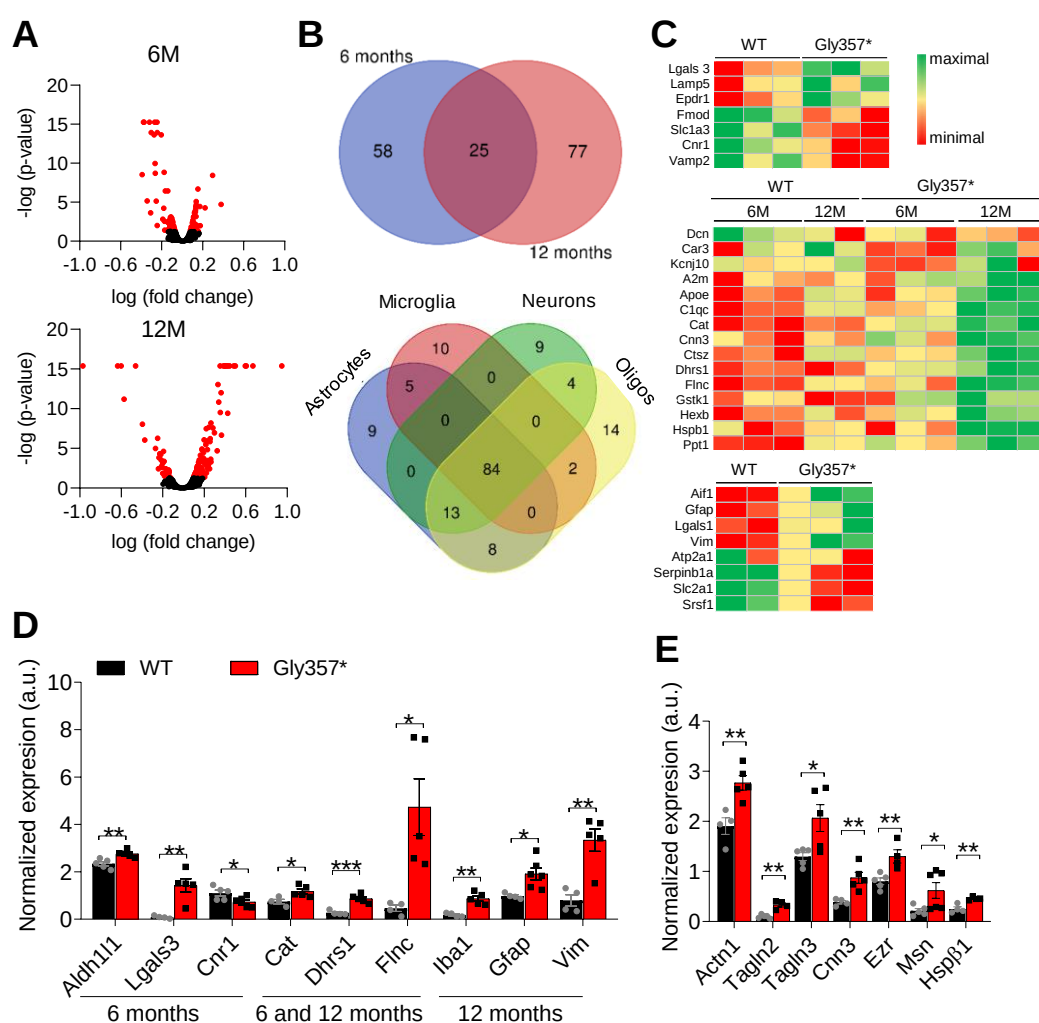


Figure 7. Proteomic profiling in *Gly357 mutant mice.** (A) Volcano plot analysis showing the differentially expressed proteins (red dots) at 6 months (upper panel) and 12 months of age (bottom panel) in *Gly357** mice. The x axis illustrates the log (fold change), and the y axis indicates as $-\log(p\text{-value})$. Up-regulated proteins have log (fold change) value > 0 and down-regulated proteins have log (fold change) value < 0 . (B) Venn diagram show the number of proteins differentially expressed at 6 months (in blue), 12 months (in pink), and at both ages (in purple; upper panel). Venn diagram showing the cell type associated to differentially expressed proteins found in proteomics (bottom panel). (C) Heat-map of selected proteins which are differentially expressed between WT and *Gly357** at 6 months (upper panel) 6 and 12 months (middle panel) and 12 months of age (bottom panel). Green colored boxes indicate high expression levels and red colored boxes indicate low expression levels. (D) Western blot quantification at 6, 6 and 12 and 12 months of age of

proteins found differentially expressed in proteomics between WT and Gly357* mice (WT n=4-5, Gly357* n=5-6). **(E)** Western Blot quantification at 12 months of age of actin related proteins found differentially expressed in proteomics between WT and Gly357* mice (WT n=4-5, Gly357* n=4-6). Data are represented as mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; unpaired parametric student's t-test.

VLCFA treatment causes alterations in the cytoskeleton and organelle dynamics in Gly357* cortical neurons

Considering that some of the identified proteins are typically expressed in neurons, we investigated how their dysregulation could cause alterations to cytoskeleton organization and dynamics.

Using an *in vitro* approach, we cultured cortical neurons from WT and Gly357* mice. To bypass the initial slow progression of the axonal pathology caused by the lack of Acbd5, neurons were exposed to methyl-ester C24:0 (meC24:0). This VLCFA promptly enters the cells and can also be used by the fatty acid elongation system to generate longer VLCFAs⁽²²³⁾, which contributes to the accumulation of VLCFA in cells with β -oxidation defects. Next, we used super-resolution microscopy and phalloidin staining to visualize the neuronal actin cytoskeleton (Figure 8A). Under control conditions, the actin cytoskeleton of Gly357* neurons had a similar structure as WT neurons. However, upon treatment with meC24:0, Gly357* neurons displayed increased actin-enriched filopodia and an abundance of actin patches (red arrows; Figure 8A, right bottom panel). Next, we investigated actin dynamics by measuring the actin retrograde flow in neurons transfected with LifeAct-GFP (Figure 8B; ⁽²⁴⁸⁾). Under control conditions, WT and Gly357* neurons had similar velocities of actin retrograde flow. However, upon exposure to meC24:0, Gly357* neurons displayed a 20% reduction in actin dynamics (Figure 8C).

The observed alterations in the axonal cytoskeleton of Gly357* mice spinal cords and the close relationship between the cytoskeleton components, i.e., actin and microtubules, also led us to analyze microtubule dynamics. We investigated microtubule dynamics by measuring the EB3 dynamics in neurons transfected with EB3-GFP (Figure 8D; ⁽²⁴⁹⁾). Under control conditions, WT and Gly357* neurons had similar microtubule growing velocities. However, upon exposure to meC24:0, Gly357* neurons showed decreased velocity (Figure 8E). This reduction seems to be related to an increased duration (i.e., EB3 comets in Gly357* neurons upon exposure to meC24:0 tend to travel for a longer period) rather than the distance traveled (Figure 8F and 8G, respectively).

The observed defect in microtubule dynamics prompted us to investigate organelle movement along MT. We investigated mitochondria dynamics by using the mitochondrial fluorescent dye MitoTracker to evaluate the organelle structure and movement. No

differences in mitochondria density (Figure 8H) and organelle area (Figure 8I) were found in neurons. However, upon exposure to meC24:0 the mitochondria circularity index was found to increase in Gly357* neurons (Figure 8J and 8L). Regarding mitochondria dynamics, WT and Gly357* neurons had similar percentages of moving mitochondria under control conditions. By contrast, upon exposure to meC24:0, Gly357* neurons displayed reduced mitochondria motility (Figure 8M). Next, we analyzed the directional mitochondria movement. No differences were found in the anterograde movement (Figure 8N). Under control conditions, WT and Gly357* neurons also had similar percentages of mitochondria moving in the retrograde direction. However, when exposed to meC24:0, Gly357* neurons displayed a reduced percentage of mitochondria moving in the retrograde direction (Figure 8O). No differences were observed in the mean velocity of mitochondria in neurons at both anterograde (data not shown) and retrograde movement directions (Figure 8P). Our results demonstrate that in neurons, VLCFA induced the reorganization of filamentous actin and defective actin dynamics, as well as defective microtubules and mitochondria dynamics.

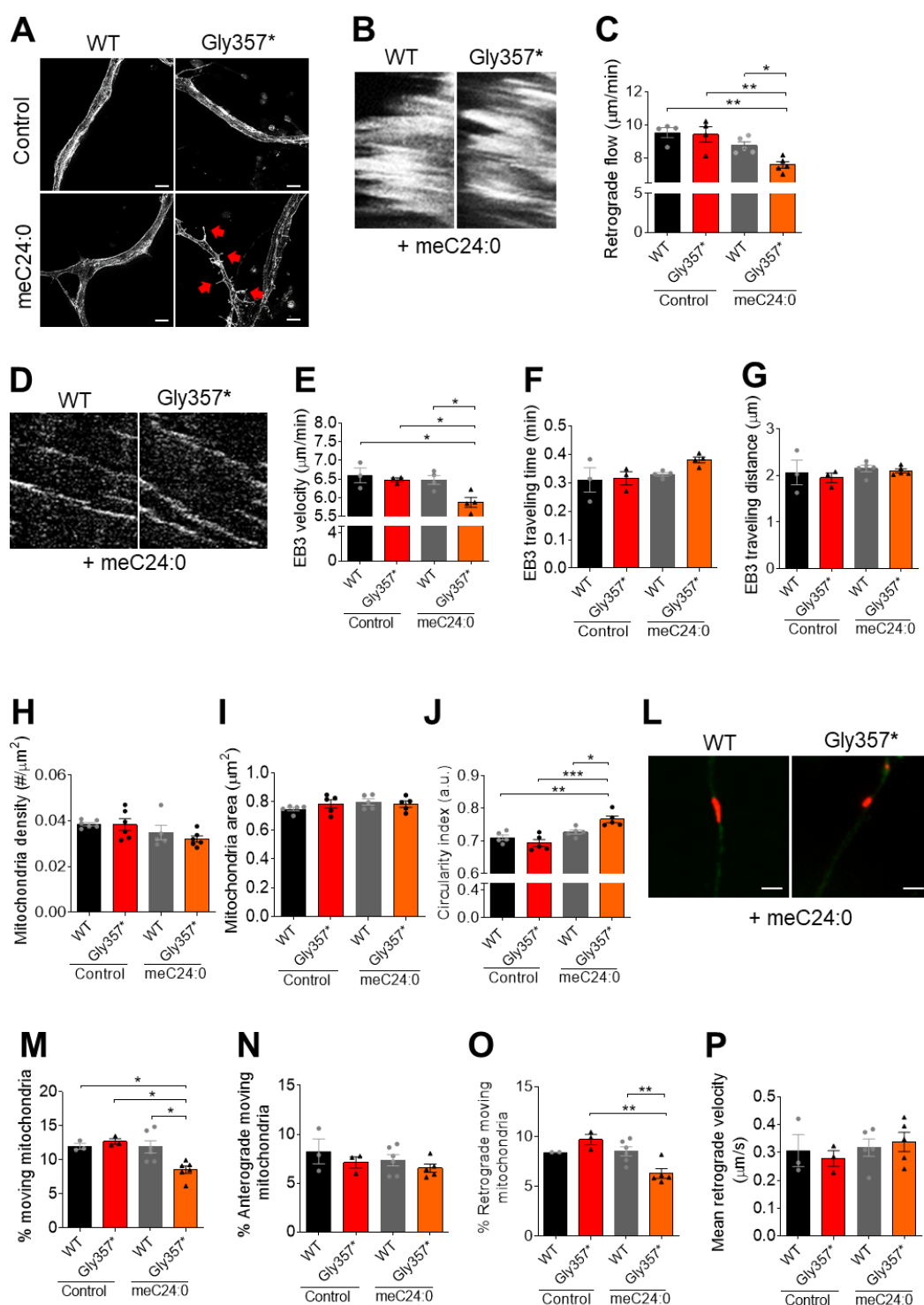


Figure 8. *In vitro* VLCFA treatment changes cytoskeleton and organelle dynamics in Gly357* cortical neurons. (A) Representative STED images from WT and Gly357* cortical neurons at DIV8 exposed to vehicle (DMSO; upper panel) or to meC24:0 (bottom panel) stained with Phalloidin 635, showing increased formation of filopodia (arrows) in the axon from mutant neurons exposed to the fatty acid (scale bars: 2μm). (B) Representative actin retrograde flow kymograph of WT and Gly357* cortical neurons exposed to meC24:0 at Div7. (C) Quantification of actin retrograde flow in the growth cone of WT and Gly357* cortical neurons exposed to vehicle (DMSO) or meC24:0 at Div7/Div8 (WT and Gly357* control n=4, WT and Gly357* treated n=5). (D) Representative EB3 dynamics kymograph of WT and Gly357* cortical neurons exposed to meC24:0 at Div7. (E-G) Quantification of

microtubule EB3 velocity **(E)**, EB3 traveling time **(F)** and EB3 traveling distance **(G)** in the axon shaft of WT and Gly357* cortical neurons exposed to vehicle (DMSO) or meC24:0 at Div7/Div8 (WT and Gly357* control n=3, WT and Gly357* treated n=4-5). **(H-J)** Quantification of mitochondria density **(H)**, mitochondria area **(I)** and mitochondria circularity index **(J)** in the axon shaft of PFA fixed WT and Gly357* cortical neurons exposed to vehicle (DMSO) or meC24:0 at Div7 (WT and Gly357* control n=5-6, WT and Gly357* treated n=5-6). **(L)** Representative image of mitochondria shape in the axon of WT and Gly357* cortical neurons exposed to meC24:0 at Div7, showing increased circularity in mutant cortical neurons (scale bars: 10µm). **(M-P)** Quantification of the percentage of moving mitochondria **(M)**, moving anterograde mitochondria **(N)**, moving retrograde mitochondria **(O)** and mean retrograde mitochondria velocity **(P)** in the axon shaft of WT and Gly357* cortical neurons exposed to vehicle (DMSO) or meC24:0 at Div11 (WT and Gly357* control n=2-3, WT and Gly357* treated n=5-6). Data are represented as mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; One-way ANOVA followed by Tukey's post hoc test.

AAV therapy reverses the axonal pathology in Gly357* mice

We reasoned that *Acbd5* gene delivery might be an appropriate approach to rescue the diverse and complex cellular defects observed in the CNS of Gly357* mice. To address this hypothesis, we designed an AAV vector with a P2A-mediated expression of green fluorescent protein (GFP) and murine *Acbd5* under the control of the CMV promoter (AAV-GFP-P2A-*Acbd5*; AAV-*Acbd5*; Supplementary Figure 5A). The control vector also expressed GFP under the CMV promoter (AAV-GFP). The functionality of the AAV-*Acbd5* vector was first assayed in transfection experiments using fibroblasts from WT and Gly357* mice (Figure 9A). *Acbd5* expression was not detected in control Gly357* cells or after the transfection with AAV-GFP. Notably, the AAV-*Acbd5* vector was able to drive the expression of *Acbd5* in Gly357* cells (Figure 9A, upper panel). Levels of GFP, the GFP-P2A fusion, and total ERK1/2 were used as controls for transfection and loading (Figure 9A, middle and bottom panels).

Using the AAV serotype PHP.B, which is capable of a highly effective CNS transduction via systemic delivery, packed AAV particles of AAV-GFP and AAV-*Acbd5* were delivered to Gly357* mice via intravenous injection at three months of age, before any visual phenotypic symptoms are present, and followed up to 6 months of age, where we analyzed gene expression and pathological phenotype (Figure 9B). The delivery of AAV-*Acbd5* was able to normalize *Acbd5* mRNA levels in Gly357* mice, whereas the control group maintained near-undetectable expression levels (Figure 9C). Histological analysis of longitudinal and cross sections of spinal cords from control-treated Gly357*-AAV-GFP mice revealed axonal disorganization and giant axonal swellings (Figure 9D). Remarkably, AAV-*Acbd5*-treated Gly357* mice showed that the therapy prevented the drastic development of the giant axonal swellings and maintained the normal organization of axons within the white matter tracts (Figure 9D, right panel). Next, we quantified the number of dystrophic axons,

i.e., axons with giant swellings or containing the accumulation of organelles (Figure 9E). After *Acbd5* gene therapy, there was a very significant reduction of dystrophic axons in Gly357*-AAV-*Acbd5* (Figure 9D and 9E), and we also observed a decreased SMI32 immunoreactivity, indicative of reduced axonal damage (Figure 9F). The evaluation of the mutant presentation was also significantly improved by the gene therapy. Notably, Gly357*-AAV-*Acbd5* presented significant improvements in limb reflexes, normal locomotion on the horizontal ladder, and normal step length and stride length (Figure 9G-I, and Supplementary Figure 5B). The histological analysis of GFP fluorescence in Gly357*-AAV-*Acbd5* mice (data not showed) also revealed that in addition to neurons, oligodendrocytes and astrocytes were also transduced. This prompted us to investigate if the AAV-*Acbd5* therapy could also have additional beneficial outcomes. The degree of myelination was improved in Gly357*-AAV-*Acbd5* mice with increased myelin thickness (Supplementary Figure 5C) and decreased g-ratio (Supplementary Figure 5D). Whereas regarding microgliosis, we did not observe any amelioration by the gene therapy, we observed an improvement in the degree of astrocytosis, with decreased Gfap immunoreactivity in Gly357*-AAV-*Acbd5* mice spinal cord (Supplementary Figure 5E-G). Overall, our results demonstrate that AAV-mediated gene therapy is able to halt the severe progression of the pathology and improve the characteristic disease presentation of Gly357* mice.

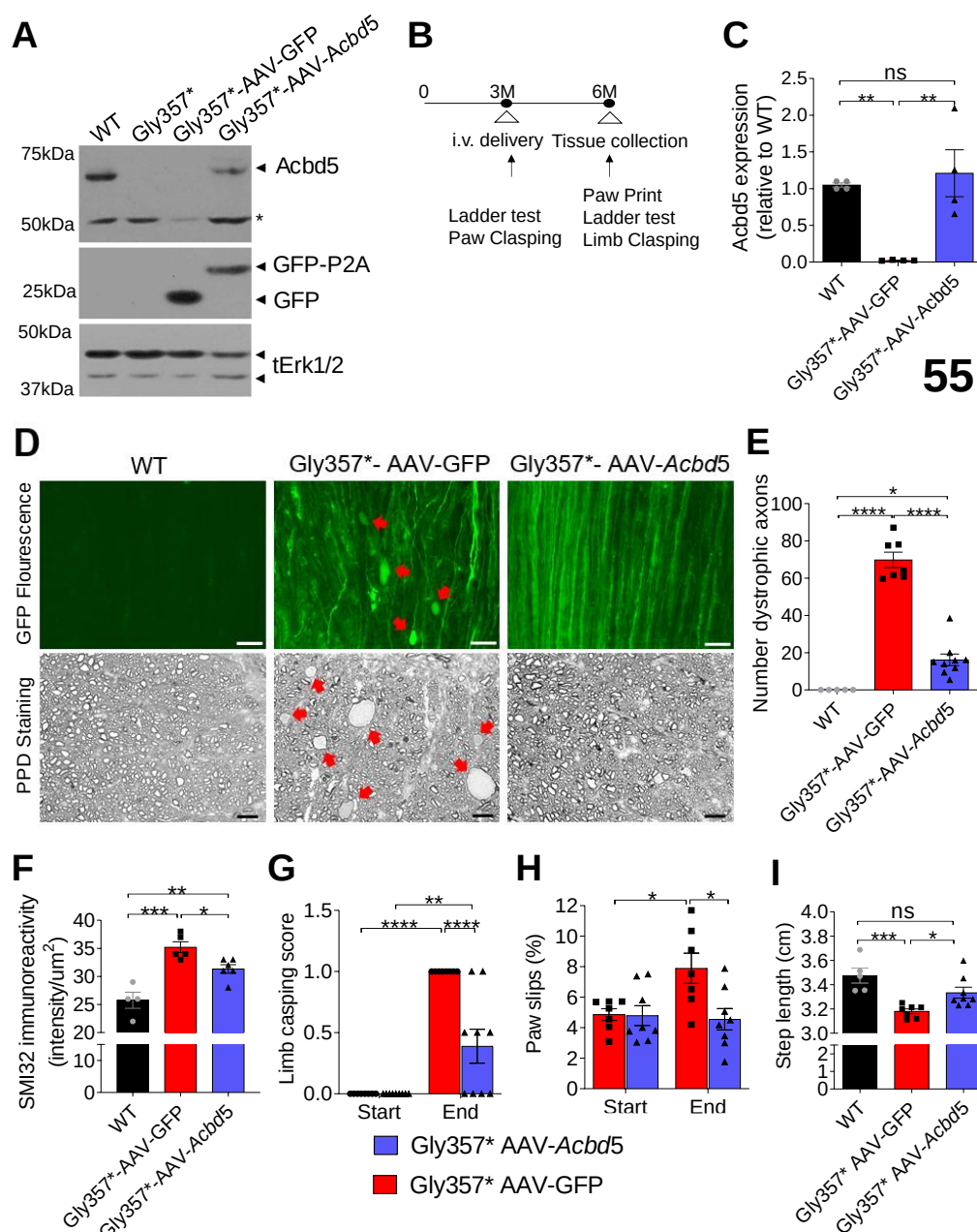


Figure 9. In vivo delivery of *Acbd5* recovers mutant mice phenotype. (A) western blot analysis of *Acbd5* and GFP in lung fibroblasts from untransfected WT and Gly357* cells and Gly357* cells transfected with AAV-GFP and AAV-*Acbd5* plasmids. Levels of tERK1/2 were used as a loading control. Asterisk indicates an unspecific cross-reacting band in *Acbd5* western blot. (B) Schematic representation of the experimental timeline and setup in the AAV-mediated gene therapy trials. (C) RT-qPCR quantification of *Acbd5* expression in brains of 6 months old untransduced WT mice, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* (WT n=4, Gly357*-AAV-GFP n=4, Gly357*-AAV-*Acbd5* n=4). (D) Representative spinal cord cryosections at 6 months of GFP-positive axons (in green) of untransduced WT and Gly357* transduced with AAV-GFP and AAV-*Acbd5* (upper panel, scale bars 50μm). Representative PPD stained spinal cord sections at 6 months of age of WT, Gly357*-AAV-

GFP, and Gly357*-AAV-*Acbd5* (bottom panel, scale bars: 20µm). Asterisks highlight dystrophic axons in each panel. **(E)** Quantification of dystrophic axons in spinal cord at 6 months of age in untransduced WT, Gly357*-AAV-GFP, and Gly357*-AAV-*Acbd5* (WT n=5, Gly357*-AAV-GFP n=7, Gly357*-AAV-*Acbd5* n=9). **(F)** Quantification of SMI32 immunoreactivity in spinal cord at 6 months of age of untransduced WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* mice (WT n=4, Gly357*-AAV-GFP n=6, Gly357*-AAV-*Acbd5* n=5). **(G)** Quantification of limb clasping at 3 months (start) and 6 months of age (end) in Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* (Gly357*-AAV-GFP n=8, Gly357*-AAV-*Acbd5* n=9). **(H)** Quantification of the percentage of paw slips at 3 months (start) and 6 months of age (end) in Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* (Gly357*-AAV-GFP n=7, Gly357*-AAV-*Acbd5* n=8). **(I)** Quantification at 6 months of age of WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* step length (WT n=5, Gly357*-AAV-GFP n=7 and Gly357*-AAV-*Acbd5* n=8). Data are represented as mean $\pm$ SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$; One-way ANOVA followed by Tukey's post hoc test (**C**, **E**, **F**, **I**); Two-way ANOVA followed by Tukey's post hoc test (**G**, **H**).

Chapter 4: Discussion

Discussion

The disruption of VLCFA metabolism and the subsequent accumulation of these fatty acids are extremely relevant to pathology in several peroxisomal disorders. A comprehensive understanding of how deficient VLCFA β -oxidation affects myelin and neuron degeneration, as well as glial activation, has been impeded by limited knowledge of the cellular mechanisms involved in the disease. In this work, the creation of a new mouse model for the disease, Gly357* mutant mice, has provided new insights into the function of the *Acbd5* gene. This work demonstrated that our mouse model develops a phenotype and neuropathology, such as in *Acbd5* deficiency, presenting retinal dystrophy, ataxia, and demyelination. We choose an engineered CRISP/Cas9 strategy to generate an exonic premature stop codon in exon 9 of the *Acbd5* gene that genetically mimics the mutations found in ACBD5 deficient patients ^{(237); (240); (241)}. Western blot analysis revealed that expression of *Acbd5* is absent in Gly357* mice, similar to what is observed with other nonsense mutations ⁽²⁴¹⁾. The lack of *Acbd5* mRNA expression in Gly357* is likely caused by active nonsense-mediated mRNA decay ⁽²⁵⁸⁾.

Studies in skin fibroblasts and plasma from *Acbd5* patients have clearly demonstrated the impact of this protein on VLCFA accumulation ⁽²⁴¹⁾. In animal models, the absence of *Acbd5* also led to the accumulation of VLCFA, as observed in the *Acbd5*^{tm1a} mouse model ⁽²⁴²⁾. We have assessed the lipidome in Gly357* and observed an accumulation of lipids enriched in VLCFA. Other lipid species not containing VLCFA were also accumulated in the spinal cords of Gly357* mice. Surprisingly, Gly357* also revealed a reduction of several species of lipids, mainly containing LCFA, which is not usually described in VLCFA accumulation-related disorders, highlighting the complexity of this dysregulation. The observed reduction in Gly357* mice may be related to the neurodegeneration and loss of myelin present in our model, which may lead to the decrease of some lipid species present in myelin. It may also be associated with an increasing replacement of LCFA by VLCFA into lipids. To prevent the effects of enriched-free VLCFA in membranes, their incorporation into phospholipids may provide alternative solutions to handle and store the excessive fatty acid content.

The major visual phenotype in Gly357* mice is the development of a severe ataxia in an early stage of the disease. Limb clasping characterizes the initial signs of impaired reflexes that progress to defects in motor coordination and locomotion, with penetrance and severity of the defects increasing with aging. The phenotype is so devastating for mutant mice that compared to *Acbd1*, *Acbd1:Acbd2*, and *Acox1* mutants, our model is the first mutant mouse with an altered lifespan. From 12 months of age onwards, the development

of tremors and hindlimb paralysis justified the establishment of a human endpoint by 14 months of age.

Our findings pinpointed an intricate conjunction of cellular alterations that may cumulatively participate in the ataxic presentation. The observed degeneration of Purkinje cells was progressive from 6 months onwards. At an early stage, our findings did not demonstrate a severe loss of these neurons; however, the neuronal defects with axonal swellings were noticeable and marked an event prior to the degeneration, and massive cell loss was observed at a later age. The presence of axonal swellings and loss of Purkinje cells was also reported in aged *Acbd5^{tm1a}* mice, which corroborates our findings and reinforces their relevance for the phenotype ⁽²⁴²⁾. Along with cerebellar degeneration, severe axonal defects accompanied by giant axonal swellings were observed in the spinal cord. Their sheer size is aggravated by their multifocal presence within a given axon. Additionally, the accumulation of organelles, neurofilaments, and cytoskeleton disorganization suggests that the axoplasmic flow is compromised, thus affecting neuronal homeostasis. Increased levels of non-phosphorylated neurofilaments, not only in axon swellings, suggest that there is neuronal damage prior to swelling formation and degeneration. Increased levels of non-phosphorylated neurofilaments have not been reported before in other impaired β -oxidation disorders, such as ALD. However, this pathologic feature is present in damaged axons in other diseases such as in Multiple Sclerosis and Charcot-Marie-Tooth diseases, and precede axonal loss and degeneration ^{(259); (260); (261)}. Axonal swellings are a feature of several neurodegenerative disorders, as observed in giant axonal neuropathy (GAN) and Charcot-Marie-Tooth disease type 2E (CMT2E) ^{(262); (263)}. In GAN, lipid alterations have been identified, including the upregulation of proteins involved in lipid metabolism and the increased presence of lipid droplets ⁽²⁶⁴⁾. It is tempting to hypothesize that the increased magnitude of axonal swellings in *Gly357** mice is partially attainable by the complex lipidic rearrangement observed. Actually, increased fluidity due to VLCFA ⁽²⁶⁵⁾, may be necessary to accommodate membrane expansion. Because of their size, VLCFA can span the lipid bilayer leaflets, which can provide increased membrane stability ⁽¹⁴⁷⁾. The accumulation of VLCFA, specifically in sphingolipids, has been reported as responsible for the formation of domains and increased stabilization of highly curved membranes or even altering the fluidity, lipid phase properties, and permeability of the membrane ⁽¹⁴⁷⁾. Nevertheless, the neuronal cytoskeleton should also provide some structural stabilization to withstand axonal integrity. Alterations in the lipid composition of the plasma membrane appear to influence the organization and dynamics of actin in *Gly357**, as evidenced in our proteomic analysis. This is reflected in increased expression levels of actin-binding proteins in the spinal cord and increased filopodia formation, along with diminished actin retrograde flow in *Gly357**

VLCFA-treated neurons. Several of these proteins are involved in actin remodeling, filament branching, formation of actin meshes and networks (e.g., Actn1, filamins), and link the actin cytoskeleton to membranes (Ezr, Msn). The complex nature of the dysregulation of actin-binding proteins was also manifested in a reduction of actin polymerization in VLCFA-treated neurons. Moreover, we observed the induced formation of filipodia in neurons from Gly357* mice, which may be a result of increased Tagl2, Tagln3, and Fmn12 ^{(266); (267)}. To date, there has been limited exploration of the association between alterations in the lipidome and changes in actin organization and dynamics. Nonetheless, some studies have highlighted this correlation. Manna and colleagues ⁽²⁶⁸⁾ demonstrated that treating *Entamoeba histolytica*, a parasitic amoebozoan, with the long-chain fatty acid C18:3 led to the disruption of the cytoskeletal network and induced F-actin polarization, thereby promoting notable filopodial extensions. Additionally, Zarrouk and colleagues ⁽²⁶⁹⁾ showed that the treatment of a human neuronal cell line with VLCFA C24:0 resulted in alterations to actin network organization and changed the interaction between actin and microtubules.

Another clear sign of the impact of Acbd5 deficiency in the CNS was the gliotic state observed from 6 months onwards. Reactive astrocytes and microglia were another feature found in the CNS of Gly357* mice. In addition to increased levels of Gfap and Iba1, the proteomic analysis also uncovered a generalized upregulation of other markers, including Aldh1l1, Dhrrs1, Lgals3, A2m, and C1qc, among others. The increased reactivity of astrocytes and microglia observed in Gly357*, and taking into account that most of the upregulated proteins found in proteomics are associated with an anti-inflammatory state, might reflect a response to the disruption of neuronal homeostasis. Alterations in astrocyte and microglia reactivity status are often associated with several CNS injuries and pathologies ^{(68); (69); (81); (85); (254)}. Actually, structural and biochemical alterations in these cells during CNS injury usually take place to minimize and repair the damaged tissue ^{(69); (85)}. Reactive gliosis can be found in many CNS diseases, but we found parallelisms between the disease markers in the spinal cords of Gly357* mice and diseases caused by VLCFA accumulation. ApoE4, a protein mainly expressed by astrocytes and microglia and a marker associated with increased severity of cerebral ALD, was found to be upregulated in Gly357* ^{(270)(270); (271)}. Hspb1 and Hspb5 were also upregulated in Gly357*, and astrocyte reactivity with upregulation of the heat shock proteins, including Hspb1 and Hspb5, was found to precede the demyelination in cerebral ALD ⁽²⁷²⁾. Moreover, microglia's response in ALD has a particular spatial and temporal progression ^{(273); (274)}. In Gly357* mice, we observed a progression from grey to white matter regions, as this phenomenon was concurrent with the onset of myelin loss. In Gly357* mice, the thinning of myelin sheaths observed by ultrastructural analysis in spinal cords and cerebellum, combined with the decreased

content of several myelin markers, denotes a relevant feature of the neuropathology. Leukodystrophy is among the main neurological defects present in patients with defective VLCFA metabolism, which include *Acbd5* deficiency and the most severe form of ALD, cALD^{(191); (198); (241)}. However, while leukodystrophy has been observed in cases of cerebral ALD (cALD), mouse models of ALD have not been able to replicate this phenotype, highlighting the importance of using Gly357* or *Acbd5*^{tm1a} mutant mice to understand how impaired VLCFA metabolism affects myelin and oligodendrocytes.

The development and efficacy of treatment options for disorders caused by VLCFA accumulation have been challenging. The field has been piloted by efforts to treat ALD using several strategies that range from metabolic modulators and antioxidants^{(206); (207); (275)} to bone marrow transplantation and gene therapy^{(216); (214); (276)}. In the last years, AAV-mediated gene delivery to CNS has been one of the most promising tools for treating neurodegenerative disorders⁽²⁷⁷⁾. AAVs are well-regarded in this field due to their safety and high transduction efficiency, and for this reason, numerous trials are currently underway, as well as others in the pipeline⁽²⁷⁸⁾. Based on this, we hypothesized that gene therapy could be an effective solution to address the cellular deficiencies caused by *Acbd5* loss. As the phenotype was present in mice at 4 months of age, we decided to start the therapy at 3 months, right before the first clinical symptoms appeared. Since we used a non-neuronal promoter (CMV promoter), there was still a possibility that other cell populations were also transduced, which would increase the possibilities for improving the neuropathology. Our findings provide the first proof of concept that gene therapy can halt or prevent the neuropathology of Gly357* mice. *In vivo* efficacy was demonstrated by the restoration of *Acbd5* expression. Efficacy was also manifested by typical axonal architecture in the spinal cord and normalization or improvement in locomotion and reflexes. The number of dystrophic axons, but also the size of axonal swellings also showed a drastic reduction. Considering the reduced size of the swellings and their frequency at 6 months of age, we hypothesize that neuronal damage began earlier, and starting the therapy at 3 months of age was not sufficient to revert the defects that were already present in neurons. In addition to the improvement seen in axonal pathology, there was also a notable enhancement in myelination and a decrease in astrocytosis. Combined, our results demonstrate that unrestricted delivery of *Acbd5* using a non-invasive approach with AAV can target the cellular defects and mitigate the severe neuropathology.

In summary, we report the generation and characterization of the *Acbd5* Gly357* mutant mice that mimic several key features of the corresponding human disease. We provide evidence that ACBD5 plays a crucial role in the maintenance of nervous tissue homeostasis. Moreover, proteomic analysis established key molecular pathways relevant

to the neuropathology, of which actin dynamics and cytoskeleton showed prominent effects on neurons. Our work has also demonstrated that by using AAV gene delivery, it is possible to recover *Acdb5* function and ameliorate the phenotype, highlighting a promising avenue for treating the disease.

Chapter 5: General Conclusions and Future Perspectives

General Conclusions and Future Perspectives

ACBD5 deficiency is the most recently identified peroxisomal disorder. With a nosologically distinct presentation, patients have been diagnosed with retinal dystrophy, leukodystrophy, and spastic paraparesis. At a biochemical level, patients revealed the accumulation of VLCFA in blood and fibroblasts.

To understand how the deficiency in ACBD5 and the consequent impaired peroxisomal functioning contributes to the pathology, the newly generated *Acbd5* Gly357* mutant mice were crucial and allowed us to reach interesting conclusions concerning the pathophysiology behind the deficiency in this protein. Our work demonstrated that our mouse model develops a phenotype and neuropathology very similar to that of the human *Acbd5* deficiency, presenting retinal dystrophy, motor impairment, and demyelination. The clearest sign of disease in our model was the progressive ataxia with impaired motor control. Histological analysis permitted us to further elucidate that mutant mice present a spinocerebellar ataxia and that loss of Purkinje cells and major axonal defects in the CNS are set to be the major contributors to the ataxic presentation. The defects comprise abnormal organelle shape and altered cytoskeletal dynamics (e.g., actin and microtubules), which we correlate with a putative alteration in the axonal transport and, consequently, accumulation of organelles and cytoskeletal components leading to the formation of gigantic axonal swellings. Our model also revealed demyelination in CNS with alterations in myelin protein content, further contributing to the motor defects observed.

Our work uncovered that the absence of ACBD5 triggers a lipidic dysregulation in CNS. We determined the lipidome in Gly357* mice and observed an accumulation of lipids enriched in VLCFA. Surprisingly, Gly357* mice also revealed a reduction of several species of lipids, mainly containing LCFA, which is not usually described in disorders that accumulate VLCFA. These results tempted us to hypothesize that the increased magnitude of axonal swellings in Gly357* mice is, in part, attainable by the complex lipidic rearrangement observed in the axonal membrane. Surprisingly, even the most gigantic swellings are myelinated, which indicates that myelin is also able to adjust to the ever-increasing radial growth. This may also suggest that the lipid composition of Gly357* myelin may have a lipid signature that confers stability and/or fluidity to accommodate the extra radial extensions.

We also aimed to evaluate a pre-clinical therapy that may be used to develop a therapy for the disorder. Using AAV gene delivery, we showed that is possible to recover *Acbd5* function and ameliorate the phenotype. Our work also suggests that this disorder is mainly caused by the disruption of the neuronal homeostasis and that by targeting this cell type, the disorder can be halted, and most of the defects observed in CNS can be reverted.

This work has provided an in-depth overview of the neuropathology and uncovered the contributors to the disease. However, some points can still be further addressed and studied. In my opinion, it would be interesting to fully analyze the lipidome in CNS to have a complete overview of the lipids altered by *Acbd5* deficiency. This would make it possible to show how the disease differs from other disorders with impaired beta-oxidation and identify possible biomarkers for early detection of the pathology. This would also elucidate us about the lipids that are incorporated in membranes and make it possible for them to exhibit the huge extension observed in the giant axonal swellings.

With a late phenotype, it would also be advantageous to properly establish an *in vitro* system to study the cellular mechanisms. This would make it possible to understand how swellings are formed and how lipidic dysregulation is changing the cytoskeleton dynamics.

Chapter 6: References

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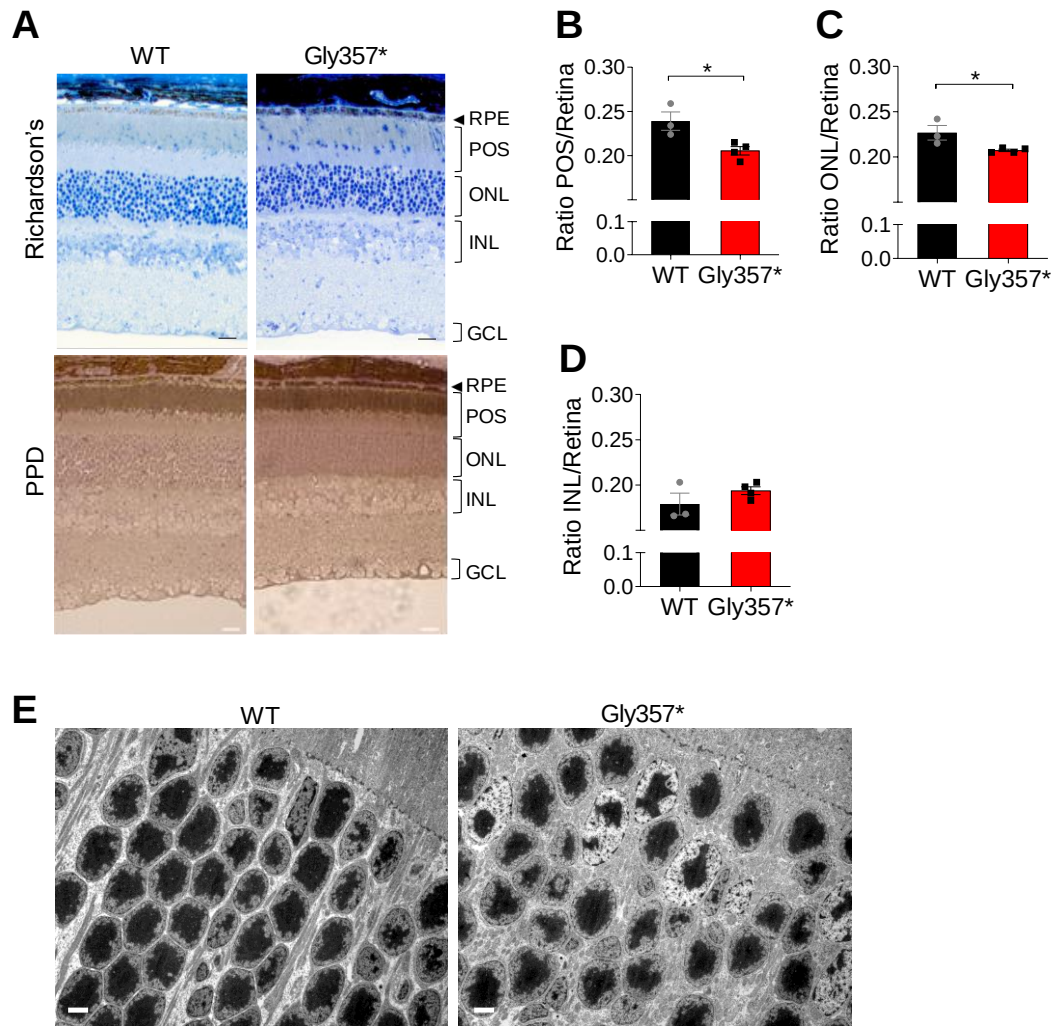
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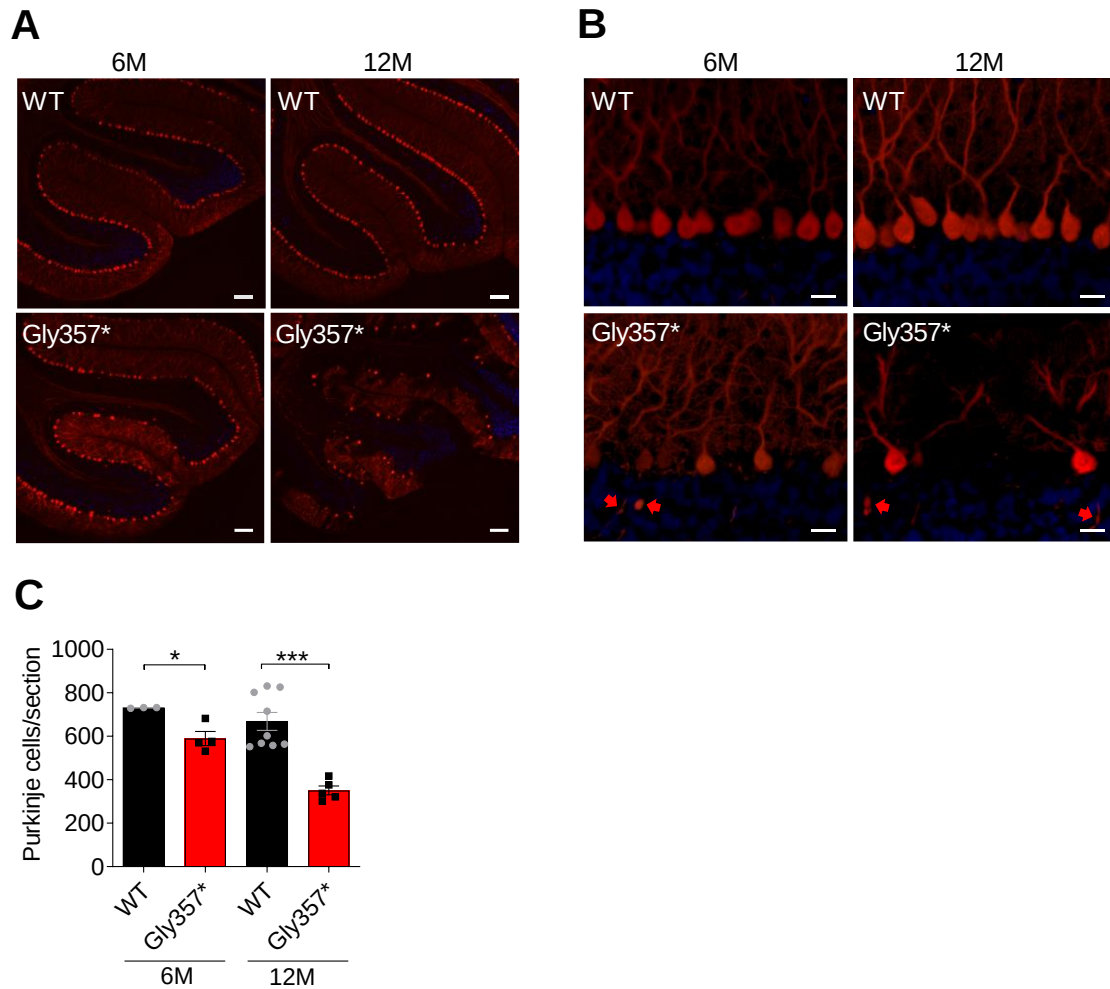
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Chapter 7: Supplementary Data

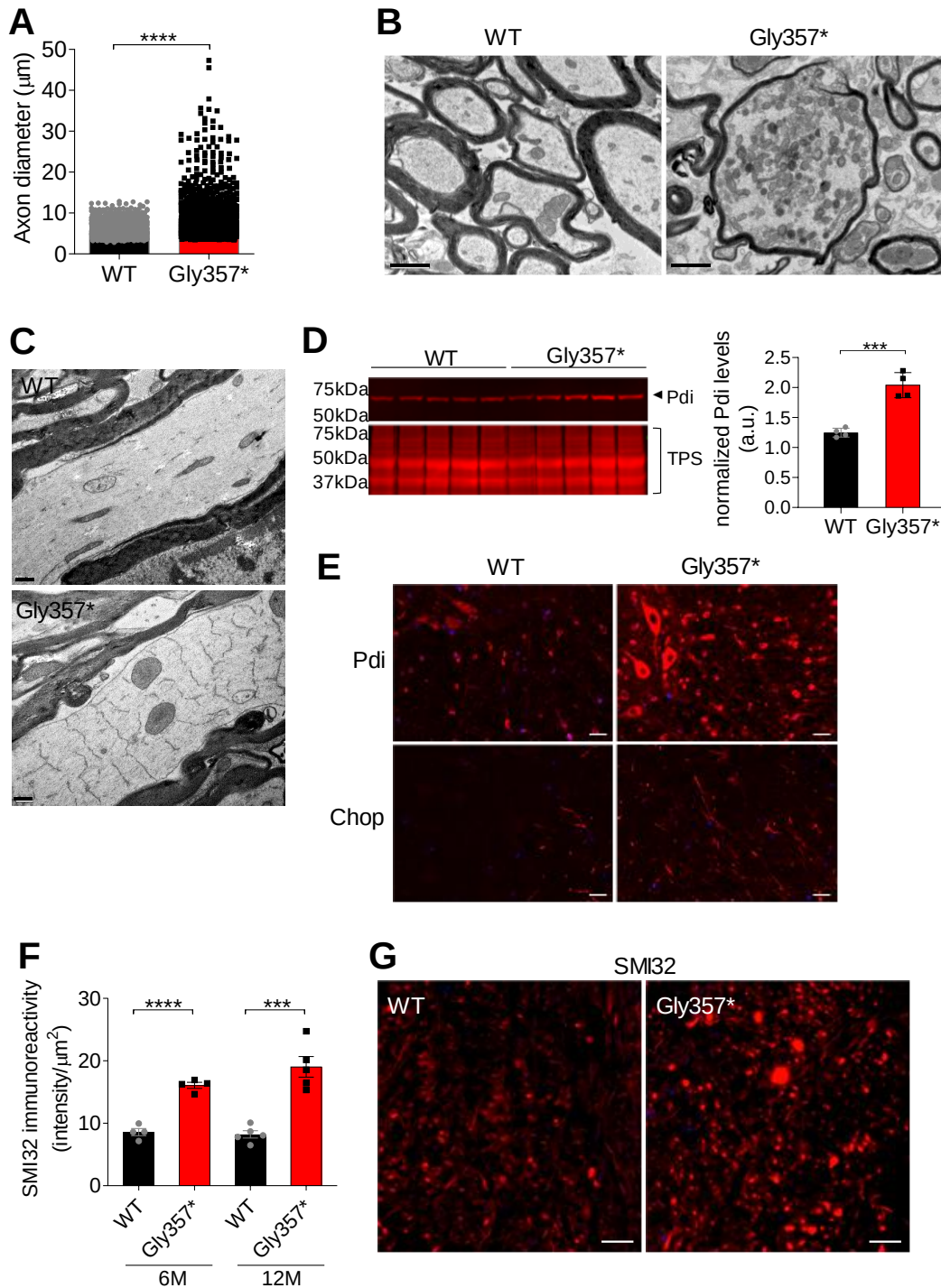
Supplementary Data



Supplementary Figure 1. *Acdb5* deficiency causes degeneration of photoreceptors in Gly357* mice. (A) Richardson's (upper panel) and PPD (bottom panel) stained retina sections from WT and Gly357* mice at 12 months of age showing shortened retina thickness in Gly357* mice (scale bars: 20 μ m). Quantification of the ratio of POS (B), ONL (C) and INL (D) normalized to retina's total thickness showed a significant reduction in POS and ONL layers in mutant mice (WT n=3, Gly357* n=4). (E) Electron microscopy images of ONL in 1-year-old WT and Gly357* mice. (scale bars: 2 μ m). RPE, Retinal pigment epithelium; POS, Photoreceptor outer segment; ONL, Outer nuclear layer; INL, Inner nuclear layer; GCL, Ganglion cell layer. Data are represented as mean $\pm$ SEM. * $P < 0.05$; unpaired parametric student's t-test.



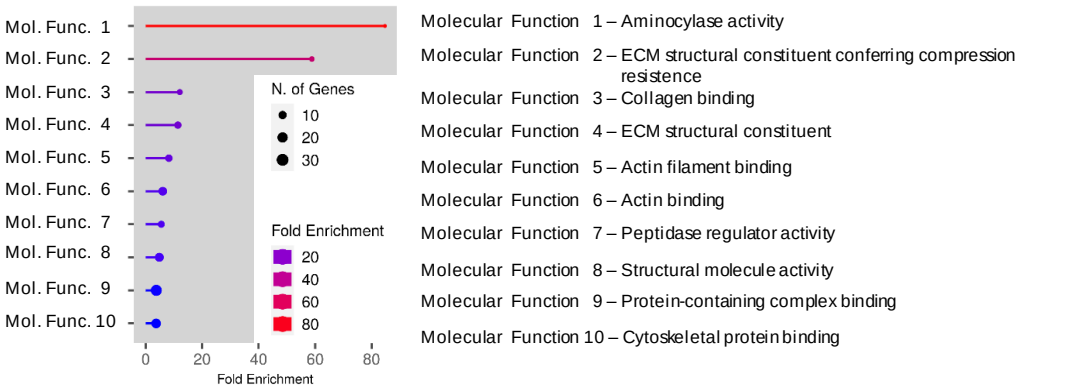
Supplementary Figure 2. Cerebellar pathology in *Acbd5* deficient mice. **(A)** Paraffin sections of WT and Gly357* cerebellum at 6 and 12 months of age stained with anti-Calbindin (in red) and DAPI (in blue) revealed a loss of Purkinje cells in mutant (scale bars: 100µm). **(B)** Zoom-in of WT and Gly357* cerebellum stained with anti-Calbindin (in red) and DAPI (in blue) at 6 and 12 months of age marked by loss of Purkinje cells and axonal swellings in mutant (scale bars: 20µm). **(C)** Quantification of the number of Purkinje cells in WT and Gly357* at 6 and 12 months (6M, WT n=3, Gly357* n= 4; 12M, WT n=9, Gly357* n=5). Data are represented as mean $\pm$ SEM. *** $P < 0.001$, * $P < 0.05$; unpaired parametric student's t-test.



Supplementary Figure 3. Loss of *Acdb5* promotes generalized axonal enlargement and alterations in the phosphorylation status of neurofilaments in CNS. (A) Quantification of the axonal diameter in WT and Gly357* spinal cord at 12 months of age (WT n=3298, Gly357* n=3539). (B) Representative electron microscopic image of WT and Gly357* cerebellum at 12 months of age showing an axonal swelling with organelle accumulation in mutant (scale bars: 1 μm). (C) Electron microscopy images of longitudinal sections of axons from the spinal cord of WT and Gly357* mice. In the mutant axons, a proliferation of ER stacks is observed in normal-looking axons and inside a giant swelling (bottom right panel). (D) Western blot analysis of Pdi expression levels in WT and Gly357* spinal cord at 12 months of age. Total Protein Staining (TPS) was used as loading control.

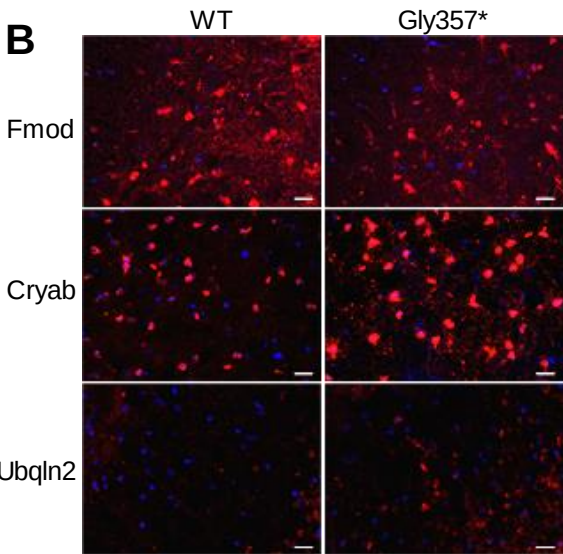
Pdi levels are increased in Gly357* (right graph; WT n=4, Gly357* n=4). **(E)** Analysis of Pdi and Chop levels (in red) in spinal cords from WT and Gly357 mice by immunofluorescence. Scale bars: 20 μ m. **(F)** Quantification of SMI32 immunoreactivity at 6 and 12 months of age in spina cord from WT and Gly357* at 6 and 12 months of age. (6M, WT n=4, Gly357* n=4; 12M, WT n=5, Gly357* n=5). **(G)** SMI32 immunoreactivity (in red) in cerebellum from WT and Gly357* at 6 months of age. Data is represented as individual points **(A)**. Data are represented as mean $\pm$ SEM **(C, D)**. **** $P < 0.0001$, *** $P < 0.001$; unpaired parametric student's t-test.

A

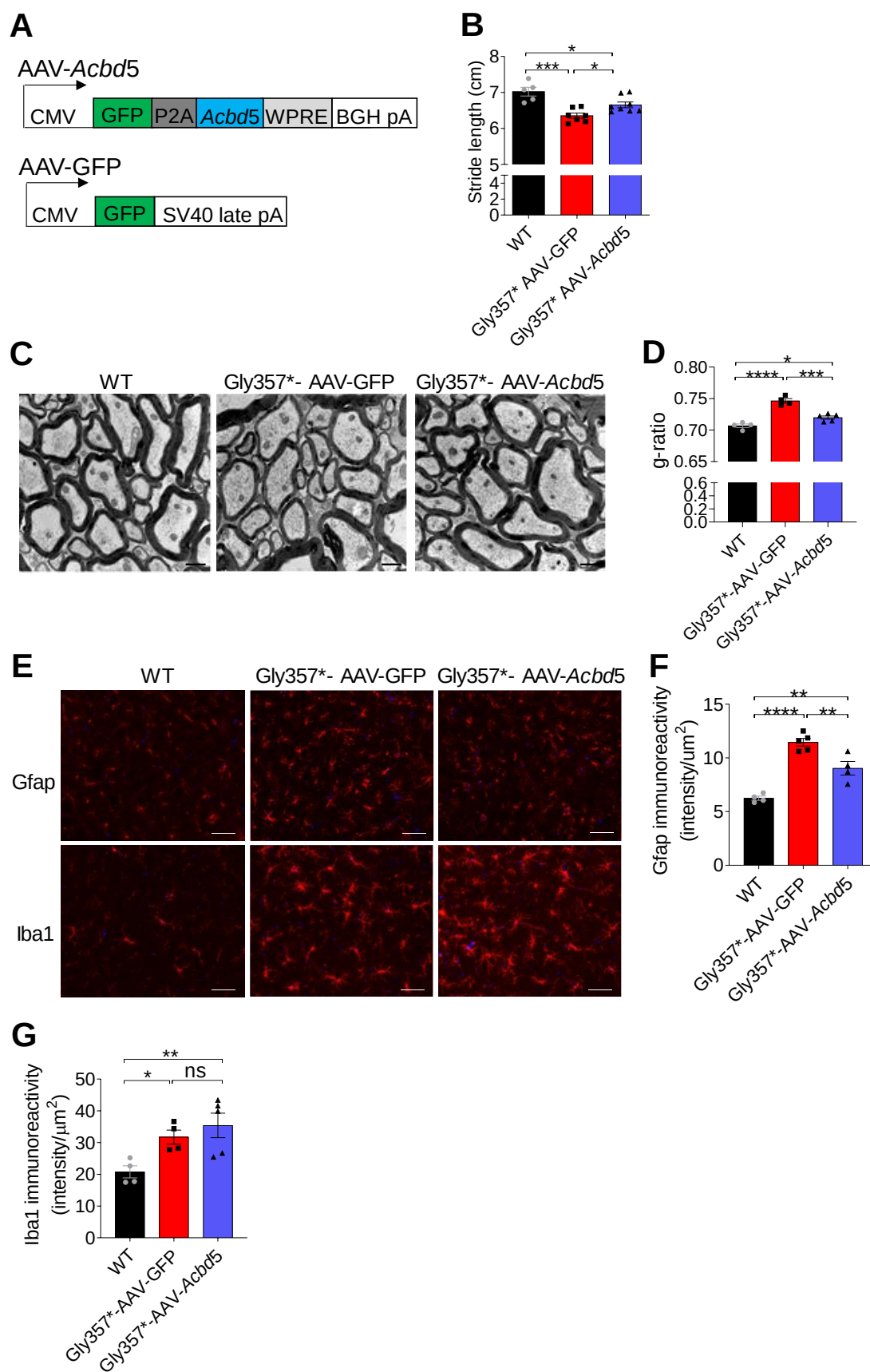


Enrichment FDR	Number of Genes	Fold Enrichment	Pathway	Gene Name
2.69x10 ⁻⁴	3	81.056	Aminoacylase activity	Aspa Acy1 Cat
1.24x10 ⁻⁶	5	56.289	ECM structural constituent conferring compression resistance	Dcn Ogn Bgn Lum Prelp
8.06x10 ⁻⁴	6	11.579	Collagen binding	Col6a1 Dcn Col6a2 Ctsb Itgb1 Lum
5.19x10 ⁻⁷	11	10.927	ECM structural constituent	Col6a1 Dcn Col6a2 Ogn Lamc1 Fbn1 Bgn Col12a1 Lum Prelp Col6a3
5.19x10 ⁻⁷	13	8.525	Actin filament binding	Actn1 Myh9 Aif1 Avil Flna Cofil1 Taglin Myo1d Fmn12 Marcks11 Ezr Capg Myh4
3.18x10 ⁻⁸	20	6.099	Actin binding	Actn1 Myh9 Aif1 Flnb Avil Itgb1 Msn Flna Cofil1 Taglin Mobp Myo1d Fmn12 Mybpc2 Marcks11 Ezr Cnn3 Capg Myh4 Flna
5.19x10 ⁻⁷	21	4.636	Structural molecule activity	Col6a1 Actn1 Dcn Col6a2 Gfap Ogn Lamc1 Vim Fbn1 Myl1f Bgn Cryab Col12a1 Mobp Lum Cldn11 Mybpc2 Prelp Col6a3 Myl1 Ahnak
6.08x10 ⁻¹²	42	3.894	Protein-containing complex binding	Col6a1 Apoe Cav1 Actn1 Fis1 Dcn Col6a2 Slc9a3r1 Vamp2 Gfap Hexb Ctsb Clu Myh9 Aif1 Avil Itgb1 Vim Fbn1 Aldh1l1 Rbm3 Flna Cofil1 Cryab Taglin Sf3b3 Atp5h Myo1d Mlc1 Fmn12 Lum Marcks11 Lgals3 Ezr Atp1f1 Capg Ide Myh4 Eno3 C4b
5.19x10 ⁻⁷	27	3.696	Cytoskeletal protein binding	Apoe Actn1 Calm3 Slc9a3r1 Vamp2 Clu Myh9 Aif1 Flnb Avil Itgb1 Msn Flna Cofil1 Cryab Taglin Mobp Myo1d Fmn12 Mybpc2 Picalm Marcks11 Ezr Cnn3 Capg Myh4 Flna
1.10x10 ⁻³	18	3.094	Protein domain specific binding	Syng3 Actn1 Srsf1 Calm3 Slc9a3r1 Myh9 Srsf7 Itgb1 Vim Arhgap1 Lamp1 Myo1d Gng12 Picalm Tceal3 Ezr Capg H4f16

B



Supplementary Figure 4. Functional characterization and validation of proteomics.
(A) Gene set enrichment analysis predicted molecular function of the differentially expressed proteins found on proteomics at 6 and 12 months of age ($P \leq 0.05$). Line color indicates the fold enrichment and dots size represent the number of genes included in each predicted molecular function (upper panel). Detailed description of the predicted molecular function analysis (bottom panel). **(B)** Paraffin sections of WT and Gly357* spinal cord stained with anti-Fibromodulin or anti-Cryab at 6 months and anti-Ubqln2 or anti-Stx18 (in red) and DAPI (in blue) at 12 months of age.



Supplementary Figure 5. *In vivo* *Acbd5* delivery ameliorates myelination and astrocytic phenotype in Gly357*. (A) Schematic representation of AAV vectors used on *in vivo* gene delivery. (B) Quantification at 6 months of age of untransduced WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* step length (WT n=5, Gly357*-AAV-GFP n=7 and Gly357*-AAV-*Acbd5* n=8). (C) Representative electron microscopic image of myelination in untransduced WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* spinal cord at 6 months of age (scale bars: 1µm). (D) Quantification of myelin in WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* spinal cord at 6 months of age by determination of g-ratio (WT n=4, Gly357*-AAV-GFP n=4 and Gly357*-AAV-*Acbd5* n=5). (E) Cryosections of untransduced WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* spinal cord stained with anti-Gfap and anti-Iba1 (in red) and DAPI (in blue) at 6 months of age (scale bars: 50µm). (F) Quantification of Gfap immunoreactivity in spinal cord at 6 months of age from untransduced WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* mice (WT n=5, Gly357*-AAV-GFP n=7, Gly357*-AAV-*Acbd5* n=9). (G) Quantification of Iba1 immunoreactivity in spinal cord at 6 months of age of WT, Gly357*-AAV-GFP and Gly357*-AAV-*Acbd5* mice (WT n=4, Gly357*-AAV-GFP n=4, Gly357*-AAV-*Acbd5* n=5). Data are represented as mean ± SEM. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$; One-way ANOVA followed by Tukey's post hoc test.