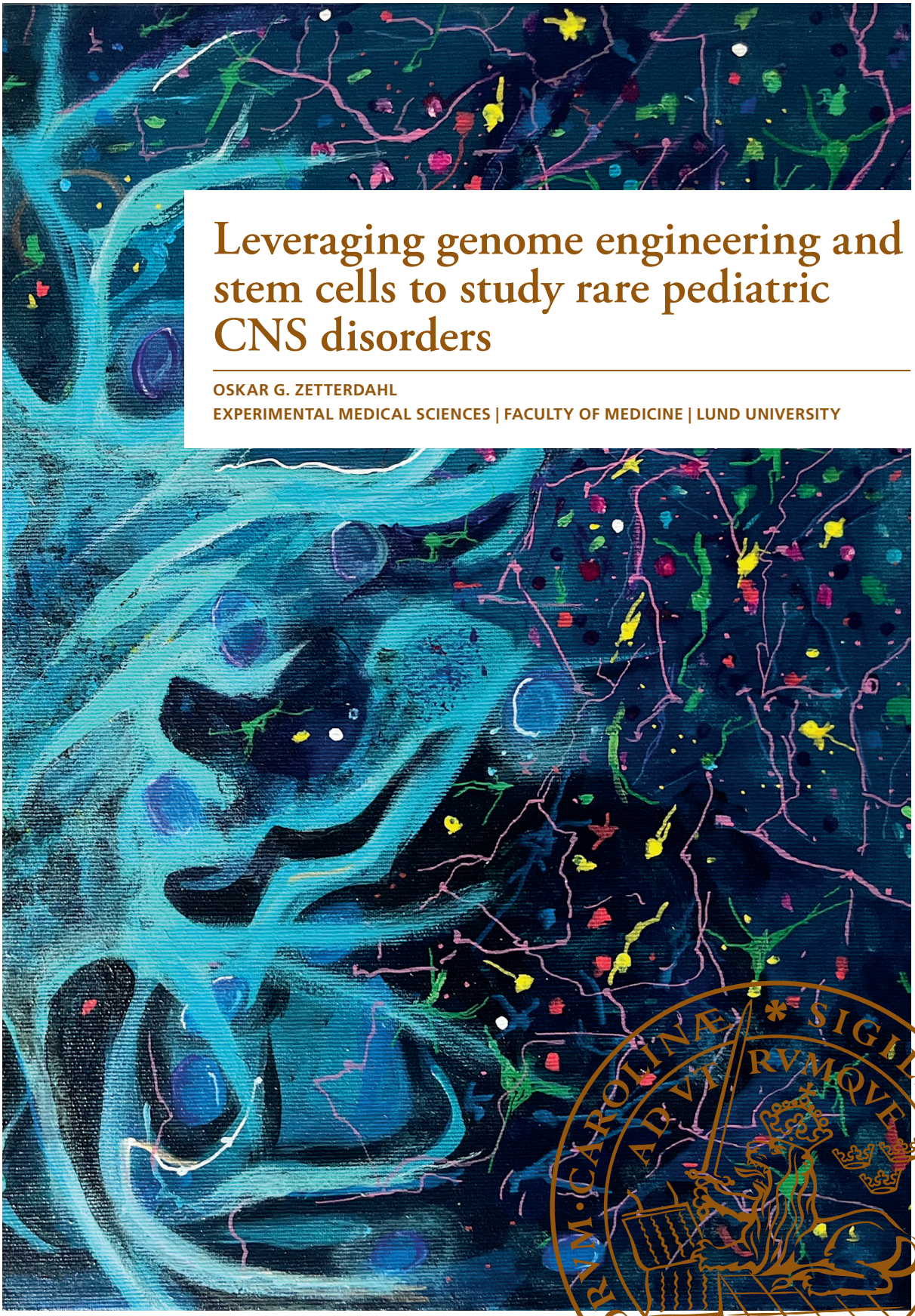
Leveraging genome engineering and stem cells to study rare pediatric CNS disorders

Zetterdahl, Oskar G.

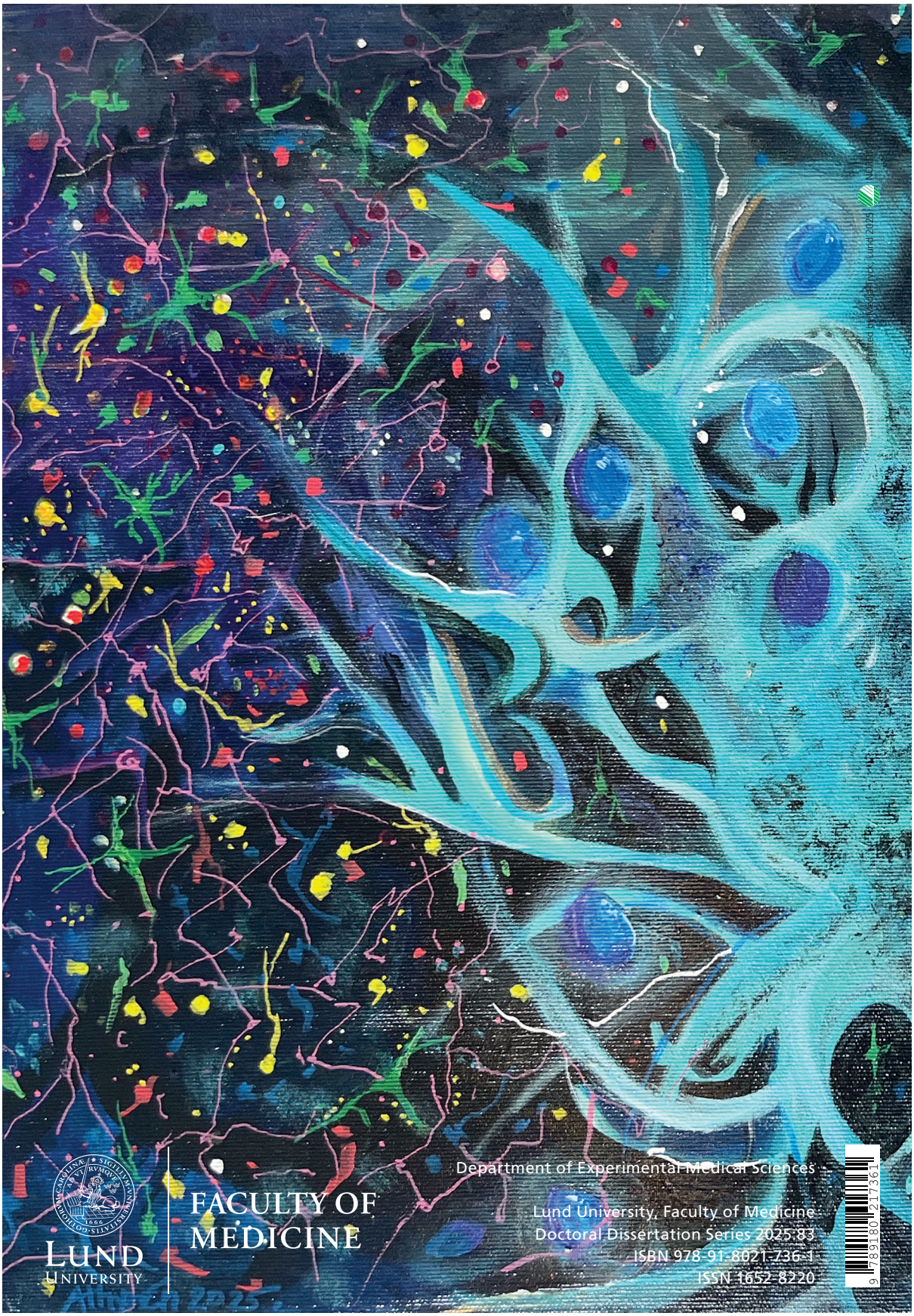
2025

Document Version:
Publisher's PDF, also known as Version of record



Leveraging genome engineering and stem cells to study rare pediatric CNS disorders

OSKAR G. ZETTERDAHL
EXPERIMENTAL MEDICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Experimental Medical Sciences

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2025:83
ISBN 978-91-8021-736-1
ISSN 1652-8220

Leveraging genome engineering and stem cells
to study rare pediatric CNS disorders

Leveraging genome engineering and stem cells to study rare pediatric CNS disorders

Oskar G. Zetterdahl

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on September 11th, 2025 at 13.00 in Segerfalksalen, Department of Experimental Medical Science, Sölvegatan 17, 223 62 Lund

Faculty opponent
Associate Professor Fredrik Sterky, University of Gothenburg

Organization: LUND UNIVERSITY

Document name: DOCTORAL DISSERTATION **Date of issue:** 2025-09-11

Author: Oskar G. Zetterdahl

Title and subtitle: Leveraging genome engineering and stem cells to study rare pediatric CNS disorders

Abstract: Rare diseases of the central nervous system (CNS) often present in early childhood, exhibit rapid progression, and result in severe neurological symptoms. Limited access to patient tissue and the lack of physiologically relevant human models have hindered efforts to investigate the underlying disease mechanisms, particularly those emerging during early neurodevelopment. Recent developments in pluripotent stem cell methodologies and genome editing techniques have enabled precise modeling of disease-relevant mechanisms in human cellular contexts.

This thesis builds upon these developments to establish in vitro models of rare pediatric CNS disorders using both patient-derived and genome-edited human pluripotent stem cells (hPSCs). These models enabled the generation of neurons and astrocytes for the analysis of cellular phenotypes, signaling dynamics, and lineage-specific vulnerabilities. Two-dimensional coculture systems enabled detailed investigation of glial-neuronal interactions and cell-type-specific dysfunction, while three-dimensional organoids introduced spatial organization and developmental complexity, recapitulating key features of brain architecture and regional identity.

Each model was applied to a distinct pathological context. Here, we present an hPSC-based model of Alexander disease, which was used to dissect GFAP-related pathology and revealed a previously undescribed defect in neural differentiation. We generated MLC1-mutated hPSC lines to enable in vitro modeling of megalencephalic leukoencephalopathy with subcortical cysts, providing a tool for studying astrocytic dysfunction in this disorder. We also developed a genome-engineered hPSC resource to visualize endogenous α-synuclein, allowing for the study of protein dynamics under physiological conditions. Furthermore, we established a novel hPSC-based organoid model of Gaucher disease, which revealed functional inhibitory deficits tied to impaired neuronal maturation.

The combined use of two-dimensional and three-dimensional systems enables investigation of early pathological mechanisms in pediatric CNS disorders. These models contribute to a deeper understanding of human neurodevelopment and support future efforts to define therapeutic targets in biologically relevant contexts.

Key words: Rare diseases, Genome engineering, Stem cells, Disease modeling, Forward Programming, Astrocytes, Organoids, Leukodystrophy, Lysosomal Storage Disease, α-synuclein

Language: English, Swedish **Number of pages**: 118

ISSN and key title: 1652-8220

ISBN: 978-91-8021-736-1

Leveraging genome engineering and stem cells to study rare pediatric CNS disorders

Oskar G. Zetterdahl

LUND
UNIVERSITY

Coverphoto by Ann Thyrén Granström

Artistic interpretation of rampant astrocytes asserting their influence on developing neurons.

Department of Experimental Medical Sciences

Lund University, Faculty of Medicine Doctoral Dissertation Series 2025:83

ISBN 1652-8220

ISSN 978-91-8021-736-1

Printed in Sweden by Media-Tryck, Lund University

Lund 2025

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

To my grandparents.
You have each played an instrumental role
in shaping this research path.

Table of Contents

Papers included in the thesis

Paper I

Zuzana Matusova, Werner Dykstra, Yolanda de Pablo, **Oskar G Zetterdahl**, Isaac Canals, Charlotte A G H van Gelder, Harmjan R Vos, Dolores Pérez-Sala, Mikael Kubista, Pavel Abaffy, Henrik Ahlenius, Lukas Valihrach, Elly M Hol and Milos Pekny.

Aberrant neurodevelopment in human iPS cell-derived models of Alexander disease

Glia, Volume 73, Issue 1, Pages 57-79, 2025.

Paper II

Oskar G. Zetterdahl, Ella Quist, Francis Paul, Isaac Canals and Henrik Ahlenius.

Generation of CRISPR/Cas9 engineered MLC1 mutated human pluripotent stem cell lines for modeling megalencephalic leukoencephalopathy with subcortical cysts

Manuscript

Paper III

Oskar G. Zetterdahl, James A. Crowe, Samira Reyhani, Miriam A. Güra, Ot Labastida-Botey, Aline Girard, D. Sean Froese, Henrik Ahlenius and Isaac Canals.

Generation of iPSC lines with tagged α-synuclein for visualization of endogenous protein in human cellular models of neurodegenerative disorders

eNeuro, Volume 12, Issue 6, 2025

Paper IV

James Crowe, **Oskar G. Zetterdahl**, Zuzana Matusova, Aline Girard, Joachim Björklund, Madalina Sacultanu, Leal Oburoglu, Ulf Ellervik, Lukas Valihrach, Henrik Ahlenius and Isaac Canals.

Patient iPSC-derived brain organoids of neuronopathic Gaucher disease identify imbalances in neural lineages and deficits in interneuron function

Manuscript

Abstract

Rare diseases of the central nervous system (CNS) often present in early childhood, exhibit rapid progression, and result in severe neurological symptoms. Limited access to patient tissue and the lack of physiologically relevant human models have hindered efforts to investigate the underlying disease mechanisms, particularly those emerging during early neurodevelopment. Recent developments in pluripotent stem cell methodologies and genome editing techniques have enabled precise modeling of disease-relevant mechanisms in human cellular contexts.

This thesis builds upon these developments to establish *in vitro* models of rare pediatric CNS disorders using both patient-derived and genome-edited human pluripotent stem cells (hPSCs). These models enabled the generation of neurons and astrocytes for the analysis of cellular phenotypes, signaling dynamics, and lineage-specific vulnerabilities. Two-dimensional coculture systems enabled detailed investigation of glial-neuronal interactions and cell-type-specific dysfunction, while three-dimensional organoids introduced spatial organization and developmental complexity, recapitulating key features of brain architecture and regional identity.

Each model was applied to a distinct pathological context. Here, we present an hPSC-based model of Alexander disease, which was used to dissect *GFAP*-related pathology and revealed a previously undescribed defect in neural differentiation. We generated *MLC1*-mutated hPSC lines to enable *in vitro* modeling of megalencephalic leukoencephalopathy with subcortical cysts, providing a tool for studying astrocytic dysfunction in this disorder. We also developed a genome-engineered hPSC resource to visualize endogenous α-synuclein, allowing for the study of protein dynamics under physiological conditions. Furthermore, we established a novel hPSC-based organoid model of Gaucher disease, which revealed functional inhibitory deficits tied to impaired neuronal maturation.

The combined use of two-dimensional and three-dimensional systems enables investigation of early pathological mechanisms in pediatric CNS disorders. These models contribute to a deeper understanding of human neurodevelopment and support future efforts to define therapeutic targets in biologically relevant contexts.

Lay Summary

Modeling the developing brain to understand rare genetic disorders

Rare childhood brain disorders often begin early in life, progress rapidly, and lead to severe neurological symptoms. Because direct study of these conditions in living patients is limited, stem cell-based models are used to recreate aspects of brain development in the laboratory and investigate how these emerge and progress.

The process begins with pluripotent stem cells, which can develop into almost any cell type in the body. These cells are either derived from early embryos or created by reprogramming adult cells, such as skin cells, back into a pluripotent state. With the help of genetic tools, a mutation linked to a specific disease can be added to healthy cells or removed from stem cells generated from patients. This makes it possible to study the effect of the individual disease-causing mutation, without influence from other genetic differences, and how it affects brain development and function.

The disorders explored in this work fall into two major categories: leukodystrophies and lysosomal storage disorders. Leukodystrophies are a group of conditions that affect the brain's white matter, which is mainly consists of myelinated nerve fibers. Damage to white matter disrupts communication between different parts of the brain and spinal cord, often leading to motor difficulties, developmental delay, and cognitive decline. Lysosomal storage disorders involve defects in cellular waste management, where substances that should be broken down instead accumulate. This buildup interferes with normal cell function and can severely impact brain development and maintenance.

Alexander disease and Megalencephalic leukoencephalopathy with subcortical cysts are examples of leukodystrophies, while Gaucher disease represents a lysosomal storage disorder. Each condition affects brain cells in distinct ways and by modeling them in the lab, it is possible to study how these changes arise during development.

Stem cells can be directed to develop into brain-specific cell types, such as neurons, which transmit signals in the brain, and astrocytes, which support and regulate neuronal function. In two-dimensional cultures, neurons and astrocytes are examined to understand changes in cell behavior, communication, and stress responses. One resource developed in this thesis enables the tracking of alpha-synuclein, a protein involved in several brain disorders, including Gaucher disease and Parkinson disease. Abnormal accumulation of alpha-synuclein is linked to neurodegeneration and impaired cellular function.

To study more complex aspects of brain structure, three-dimensional structures known as organoids are utilized. These miniature brain-like tissues mimic early stages of development and contain multiple cell types arranged in layers and regions, similar to the architecture of the human brain. Organoids make it possible to observe how brain cells move, organize, and form patterns over time, allowing for the identification of early disruptions that may contribute to disease development.

Together, these models provide a robust framework for studying rare pediatric brain disorders and contribute to a deeper understanding of how diseases begin, progress, and might be treated in the future.

Populärvetenskaplig sammanfattning

Modellering av den utvecklande hjärnan för att förstå sällsynta genetiska sjukdomar

Sällsynta hjärnsjukdomar hos barn uppstår ofta tidigt i livet, utvecklas snabbt och leder till allvarliga neurologiska symtom. Eftersom möjligheten att studera dessa sjukdomar bland patienter är begränsad, används stamcellsbaserade modeller för att återskapa hjärnans utveckling i laboratoriemiljö och undersöka hur sjukdomarna uppkommer och utvecklas.

Processen börjar med pluripotenta stamceller, som kan utvecklas till nästan vilken celltyp som helst i kroppen. Dessa celler kommer antingen från tidiga embryon eller skapas genom att omprogrammera vuxna celler, till exempel hudceller, så att de återfår sin pluripotenta egenskap. Med hjälp av genetiska verktyg kan en mutation som är kopplad till en specifik sjukdom läggas till i friska celler eller tas bort från stamceller som har skapats från patientmaterial. Detta gör det möjligt att studera effekten av just den enskilda sjukdomsorsakande mutationen, utan påverkan från andra genetiska skillnader, och hur den påverkar hjärnans utveckling och funktion.

De sjukdomar som behandlas i detta arbete tillhör två huvudgrupper: leukodystrofier och lysosomala lagringssjukdomar. Leukodystrofier är en grupp sjukdomar som påverkar den vita hjärnsubstansen, som främst består av myeliniserade nervfibrer. Skador på den vita substansen stör kommunikationen mellan olika delar av hjärnan och ryggmärgen, vilket ofta leder till motoriska svårigheter, utvecklingsförseningar och kognitiv försämring. Lysosomala lagringssjukdomar innebär att cellernas avfallshantering inte fungerar som den ska, vilket leder till att ämnen som borde brytas ner istället ansamlas. Denna ansamling stör cellernas normala funktion och kan ha stor påverkan på hjärnans utveckling och underhåll.

Alexanders sjukdom är ett exempel på en leukodystrofi, medan Gauchers sjukdom är en lysosomal lagringssjukdom. Dessa sjukdomar påverkar hjärnceller på olika sätt, och genom att modellera dem i laboratoriet går det att noggrant studera hur dessa förändringar uppstår under utvecklingen.

Stamcellerna kan styras till att utvecklas till hjärnspecifika celltyper, såsom nervceller som skickar signaler i hjärnan och astrocyter som stödjer och reglerar nervcellernas funktion. I tvådimensionella cellkulturer studeras nervceller och astrocyter för att förstå förändringar i cellernas beteende, kommunikation och stressreaktioner. Ett verktyg som utvecklats inom ramen för denna avhandling gör det möjligt att följa proteinet alfa-synuklein, som är involverat i flera hjärnsjukdomar, inklusive Gauchers sjukdom och Parkinsons sjukdom. Onormal ansamling av alfa-synuklein är kopplad till neurodegeneration och försämrad cellfunktion.

För att studera mer komplexa aspekter av hjärnans struktur används tredimensionella modeller som kallas organoider. Dessa små hjärnliknande vävnader efterliknar tidiga stadier av hjärnans utveckling och innehåller flera celltyper som är organiserade i lager och regioner, liknande den mänskliga hjärnans arkitektur. Organoider gör det möjligt att observera hur hjärnceller rör sig, organiserar sig och bildar mönster över tid. Detta hjälper till att identifiera tidiga avvikelser i dessa processer som kan bidra till sjukdomsutveckling.

Tillsammans utgör dessa modeller en kraftfull plattform för att studera sällsynta hjärnsjukdomar hos barn och bidrar till en djupare förståelse för hur de uppstår, utvecklas och i framtiden kan behandlas.

Abbreviations

2D – Two-dimensional
3D – Three-dimensional
α-syn – Alpha-synuclein
AxD – Alexander disease
BBB – Blood-brain barrier
CNS – Central nervous system
CRISPR – Clustered regularly interspaced short palindromic repeats
Cas9 – CRISPR associated protein 9
Cas9n – CRISPR associated protein 9 nickase
DEA – Differential expression analysis
DEGs – Differentially expressed genes
DSB – Double-stranded breaks
GCase – β-Glucocerebrosidase
GD – Gaucher disease
GFAP – Glial fibrillary acidic protein
hCO – Human cortical organoid
hESC – Embryonic stem cell
hiPSC – Human induced pluripotent stem cell
hPSC – Human pluripotent stem cell
hSO – Human Subpallial Organoid
iAs – Induced astrocyte
ICC – Immunocytochemistry
iN – Induced neuron
HDR – Homology directed repair
LSD – Lysosomal storage disorder
PAM – Protospacer adjacent motif
PD – Parkinson disease
RDs – Rare diseases
scRNAseq – Single-cell RNA sequencing
sgRNA – Single guide RNA
TF – Transcription factor

Glossary

Ataxia: Impaired control and coordination of voluntary movements.

Astrocytopathy: A condition involving abnormal function or pathology of astrocytes.

Astrogliosis: : A reactive process in which astrocytes undergo structural and functional changes in response to central nervous system injury or disease.

Bulbar symptoms: Impairments in speech, swallowing, and tongue movement resulting from brainstem pathology.

***De novo**:* In context of this thesis, it refers to a genetic mutation that arises spontaneously, rather than being inherited.

Demyelinating: Pathological loss of myelin sheaths that normally insulate nerve fibers.

Dysmeylinating: Pathological failure to form normal myelin sheaths that insulate nerve fibers.

Leukodystrophy: A group of inherited disorders involving degeneration of brain white matter due to myelin defects.

Hyperreflexia: Exaggerated reflex responses due to disruption of descending inhibitory pathways.

Hypertrophy: A cellular process involving increased cell volume through expansion of cytoplasmic components such as organelles and structural proteins, without an increase in cell number.

Macrocephaly: : A condition characterized by an abnormally large head circumference relative to age and sex.

Microcephaly: A condition characterized by an abnormally small head circumference relative to age and sex.

Palatal myoclonus: A movement disorder marked by involuntary, rhythmic contractions of the soft palate muscles, sometimes producing audible clicks.

Spastic paraparesis: A motor disorder involving partial weakness and stiffness in both legs due to central nervous system lesions.

Spasticity: A motor disorder involving increased muscle tone and exaggerated reflexes, leading to stiffness and reduced voluntary movement due to impaired inhibitory control.

Introduction

Rare diseases: a global health challenge

Rare diseases (RDs) comprise a heterogeneous group of over 9000 medical conditions that individually affect a small percentage of the population but collectively impact more than 400 million people worldwide (Ferreira, 2019; Kernohan & Boycott, 2024). Despite their diversity, most RDs are chronic, progressive, and frequently life-limiting, with very few effective or disease-modifying treatments (Kaufmann et al., 2018; Lopes-Júnior et al., 2022). Their low prevalence contributes to delayed diagnoses, limited clinical awareness and research expertise, thereby placing a significant burden on patients and their families (Marwaha et al., 2022; Wright et al., 2018).

There is no single global definition of a rare disease; instead, definitions are determined by regional legislation. In the European Union, a disorder is considered rare if it affects fewer than 1 in 2000 individuals (Regulation (EC) No 141/2000). In the United States, the Orphan Drug Act defines rare diseases as those affecting fewer than 200,000 individuals nationwide, which today constitutes roughly 1 in 1700 citizens (Nguengang Wakap et al., 2020). These definitions have enabled frameworks for orphan drug designation, encouraging the development of therapeutics for otherwise neglected diseases.

Although many RDs are caused by mutations in a single gene, they represent a clinically and genetically diverse group. This heterogeneity often results in variable disease progression, symptom severity and age of onset even among individuals with the same genetic diagnosis. In many cases, these disorders share overlapping features with more common conditions, which can hinder and delay proper diagnosis. A significant proportion of RDs begin in early childhood and follow a chronic, progressive course. This highlights the need for early and accurate diagnosis to ensure patients receive appropriate care and, when possible, access to targeted therapies that may alter the course of the disease (Kernohan & Boycott, 2024; Taruscio & Gahl, 2024). Small patient populations and limited access to dedicated research funding significantly constrain research into rare diseases. These factors make it difficult to identify novel therapeutic targets and conduct sufficiently powered clinical trials. As a result, therapeutic options remain scarce, with fewer than 5% of RDs having any effective treatment, and even fewer with any disease-modifying effect (Kaufmann et al., 2018; Lopes-Júnior et al., 2022).

Rare diseases and the central nervous system

More than half of known rare diseases exhibit central nervous system (CNS) involvement, frequently with neurodevelopmental or neurodegenerative features. Access to disease-relevant tissue, particularly during early developmental stages, is extremely limited due to the invasive nature of biopsies in the CNS, making it challenging to study disease mechanisms in a physiologically relevant context. Consequently, there is an urgent need for robust disease-relevant models of RDs (Freel et al., 2020; Lee, 2024; Reinhard et al., 2021).

Historically, rodent models have served as the primary system for investigating rare CNS disorders. Genome editing has expanded the utility of these models by enabling the introduction of patient-specific mutations into transgenic animals, allowing for interrogation of genotype-phenotype relationships, immune and behavioral responses, and disease progression in a controlled genetic environment. However, fundamental interspecies differences constrain the translational relevance of these systems. Key differences in cellular complexity, cell type diversity, and neurodevelopmental timing limit the extent to which rodent models can recapitulate CNS pathology of the human brain. For example, a rodent astrocyte is many times smaller and far less complex than its human counterpart (Oberheim et al., 2009), and the neuronal subtype diversity differs between mice and men (Luo et al., 2017). Moreover, many disease models depend on artificially induced pathology, which may limit their ability to recapitulate human disease mechanisms accurately. Consequently, therapeutic candidates for CNS disorders that show promise in animal models frequently fail in human trials (Eaton & Wishart, 2017; Geerts, 2009; Jucker, 2010; Vaquer et al., 2013).

In recent years, changes in regulatory policy coupled with growing attention to the 3R principles of ethical animal use (Replacement, Reduction, Refinement) have contributed to the development of alternative research platforms that more accurately reflect human physiology. The European Union continues to uphold its firm policy commitment to the 3R principles through Directive 2010/63/EU, as well as more recent strategic initiatives aimed at reducing and phasing out animal testing. The EU-Innovation Network Horizon Scanning Report represents a key development in this effort, which outlines priorities for integrating New Approach Methodologies into the regulatory framework. Among these methodologies, human induced pluripotent stem cell (hiPSC)-derived models are increasingly recognized for their potential to improve the translational relevance of drug development. In the United States, recent regulatory reforms such as the FDA Modernization Act 2.0 have supported the transition away from animal testing in preclinical research. This legislation removes the requirement of animal studies for specific drug approval pathways, promotes the use of human *in vitro* and computational models, and supports expedited review processes for data generated using validated human-specific systems. This regulatory acceleration is mirrored by a growing shift within

the pharmaceutical industry towards more predictive and human-relevant research models. Drug developers and contract research organizations are increasingly integrating platforms such as hiPSC-based systems and computational approaches to improve translational accuracy, shorten development timelines, and reduce clinical trial failure rates. These strategies are particularly relevant for neurological disorders, as complex disease mechanisms and limited success in late-stage therapeutic development continue to impede progress (Luce & Duclos-Vallee, 2025).

Within this evolving research landscape, hiPSC-derived models offer a promising framework for investigating rare CNS disorders. These systems enable the derivation of region-specific cell types that are relevant to early-stage disease pathology. By integrating tools such as genome editing, multicellular cocultures, and organoids, hiPSC technologies support the reconstruction of human neural circuits in a controlled and genetically tailored context. Collectively, these innovations offer a scalable and ethically favorable solution with increased biological relevance for investigating disease mechanisms and advancing translational research of rare neurological disorders.

Pluripotent stem cells and genome editing

Human pluripotent stem cells (hPSCs), which encompass both human embryonic stem cells (hESCs) and induced pluripotent stem cells, are cells that, in theory, have unlimited capacity for self-renewal and can be differentiated into virtually any somatic cell type. The fundamental difference between hESCs and hiPSCs lies in their cellular origin and method of derivation. Embryonic stem cells, like their name implies, are derived from blastocyst-stage embryos (5-6 days post-fertilization), typically from surplus *in vitro* fertilization embryos donated to research following informed consent (A. E. Chen et al., 2009). hiPSCs, on the other hand, are derived from somatic cells, such as fibroblasts or blood cells, that have been reprogrammed to reacquire a pluripotent state through exposure to key transcription factors (Takahashi et al., 2007). This process requires only minimally invasive sampling procedures and enables the generation of patient-specific *in vitro* models that recapitulate key disease phenotypes.

Advances in induced pluripotent stem cell (iPSC) technology, together with genome editing methods such as CRISPR/Cas9, have significantly expanded the experimental toolkit available for investigating rare central nervous system disorders. Leveraging these complementary approaches allows for the generation of disease-specific iPSC lines and isogenic controls, ensuring that any observed differences is directly linked to the mutation of interest, and thereby enabling the investigation of mutation-specific phenotypes.

Conventional genome engineering relies on programmable nucleases to induce double-stranded breaks (DSBs) in DNA, thereby activating the cell's endogenous repair machinery. DNA breaks can be repaired via non-homologous end joining, which directly ligates the broken ends and often disrupts gene function, or through homology-directed repair (HDR), which uses a homologous sequence to restore the targeted region. Although HDR typically utilizes the sister chromatid as a template, the repair process can be redirected by introducing an exogenous donor sequence to enable precise mutation correction or insertion. This approach also permits the integration of larger genetic cassettes, allowing the generation of reporter lines for tracking gene expression, cellular identity, or lineage commitment (Chandrasegaran & Carroll, 2016).

The CRISPR/Cas9 system, which is the most widely used genome editing technology today, is adapted from a bacterial immune response against bacteriophages. The technique relies on RNA-guided Cas9 nucleases to introduce DNA breaks at a specific genomic sequence, which can be readily programmed by designing 20-nucleotide (nt) single-guide RNA (sgRNA) complementary to the target site (Fig. 1). This sgRNA must be positioned adjacent to the protospacer adjacent motif (PAM) sequence, typically NGG, and is cloned into the Cas9 vector prior to transfection into the cells of interest (Hsu et al., 2013). To improve target specificity and minimize off-target cleavage, a modified form of the enzyme, Cas9 nickase, can be employed. The nickase variant contains a point mutation to one of the nuclease domains, disabling its function, resulting in single-strand cleavage activity known as nicking. When two sgRNAs guide Cas9 nickases to opposite DNA strands, they generate a staggered double-strand break at the target site, offering greater precision and minimizing unintended genomic disruption compared to the wild-type Cas9 enzyme. However, this comes at the expense of efficiency and may be limited by a lack of nearby PAM sites (Ran et al., 2013).

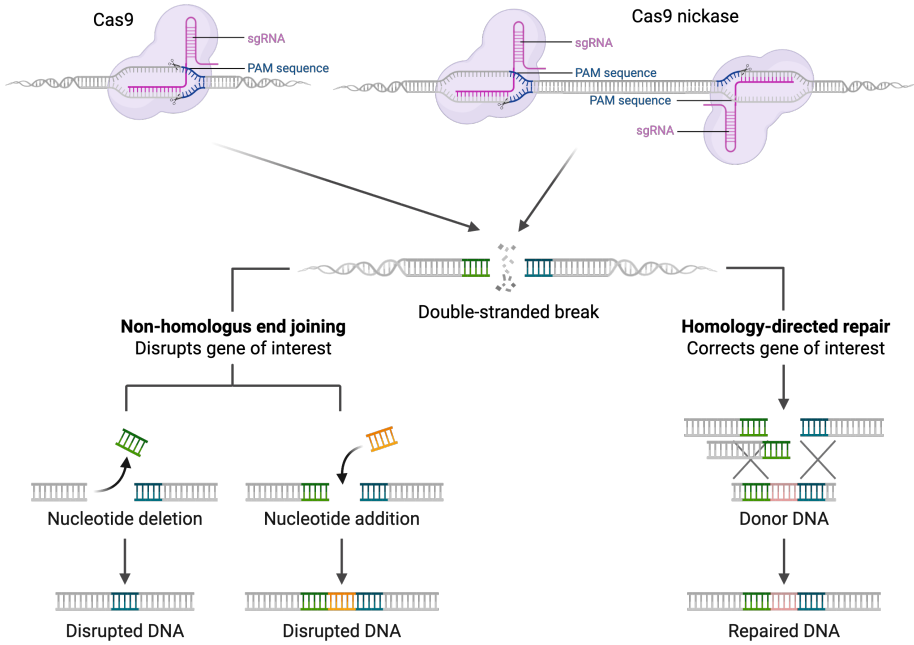


Figure 1: Genome editing using CRISPR/Cas9.
Schematic overview of CRISPR-based editing mechanisms. CRISPR/Cas9 and Cas9 nickase are guided by sgRNAs to specific genomic sites, where they induce double-stranded DNA breaks. Repair via non-homologous end joining can disrupt gene function through insertions or deletions and is commonly used to generate gene knockouts. In contrast, homology-directed repair (HDR) with a donor template enables precise editing of the genome. Created with BioRender.com.

Prime editing is a more recent development that builds upon the CRISPR platform, enabling small but very precise edits, like the introduction of point mutations, small insertions or deletions, without the need for double-stranded DNA breaks. This reduces the risk of undesired edits or chromosomal rearrangements that are common with a traditional CRISPR approach, while also improving the editing fidelity. This genome editing technique operates using a system of three separate components: a Cas9 nickase fused together with an engineered reverse transcriptase, a prime editing guide RNA (pegRNA) that guides the complex to the target site and provides a template for the edit, and an optional, secondary nicking sgRNA (ngRNA) that enhances editing efficiency. The reverse transcriptase uses the RNA-encoded template to synthesize the edited sequence at the target locus, enabling precise changes without the need for DSBs (Anzalone et al., 2019).

However, prime editing is a more technically challenging technique to implement, it comes with a significantly reduced editing efficiency, limitations to the size of the edit and overall versatility in its current form when compared to CRISPR/Cas9. Thus, CRISPR remains the go-to choice for any large-scale genetic manipulations, but prime editing holds high promise for therapeutic contexts where high precision is essential.

In vitro modeling strategies for rare CNS disorders

Pluripotent stem cell-based systems now enable a versatile platform for modeling rare CNS disorders using disease-relevant cells (Fig. 2A and 2B). These models provide a reproducible *in vitro* environment for studying early cellular development and disease progression, offering insights into the underlying mechanisms of disease. This enables biomarker identification, toxicological assessment, and preclinical drug screening, allowing for the early detection of adverse effects prior to first-in-human trials and supporting therapeutic advancement (Anderson & Francis, 2018; Haggarty et al., 2016).

Compared to traditional approaches relying on animal models or peripheral tissues, hPSC-based platforms offer superior physiological relevance. For example, Sanfillipo patient-derived hiPSC-based neurons did not respond to an siRNA-based treatment previously found effective in patient fibroblasts, underscoring the importance of using CNS-relevant cell types for evaluating therapeutic efficacy (Benetó et al., 2020). Furthermore, rare CNS disease models offer mechanistic insights that extend beyond their immediate context, as impairments in astrocyte biology, lysosomal function, and mitochondrial metabolism are increasingly recognized in both rare and common neurodegenerative conditions (A. Sosunov et al., 2018; Hertz et al., 2024).

Following reprogramming, hPSC lines can be differentiated into two-dimensional (2D) neural lineages using various protocols. Chemical induction protocols direct hiPSC differentiation by modulating intracellular signaling with small molecules. These approaches yield neurons with varying degrees of molecular identity and functional maturity, depending on the cell line, chemical composition, and culture setup (Telias, 2023). In contrast, forward programming accelerates differentiation by overexpressing lineage-specific transcription factors that drive hPSCs towards mature neural fates. This strategy enables rapid and scalable production of pure cell populations, such as neurons and astrocytes, in a shorter timeline than many developmental cue-based protocols (Zhang et al., 2013; Yang et al., 2017; Canals et al., 2018).

While forward programming is well-suited for targeted applications that require rapid and efficient generation of mature neurons and astrocytes, it also presents certain trade-offs. By bypassing intermediate developmental stages, these methods limit the model's utility for studying early disease mechanisms or developmental pathogenesis. Forced transcription factor (TF) overexpression can also disrupt intrinsic gene expression, potentially masking subtle disease phenotypes. Furthermore, integrating lentiviral vectors poses risks of insertional mutagenesis and compromises long-term genomic integrity, thereby restricting their applicability in translational and therapeutic contexts (Pauwels et al., 2009).

An alternative to hPSC-based differentiation is the direct conversion of fibroblasts into neural cell types through TF-mediated forward reprogramming. In contrast to hiPSC-based methods, this approach has been shown to preserve donor-specific, age-dependent transcriptomic signatures, enabling more physiologically relevant modeling of age-associated phenotypes, particularly valuable for investigating late-onset CNS disorders. However, this method presents several limitations. Direct conversion is limited by low reprogramming efficiency, restricted passage potential of fibroblasts, and the difficulty of generating isogenic controls, which limits scalability and complicates mechanistic studies (Mertens et al., 2015; Chanoumidou et al., 2021; Quist et al., 2022).

Organoid approaches take neural differentiation one step further by introducing a third spatial dimension, allowing hPSCs to undergo stage-specific signaling and self-organize into three-dimensional (3D) structures that model aspects of human brain development. These models recapitulate key features of spatial architecture and intercellular signaling present during brain development, thereby enabling the investigation of neurodevelopmental mechanisms and structural impairments linked to rare CNS disorders. Nonetheless, these systems present specific experimental and technical challenges. Prolonged culture durations are typically needed to generate specific cell types of interest for RDs, such as astrocytes and oligodendrocytes (Lancaster et al., 2013; Mayhew & Singhania, 2022). Organoid models can display considerable batch-to-batch variability in size, cellular composition, and tissue architecture, which poses challenges for reproducibility and data interpretation. Together with the technical complexity, this variability presents a barrier to standardization and limits the scalability of organoid platforms across research settings.

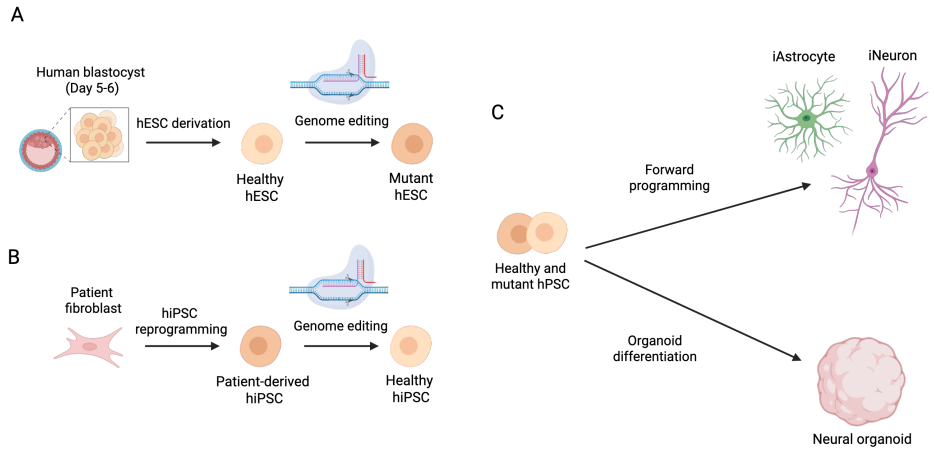


Figure 2: Schematic of two alternative hPSC-based approaches for CNS disease modeling using isogenic cell lines.
(A) Healthy hESCs derived from embryos are genetically modified using genome editing to introduce disease-causing mutations, generating mutant hESC lines. (B) Patient-derived iPSCs are generated by iPSC reprogramming of somatic cells, such as fibroblasts. Genome editing is applied to correct the disease-causing mutation, generating isogenic healthy iPSC lines. (C) Healthy and mutant hPSC lines can be differentiated by forward programming to generate 2D cultures of induced neurons and astrocytes, and by 3D differentation protocols to generate neural organoids. Created with BioRender.com.

Given the respective advantages and limitations of available differentiation methods, strategic selection or integration of complementary approaches is essential for the development of *in vitro* models of rare CNS disorders (Fig. 3C). While rapid, lineage-specific forward programming provides an efficient and scalable means of producing mature cell types, organoid-based approaches provide the spatial architecture and multicellular interactions needed to model early developmental processes. Therefore, the integration of 2D and 3D culture systems offer a more comprehensive framework for modeling neural development and function, capturing both cellular diversity and spatial architecture within in vitro environments.

The utility of *in vitro* CNS models relies not only on accurate cell type specification and maturation, but also on their capacity to reflect essential intercellular interactions. These interactions are fundamental to the coordination of neural development, circuit formation, and tissue maintenance. Among these, neuron-astrocyte communication plays a central role in maintaining synaptic function, metabolic balance, and tissue homeostasis. Disruption of this signaling axis has been implicated in multiple CNS disorders, reinforcing the need for models that capture both cell-intrinsic properties and intercellular dynamics.

Neurons and astrocytes: cellular interplay in health and disease

Neurons are the principal signaling unit of the CNS, specialized in rapid electrical and chemical communication. Structurally, they are polarized cells composed of three main components: dendrites, which receive input from other cells; the soma, which contains the nucleus and integrates incoming signals; and axons, the long projections that conduct action potentials away from the cell body towards target cells. This cellular architecture enables propagation of long-range signals and the formation of complex neural networks (Spruston, 2008).

Axons can either be unmyelinated or myelinated. Unmyelinated axons conduct signals slowly through continuous propagation, whereas myelinated axons are ensheathed by a lipid-rich myelin sheath that insulates the axon, dramatically increasing signaling velocity. In the CNS, myelination is carried out by oligodendrocytes, while in the peripheral nervous system (PNS), Schwann cells perform this role (Momenzadeh & Jami, 2021).

Action potentials are rapid, all-or-nothing electrical impulses mediated by voltage-gated ion channels. They provide the foundation for neuronal excitability and directional signal transmission throughout the nervous system. Neuronal impulses terminate at synapses, which are specialized junctions facilitating communication between neurons and their target cells. Synapses are usually classified as either chemical or electrical, each distinguished by its mechanism of signal transmission. At chemical synapses, which are the predominant form in the human brain, neurotransmitters are released from presynaptic terminals into the synaptic cleft and bind to receptors on the postsynaptic cell. This process modulates the excitability of the receiving cell, allowing for signal modulation, integration, and plasticity. In contrast, electrical synapses form direct cytoplasmic bridges between adjacent neurons through gap junctions, which permit the passive diffusion of ions and small signaling molecules. This enables near-instantaneous, bidirectional signal transmission and is particularly important in circuits requiring synchronized activity, such as those governing reflexes and rhythmic oscillations (Pereda, 2014; Südhof, 2017).

Neurons play a central role in encoding, integrating, and relaying sensory, motor, and cognitive information, forming neural circuits that maintain homeostasis, enable perception, and coordinate complex behaviors. However, they do not function in isolation. Neurons rely on neighboring astrocytes for metabolic support, ion buffering, and synaptic regulation (Lalo et al., 2021).

Astrocytes are key to the structural and functional organization of the CNS, contributing to the maintenance of brain homeostasis. Once regarded as passive support cells, they are now recognized as active regulators of CNS function and

neuronal health. During embryonic development, astrocytes arise from radial glial cells, which are bipolar neural stem cells that initially generate excitatory neurons before undergoing a gliogenic switch to give rise to glial cells, including both astrocytes and oligodendrocytes (Fu et al., 2021; Jovanovic et al., 2023)

In vivo, mature astrocytes exhibit a stellate morphology characterized by numerous fine, branched processes extending from the soma. These processes envelop synapses and contribute to neurotransmission as part of the tripartite synapse, where they modulate synaptic signaling and maintain ionic homeostasis. Astrocytes are extensively interconnected via gap junctions, creating functional networks that facilitate intercellular communication and metabolite distribution (Lalo et al., 2021). Their perivascular endfeet establish contacts with blood vessels to form neurovascular units, regulating cerebral blood flow and coordinating metabolic exchange. Through their neurovascular interactions and proximity to synapses, astrocytes play a central role in maintaining neuronal energy homeostasis. While neurons primarily rely on oxidative phosphorylation, they possess limited metabolic reserves and remain dependent on astrocyte-mediated support. Astrocytes absorb glucose from the bloodstream via their endfeet, convert it to lactate through aerobic glycolysis, and supply it to neurons during periods of heightened synaptic activity. This lactate shuttle ensures sustained ATP production necessary for synaptic transmission and neuronal viability (Andersen et al., 2021; Sofroniew & Vinters, 2010).

In parallel, astrocytes play a crucial role in maintaining the structural and functional integrity of the blood-brain barrier (BBB). Through the secretion of signaling molecules, they modulate the expression of tight junction proteins in capillary endothelial cells, thereby controlling BBB permeability and maintaining structural integrity. Disruption of this regulatory role, whether due to genetic mutations, oxidative stress, or chronic inflammation, can impair barrier function, leading to leukocyte infiltration, cerebral edema, and progressive neurodegeneration (Abbott et al., 2006; Schiera et al., 2024).

The structural integrity of astrocytes is supported by a dynamic cytoskeletal network of intermediate filaments, primarily composed of glial fibrillary acidic protein (GFAP), alongside vimentin, synemin, nestin, and lamin, depending on developmental stage and state of the cell. These filaments maintain the cellular architecture, stabilize organelle position, and facilitate intracellular transport. Beyond their structural function, intermediate filaments are increasingly recognized as dynamic participants in intracellular signaling and transcriptional regulation (Potokar et al., 2020). Disruption of intermediate filament network, particularly involving GFAP, has been associated with severe neuropathological conditions like Alexander disease (AxD), a leukodystrophy caused by dominant mutations in the *GFAP* gene. The pathophysiological features and disease mechanisms of AxD will be further explored later in this thesis.

Astrocytes are highly reactive and capable of undergoing profound functional and morphological remodeling in response to injury, inflammation, or degenerative insult, a process referred to as reactive astrogliosis. This state is characterized by the upregulation of intermediate filaments, proliferation and hypertrophy, which collectively alter cell stiffness, cytoskeletal architecture and protein trafficking. While moderate transient gliosis can serve a protective role by containing damage and preserving homeostasis, chronic reactivity may impair glutamate clearance, reduce metabolic support, and promote the release of proinflammatory cytokines, contributing to a maladapted and toxic environment (Pekny & Pekna, 2014).

In many neurodegenerative conditions, neuronal impairment preceeds the onset of overt neurodegeneration. For instance, in Alzheimer Disease, synaptic deficits manifest before pathological accumulation of amyloid-beta and tau (K. Li et al., 2018). Similarly, in Parkinson Disease (PD), mitochondrial dysfunction, α-synuclein (α-syn) aggregation, and defective autophagy occur years before the selective degeneration of dopaminergic neurons in the substantia nigra (Morris et al., 2024). These early pathological events are commonly accompanied by excitotoxicity, arising from excessive glutamate release, impaired astrocytic uptake, or both, as well as oxidative stress, intensifying neuronal vulnerability. Importantly, such mechanisms are not solely neuron-intrinsic; astrocytic defects play a pivotal role in amplifying these insults (Andersen et al., 2021). While neuropathology is central to these disorders, growing evidence suggests that impaired astrocytic function, including deficient glutamate clearance, metabolic failure, or inflammatory signaling, can accelerate and, in some cases initiate, disease pathogenesis.

Beyond neurodegeneration, astrocytic impairments have also been implicated in psychiatric and neurodevelopmental disorders. Genetic mutations affecting astrocytic ion channels have been associated with conditions such as epilepsy and autism spectrum disorder (Sicca et al., 2016). Recent studies further suggest that dysfunctional astrocytic modulation of synaptic signaling plays a role in the pathophysiology of schizophrenia and depression-related disorders (Kruyer et al., 2023).

Historically, neurological research has focused primarily on neuronal pathology. However, growing recognition of neuron-glia interaction has led to a hypothesis of co-pathogenesis, where pathological changes in both cell types contribute synergistically to drive the progression of neurological diseases. Recognizing the bidirectional influence between these cell types highlights the necessity of astrocyte-inclusive models to understand the cellular mechanisms underlying CNS disorders. Among the shared vulnerabilities contributing to this is lysosomal dysfunction, which impacts homeostasis of both cell types and amplifies circuit-wide stress responses.

Lysosomal function

A central pathway supporting cellular homeostasis in both neurons and astrocytes is the endosomal-autophagic-lysosomal pathway, which governs the degradation of intracellular waste, misfolded proteins, and damaged organelles. At the center of this process lies lysosomal degradation, which relies on highly acidic vesicles filled with hydrolytic enzymes that fuse with either endosomes or autophagosomes to break down internalized cargo. Autophagy, the selective degradation of intracellular components, begins with the formation of a double-membraned autophagosome that engulfs cytoplasmic material. These vesicles then fuse with lysosomes or late-endosomes to form autolysosomes in which the cargo is degraded. In parallel, the endosomal pathway manages the sorting, trafficking, and degradation of extracellular material and membrane proteins internalized via endocytosis. Early endosomes undergo progressive maturation into late endosomes, characterized by alterations in membrane lipid composition, luminal acidification, and cargo sorting. Multivesicular bodies represent an intermediate organelle in the endosomal maturation pathway. They are defined by the presence of internal vesicles formed through invagination and fission of the endosomal membrane. Depending on the context, they either fuse with the plasma membrane to release their contents or merge with lysosomes to facilitate cargo degradation. This is not an isolated pathway; autophagosomes and endosomes intersect repeatedly throughout their maturation, converging at multiple stages to share lysosomal hydrolytic enzymes, particularly so in axons and synaptic terminals where high turnover and spatial constraints require efficient degradation (Saftig & Klumperman, 2009; Woodman & Futter, 2008; Diao et al., 2024).

Beyond degradation, the endosomal-autophagic-lysosomal system also contributes to neurodevelopment, synaptic remodeling, and metabolic adaptation, highlighting its integral role in neuronal function. Disruptions to this multifaceted pathway are increasingly associated with a range of neurodevelopmental and neurodegenerative diseases. In PD, Alzheimer Disease, Huntington Disease, and frontotemporal dementia, defects in autophagosome formation, lysosomal trafficking, and cargo clearance have been reported, which contribute to pathological accumulation of proteins and dysfunctional organelles (Malik et al., 2019). Genetic mutations in several key genes further exacerbate these defects. Mutations in *GBA1*, which encodes the lysosomal enzyme β-glucocerebrosidase (GCase), are associated with PD and cause Gaucher disease (GD), a lysosomal storage disorder explored further in this thesis. Mutations in *ATP13A1* are linked to early-onset PD and cause the extremely rare CNS disorder Kufor-Rakeb syndrome. Additionally, mutations in autophagy-related genes such as *LRRK2*, *PINK1*, and *ATG5,* are implicated in Parkinson phenotypes, causing mitochondrial dysfunction, and impaired protein turnover. These mutations impair degradative capacity, causing cellular stress, energy imbalance and neuronal death. Compromised lysosomal acidification and

enzymatic activity further aggravate debris accumulation, promoting neuroinflammation and accelerating disease progression (Colacurcio & Nixon, 2016; Fujikake et al., 2018; Malik et al., 2019).

Considering the role of astrocytes in maintaining brain homeostasis, a process that relies heavily on autophagy and endocytosis, the impact of a disrupted endosomal-autophagic-lysosomal pathway extends beyond neurons, affecting broader cellular networks and contributing to widespread neuropathology. Understanding this pathway in both healthy and disease states may offer new therapeutic approaches aimed at restoring lysosomal function and degradative capacity, which holds promise of alleviating symptoms and slowing down the progression of a wide range of neurological diseases (Zeng et al., 2025; Zhou et al., 2022).

Leukodystrophies and astrocytopathies

Pathogenic mechanisms underlying leukodystrophies

Leukodystrophies are a heterogeneous group of rare neurological disorders, primarily affecting the white matter of the CNS. Although genetically and clinically diverse, these disorders share a common pathological feature: disruption of myelin, the lipid-rich sheath that insulates specific axonal populations. More than 50 genetically distinct leukodystrophies have been described, reflecting the complexity of CNS myelination and the mechanisms required for its maintenance. These conditions can be classified into two categories: dysmyelinating, characterized by impaired myelin development, and demyelinating, which involves progressive myelin degeneration. Underlying mutations are linked to genes crucial for membrane transport (*ABCD1* in X-linked adrenoleukodystrophy), structural myelin integrity (*PLP1* in Pelizaeus-Merzbacher disease), or lipid metabolism (*GALC* in Krabbe disease and *ARSA* in Metachromatic leukodystrophy (MLD)). Other mutations affect genes such as *MLC1* and *GFAP*, which regulate astrocyte homeostasis and function. Table 1 provides an overview of selected disorders, highlighting genetic diversity, pathogenic mechanisms, and myelin pathology. This reflects the broad pathogenic diversity of leukodystrophies, extending beyond myelin disruption to include astrocytic dysfunction and other cellular abnormalities that compromise white matter integrity (van der Knaap & Bugiani, 2017, 2018; Bugiani & Breur, 2018). Beyond their classification as leukodystrophies, both Krabbe disease and MLD are also categorized as lysosomal storage disorders (LSD), a group of conditions examined further in this thesis (Babcock et al., 2021).

Astrocyte dysfunction and white matter pathology

Astrocytopathies have emerged as an intersecting disease category of particular interest, in which astrocyte dysfunction is the primary contributor to disease pathology. In some cases, such as in Alexander disease and Megalencephalic leukoencephalopathy with subcortical cysts (MLC), they present with leukodystrophic features, where compromised astrocytic support disrupts oligodendrocyte health and function, leading to defective myelination and progressive white matter degeneration (Jorge & Bugiani, 2019; Zhou et al., 2022)

The study of astrocytopathies offers a unique window into the cellular mechanisms linked to white matter integrity and dysfunction. These conditions illustrate how primary astrocyte pathologies can disrupt glial crosstalk, impair myelin maintenance and trigger neuroinflammation, stressing the pathological significance of astrocytes in white matter degeneration.

Insights from astrocyte-driven leukodystrophies, such as AxD and MLC, have contributed to a broader understanding of neurological conditions where astrocyte dysfunction may play a role. Disorders such as epilepsy, Alzheimer disease and, multiple sclerosis are increasingly studied for their glial involvement. (de Waard & Bugiani, 2020; Jorge & Bugiani, 2019; Siracusa et al., 2019). Deciphering the underlying cellular mechanisms contributing to leukodystrophy pathology may lead to novel, glia-targeted interventions for a broad spectrum of CNS disorders (Liu et al., 2017; Valori et al., 2021).

Clinical features of leukodystrophies

The clinical presentation of leukodystrophies varies, but a defining feature of the disease group is progressive white matter degeneration. Most cases first exhibit in infancy or early childhood, and are marked by severe CNS symptoms, including seizures, spasticity, developmental delay or regression, and cognitive decline. While less common, adult-onset variants do occur and present with psychiatric symptoms, motor dysfunction, and severe cognitive impairment. Other usual disease features include visual and auditory impairments, ataxia and, micro- or macrocephaly (Ceravolo et al., 2024; Muthusamy et al., 2023).

Magnetic resonance imaging is central to the diagnostic workup of leukodystrophies. Distinct patterns of white matter abnormalities help narrow the differential diagnosis and guide targeted genetic testing. Next-generation sequencing has significantly improved diagnostic accuracy and enabled the identification of an increasing number of distinct leukodystrophies. However, despite these improved diagnostic capabilities, a subset of patients remains without a definitive molecular diagnosis, highlighting the complexity and heterogeneity of these disorders (Ceravolo et al., 2024).

Table 1: Genetic and pathogenic diversity in leukodystrophies
Overview of selected leukodystrophies accentuating variation in affected genes, disease mechanisms, clinical onset, and primary astrocytic and lysosomal involvement. The final two columns denote astrocytopathy and lysosomal pathology, respectively: **A*** indicates predominant astrocytic dysfunction, **LSD*** marks disorders classified as lysosomal storage diseases, and X denotes that the feature applies to the corresponding condition.

Disorder	Affected gene(s)	Pathogenic mechanism	Typical onset	Myelin Pathology	A*	LSD*
Alexander disease	*GFAP*	Astrocyte cytoskeletal disruption	Infantile to adult	Secondary myelin loss	X	
X-linked adrenoleukodystrophy	*ABCD1*	Impaired fatty acid transport	Childhood to adulthood	Demyelination		
Pelizaeus-Merzbacher disease	*PLP1*	Defective myelin structure	Early infancy	Dysmyelination		
Krabbe disease	*GALC*	Galactolipid accumulation	Infantile to juvenile	Demyelination		X
Metachromatic leukodystrophy	*ARSA*	Sulfatide accumulation	Infantile to adult	Demyelination		X
Megalencephalic leukoencephalopathy with subcortical cysts	*MLC1, HEPACAM, GPRC5B, AQP4*	Astrocyte ion/water imbalance	Early childhood	Myelin vacuolization	X	

Current and future treatment approaches

Therapeutic options for leukodystrophies remain limited, primarily focusing on symptom management and supportive care. Nonetheless, emerging disease-modifying treatments are showing promise in selected subtypes. Advances in gene therapy, enzyme replacement therapy, and hematopoietic stem cell transplantation have demonstrated meaningful clinical benefits in disorders such as metachromatic leukodystrophy (MLD) and X-linked adrenoleukodystrophy (Fumagalli et al., 2022; Wolf et al., 2025). For instance, an *ex vivo* gene therapy for metachromatic leukodystrophy is now available at specialized centers, including the Nordic treatment hub at Skåne University Hospital.

Given the rarity and genetic heterogeneity of these disorders, traditional preclinical models often fail to capture the full scope of the disease-specific pathology. To overcome these limitations, patient-derived stem cell models provide a robust platform for investigating leukodystrophy mechanisms in a personalized context, allowing for critical insights into cellular pathogenesis as well as a scalable system for therapeutic screening, target validation, and development of precision medicine approaches targeted to individual disease subtypes (Lanciotti et al., 2022).

Alexander disease

Alexander disease is a rare, progressive leukodystrophy caused by heterozygous gain-of-function mutations in the *GFAP* gene, which encodes glial fibrillary acidic protein, an intermediate filament protein expressed almost exclusively in astrocytes. The disorder follows an autosomal dominant inheritance pattern, but most cases arise from *de novo* mutations. Unlike many leukodystrophies, where oligodendrocyte dysfunction is central, AxD is defined by astrocyte pathology and is considered a prototypical primary astrocytopathy (Hagemann et al., 2021). To date, over 70 AxD-causing GFAP mutations have been identified, reflecting the considerable genetic heterogeneity and contributing to the broad clinical spectrum of the disorder (Battaglia et al., 2019).

Clinical presentation and pathological hallmarks

AxD presents with a heterogeneous clinical phenotype, stratified by age of onset into infantile, juvenile, and adult subtypes, each associated with distinct neurological features and progression patterns. The infantile form, which typically presents before two years of age, manifests with macrocephaly, seizures, developmental delay and progressive deterioration. The juvenile variant typically follows a milder disease progression with preserved motor and cognitive function, and is characterized by bulbar symptoms, hyperreflexia, and ataxia. Adult AxD, ranging from late adolescence and above, displays similarly to the juvenile form, and is characterized by spastic paraparesis, palatal myoclonus, ataxia, and bulbar symptoms. Magnetic resonance imaging (MRI) findings are crucial for diagnosis, revealing distinctive white matter abnormalities in the frontal lobe, brainstem, and basal ganglia. A definitive diagnosis is established through the identification of a pathogenic GFAP mutation (Prust et al., 2011).

The defining histopathological feature of AxD is the accumulation of Rosenthal fibers (RFs), which are cytoplasmic inclusions within astrocytes composed of aggregated mutant GFAP, small heat shock proteins, and other stress-associated proteins (Wippold et al., 2006). Their formation is indicative of profound astrocytic stress and has been associated with disrupted vesicular trafficking, protein homeostasis impairments, and dysregulated glutamate and calcium homeostasis (A. A. Sosunov et al., 2017). AxD astrocytes also exhibit reactive features, such as multinucleation and mitotic arrest (A. A. Sosunov et al., 2013), as well as altered post-translational modification of GFAP, including hyperphosphorylation (Battaglia et al., 2019; Viedma-Poyatos et al., 2022). Post-mortem investigations reveal widespread astrogliosis and white matter degeneration, highlighting the central role of astrocytic dysfunction in the pathophysiology of Alexander disease (Olabarria et al., 2015; Walker et al., 2014).

Insights from animal and hPSC-based AxD models

Animal models have been instrumental in advancing our understanding of Alexander disease pathogenesis, providing critical insights into astrocyte dysfunction and associated white matter abnormalities. Transgenic rats and mice engineered to overexpress mutant *GFAP* reliably recapitulate the hallmark features of the disorder, including the accumulation of RFs, astrogliosis, and white matter degeneration. However, as these models rely on overexpression paradigms of mutant GFAP to elicit the AxD pathology, their translational relevance is limited (Hagemann, 2022). A notable exception is a knock-in rat model that reproduces AxD pathology without relying on overexpression and has been integral in demonstrating the therapeutic potential of antisense oligonucleotides against *GFAP* transcripts, which significantly reduce protein aggregation and reverse disease phenotypes after a single treatment (Hagemann et al., 2021).

In parallel, patient-derived hiPSC models provide a human-specific approach to study Alexander disease and have proven effective in capturing phenotypes that reflect key aspects of astrocyte pathology. For example, hiPSC-derived astrocytes from AxD patients display RF-like inclusions and elevated levels of inflammatory cytokines, recapitulating pathological features of patient tissue (Kondo et al., 2016). Furthermore, coculture experiments have shown that patient-derived AxD astrocytes secrete inhibitory factors, such as CHI3L1, which impair the proliferation and myelination capabilities of oligodendrocyte precursor cells (L. Li et al., 2018). This provides valuable mechanistic insight into the disrupted astrocyte-oligodendrocyte signaling and cell-non-autonomous dysfunction of astrocytes in AxD.

Other studies have expanded on the phenotypic spectrum of hPSC-derived AxD models. These findings include abnormal organelle distribution and morphology (Jones et al., 2018), altered expression of astrocytic ion transporters (Canals et al., 2018), and disease-associated post-translational modification of GFAP, recapitulating key characteristics of *in vivo* AxD astrocytes (Battaglia et al., 2019). In addition, a recent study has shown that patient-derived AxD astrocytes exhibit altered composition and remodeling of the extracellular matrix (ECM) (Yi et al., 2025), suggesting that ECM dysfunction may contribute to the disease progression.

Beyond recapitulating key pathological features of AxD, hPSC-based models offer an important translational platform, bridging the gap between fundamental disease mechanisms and preclinical therapy development by enabling a patient-specific, human-relevant screening for targeted interventions.

Megalencephalic leukoencephalopathy with subcortical cysts

Megalencephalic leukoencephalopathy with subcortical cysts is a rare genetic leukodystrophy characterized by infantile-onset white matter edema, microencephaly, and progressive neurological decline. Brain MRI typically reveals diffuse white abnormalities, swelling and subcortical cysts (Ilja Boor et al., 2006; van der Knaap et al., 1995).

MLC is genetically heterogeneous. The classical form is caused by biallelic pathogenic variants in *MLC1* or *HEPACAM*, also known as *GLIALCAM*, and is inherited in an autosomal recessive manner. In contrast, heterozygous dominant mutations in *HEPACAM* are associated with a remitting form of the disease, characterized by early-onset presentation that stabilizes or improves without long-term clinical regression (Passchier et al., 2024). Recent genetic studies have identified rare dominant variants in *GPRC5B* and recessive mutations in *AQP4* in individuals presenting with MLC-like features, thereby expanding the known genetic landscape of megalencephalic leukoencephalopathy with subcortical cysts (Brignone et al., 2015; Passchier et al., 2023).

Astrocytic proteins and MLC pathophysiology

The *MLC1* gene, located on chromosome 22, encodes a 377-amino-acid transmembrane protein with eight predicted membrane-spanning domains. The MLC1 protein is highly expressed in astrocyte endfeet in contact with the BBB and the pial membrane. Within these domains, MLC1 and HepaCAM form a membrane-bound complex vital to the functional and structural integrity of perivascular astrocytes. The function of the protein remained elusive for a long time, but recent studies indicate that MLC1 is a Ca^{2+}-regulated protein involved in astrocytic volume regulation and ion homeostasis, connecting the protein to fundamental functions of astrocytes (Passchier et al., 2024).

HepaCAM, belonging to the immunoglobulin superfamily, serves a dual role as a molecular chaperone and a cell adhesion molecule. It stabilizes MLC1 and the chloride channel CIC-2 by guiding them to the astrocytic plasma membrane (Hoegg-Beiler et al., 2014; López-Hernández et al., 2011). Mutations to *HEPACAM* disrupt this interaction, leading to protein mislocalization and compromised astrocytic signaling, which is central to MLC pathology.

Aquaporin-4, encoded by *AQP4*, is also concentrated at astrocyte endfeet, acting as the primary water channel of the CNS. The protein is essential for fluid transport, potassium buffering, and clearance of metabolic waste. Similarly, the orphan G protein-coupled receptor GPRC5B is found in the same astrocytic domains and has been shown to interact with ion channels related to astrocytic volume regulation, further implicating astrocytic endfeet dysfunction in MLC pathogenesis (Brignone et al., 2015; Passchier et al., 2023).

Genetic landscape of MLC mutations

Pathogenic variants in *MLC1* account for approximately 75% of genetically confirmed cases of MLC, with over 150 mutations reported across the coding region of this gene. These variants show no clear association between their genomic location and clinical severity. Mutations in *HEPACAM* are identified in 20-25% of patients, primarily in individuals lacking *MLC1* involvement. The remaining cases are explained by rare variants in *AQP4* and *GPRC5B* or remain genetically unresolved (Passchier et al., 2023, 2024).

Modeling MLC

Although animal models have provided valuable insights into the function of *MLC1* and *HepaCAM* and their role in maintaining BBB integrity (Morales et al., 2022), they do not fully capture key features of human MLC, such as persistent white matter edema and signaling pathways unique to human astrocytes. As both an astrocytopathy and a leukodystrophy, MLC illustrates how primary astrocyte dysfunction can disrupt ion and water homeostasis and drive white matter pathology. Stem cell-based approaches now offer a promising means of generating human-relevant MLC models, enabling more detailed investigation of disease mechanisms and the development of glial-targeted therapies.

Lysosomal storage disorders

Molecular basis and pathogenesis

LSDs comprise a group of over 70 rare, inherited metabolic diseases linked by impaired lysosomal function. The lysosome relies on a coordinated network of more than 60 lysosomal acid hydrolases, membrane transporters, and assessor proteins to maintain cellular homeostasis (Staudt et al., 2017). Mutations in genes encoding these lysosomal proteins disrupts lysosomal integrity and function, leading to progressive accumulation of undegraded or partially degraded substrates. Aberrant lysosomal accumulation disrupts organelle architecture and interferes with key cellular processes, including inter-organelle communication, membrane trafficking, and intracellular signaling. These disruptions contribute to cellular dysfunction and drive disease pathogenesis (Ballabio & Gieselmann, 2009; Platt et al., 2012).

Clinical presentation and disease spectrum

Despite their rarity, collectively LSDs affect approximately 1 in 5000 to 5500 newborns. The clinical manifestations are highly variable, both between and within disease types. Most LSDs present in infancy or early childhood, though adult-onset forms are increasingly being recognized. The clinical phenotype of lysosomal storage disorders reflects the progressive accumulation of undegraded substrates and the differential vulnerability of specific tissues to lysosomal dysfunction. Neurons are especially susceptible due to their post-mitotic nature and reliance on efficient vesicular trafficking, rendering neurological involvement a defining feature of many LSDs. Nearly two-thirds of LSDs exhibit some form of CNS pathology, ranging from developmental delay and seizures to neurodegeneration and cognitive decline. In addition to the CNS involvement, LSD often affects visceral organs, bone, connective tissue, and the immune system, resulting in hepatosplenomegaly, skeletal deformities, cardiomyopathy, and systemic inflammation (Platt et al., 2018; Sevin & Deiva, 2021; Walkley, 2021).

Secondary aggregation and cellular crosstalk

The pathogenesis of LSD extends beyond primary substrate accumulation. Impaired lysosomal clearance can lead to secondary aggregation of unrelated macromolecules, compounding lysosomal congestion and cellular stress. In parallel, lysosomal dysfunction perturbs autophagic flux, mitochondrial dynamics, and calcium signaling, contributing to a widespread disruption of cellular homeostasis. LSDs are systemic diseases, characterized by lysosomal dysfunction, which disrupts multiple organelles and pathways. These widespread effects compromise homeostasis and contribute to multisystem pathology, particularly in neurodegenerative forms where glial-neuronal interactions and synaptic integrity are affected (Darios & Stevanin, 2020; Platt et al., 2012).

Therapeutic strategies

Therapeutic strategies for LSDs have expanded significantly in recent years. Enzyme replacement therapy remains the standard treatment for several systemic lysosomal storage disorders, including Gaucher and Fabry disease, and has demonstrated efficacy in alleviating peripheral manifestations. However, its limited ability to penetrate the BBB restricts its effectiveness in treating neurological symptoms. To mitigate this limitation, substrate reduction therapy was developed to decrease the synthesis of accumulating substrates and reduce lysosomal load. Pharmacological chaperones represent an additional therapeutic strategy, acting to stabilize misfolded lysosomal enzymes and facilitate their proper folding and trafficking to the lysosome. More recently, gene therapy and genome editing strategies have entered clinical trials, showing promise towards correcting the underlying mutations and restoring lysosomal function (Ballabio & Gieselmann, 2009; Platt et al., 2018; Massaro et al., 2021).

Modeling of lysosomal storage disorders

Despite therapeutic advances, many LSDs remain without effective treatment, particularly those affecting the CNS. Although animal models have provided valuable mechanistic insights, fundamental differences in lysosomal biology and neurodevelopment between species limit their translational relevance. Human-relevant models are essential for accurately dissecting pathogenesis, identifying therapeutic targets, and developing interventions that reflect the complexity of human CNS involvement.

Gaucher disease

GD is a lysosomal storage disorder caused by autosomal recessive mutations in the *GBA1* gene, which encodes for the lysosomal enzyme GCase. Enzymatic deficiency results in the accumulation of glucosylceramide and its deacetylated derivative, glycosylsphingosine, within lysosomes, disrupting cellular homeostasis and contributing to multisystem pathology (Revel-Vilk et al., 2020; Roshan Lal & Sidransky, 2017). The *GBA1* gene is located on chromosome 1, adjacent to a highly homologous pseudogene (*psGBA*) that shares 96% sequence identity across coding regions, which complicates molecular diagnostics. The high sequence also enables non-allelic homologous recombination events, giving rise to complex recombinant alleles such as RecNcil, which are known to be pathogenic and are associated with severe neuronopathic forms of GD (Woo et al., 2021). To date, over 500 pathogenic *GBA1* variants have been identified, including missense mutations, insertions, deletions, and complex rearrangements, each associated with variable decreases in GCase activity and clinical severity (Y. Lin et al., 2024).

Clinical spectrum and neurological involvement

GD encompasses a broad phenotypic spectrum, ranging from asymptomatic carriers to patients with severe, multisystem involvement (Fig. 3). Historically, it has been classified into three clinical subtypes based on CNS involvement: non-neuronopathic (type I), acute neuronopathic (type II), and chronic neuronopathic (type III). A perinatal-lethal GD (type 0) has also been recognized, and the classification is increasingly viewed as a continuum rather than distinct subtypes. The non-neuronopathic form is the most prevalent and lacks overt CNS involvement, but subtle neurological features and associations with PD have been reported. In contrast, the acute and chronic neuronopathic forms of Gaucher disease are characterized by primary neurological involvement. The acute variant typically presents in early infancy with bulbar dysfunction, spasticity, and rapidly progressive neurodegeneration, often resulting in death before two years of age. The chronic neuronopathic variant follows a more protracted course, with symptoms such as gaze palsy, ataxia, and seizures emerging later in childhood or adolescence. Currently, no disease-modifying treatments exist for the neurological features of GD (Nalysnyk et al., 2017; Roshan Lal & Sidransky, 2017).

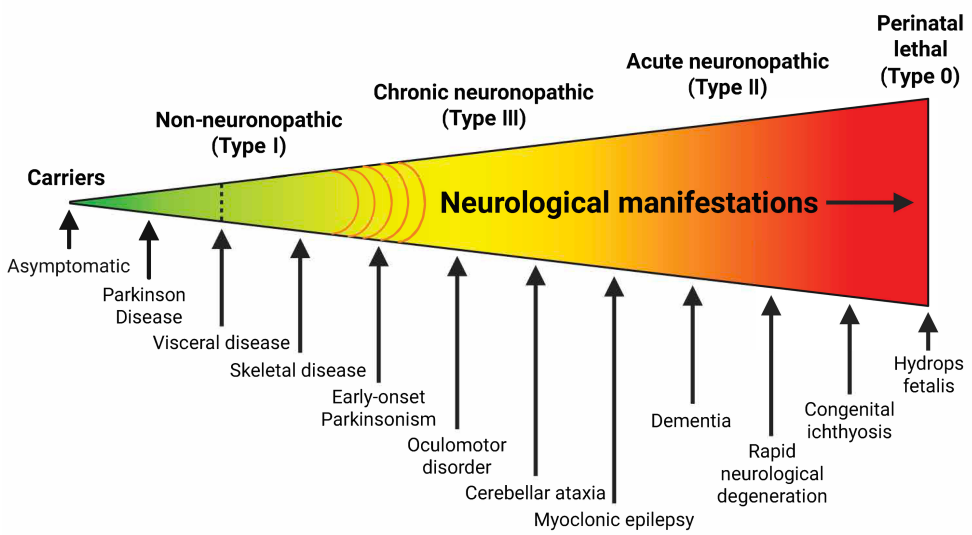


Figure 3: Clinical continuum of Gaucher disease.
Schematic of the Gaucher disease spectrum. Ranging from *GBA1* mutation carriers, with increased risk for Parkinson disease, toward phenotypes with progressively earlier onset and increased neurological involvement. Adapted from Eblan, M. J., Goker-Alpan, O., & Sidransky, E. (2005). Perinatal lethal Gaucher disease: A distinct phenotype along the neuronopathic continuum. Fetal and Pediatric Pathology, 24(4-5), 205-222. (Eblan et al., 2005). Created with BioRender.com.

Pathogenic mechanism

Neurodegeneration is a defining feature of neuronopathic GD (nGD), yet its underlying disease mechanisms remain poorly understood. Early studies emphasized neuronal vulnerability, particularly the toxic accumulation of glycosphingolipids such as glycosylsphingosine and gangliosides. These lipids disrupt membrane architecture and synaptic integrity, contributing to neuronal dysfunction and loss. Ganglioside accumulation, in particular, has been implicated in altered neurotropic signaling and impaired neuronal survival, suggesting a direct role in disease pathogenesis (Orvisky et al., 2000; Kim et al., 2006; Revel-Vilk et al., 2020).

Neuropathological studies of nGD have documented degeneration across multiple brain regions, including cortical layers III and V, the hippocampus, thalamus, and brainstem (Wong et al., 2004). These selective patterns may reflect differences in neuronal maturity, metabolic burden, or lipid processing and reinforce the need for models that accurately recapitulate cortical architecture.

Glial involvement in the central nervous system pathology of Gaucher disease has been increasingly recognized. Postmortem analyses of human brain tissue have revealed astrocytic alterations, including gliosis and lysosomal dysfunction, which are indicative of compromised neurotrophic support and a potential contributory role in neuroinflammatory processes (Wong et al., 2004). In parallel, studies using murine models with cell-specific *GBA1* deletion and rescue have demonstrated microglial activation, which sustains inflammatory signaling and exacerbates neurodegenerative outcomes (Boddupalli et al., 2022). In systemic Gaucher disease, macrophages accumulate undegraded glucosylceramide, transforming into Gaucher cells; enlarged, lipid-laden cells with characteristic cytoplasmic striations. These cells are prominent in bone marrow, liver, and spleen, and contribute to organomegaly, cytopenias, and inflammatory tissue damage (Stirnemann et al., 2017; Zimran et al., 2020). In the central nervous system, microglia serve as the resident immune cells and functional counterparts to peripheral macrophages. Despite their central role in neuroinflammation and neurodegeneration, particularly in neuronopathic Gaucher disease, mechanistic insights into microglial dysfunction remain limited due to the scarcity of human-based models. Advancing these model systems is critical to clarify cell-type-specific roles in disease pathogenesis and for guiding the development of targeted therapeutic interventions in the CNS.

Taken together, these findings support a multifactorial model of neurodegeneration in neuronopathic Gaucher disease, characterized by both neuronal lipid dysregulation and glial impairment. Histopathological studies have revealed neuronal loss in cortical layers III and V, as well as in the hippocampus, thalamus, and brainstem, suggesting a regionally selective and cell-type-specific vulnerability. Systematic investigation of spatial and cellular patterns in human-derived models could clarify disease mechanisms and support the development of treatment strategies with greater precision and clinical relevance. Further studies are needed to characterize how glycosphingolipid accumulation alters astrocytic and microglial function, and to determine the extent to which these glial disturbances affect neuronal viability and cortical organization.

GBA1 and Parkinson disease: a converging pathway

The identification of heterozygous *GBA1* mutations as a significant genetic risk factor for Parkinson disease has established an important link between GD and synucleinopathies. Variants in the GBA1 gene are now recognized as among the most common genetic risk factors for PD. Carriers exhibit increased risk of developing the disorder, typically presenting with earlier disease onset and faster cognitive decline (S. T. Kumar et al., 2020; Sidransky et al., 2009). Mechanistically, reduced glucocerebrosidase (GCase) activity impairs lysosomal degradation, promoting the accumulation and aggregation of α-synuclein. This relationship is bidirectional, as α-synuclein aggregates further inhibit GCase function, reinforcing

a pathogenic cycle of lysosomal dysfunction and progressive neurodegeneration (Mazzulli et al., 2011). In GD, this secondary aggregation of α-synuclein is increasingly recognized as a contributor to neuronal vulnerability. Studies using GBA1-associated Parkinson disease (*GBA*1-PD) and GD patient-derived neurons have demonstrated that *GBA1* mutations lead to autophagic defects, impaired calcium homeostasis, and enhanced α-synuclein pathology, reinforcing the central role of lysosomal dysfunction in both disorders (Schöndorf et al., 2014). Recent findings also suggest that iPSC-derived astrocytes carrying the *GBA1* N370S mutation exhibit mitochondrial fragmentation and lysosomal impairment, indicating that astrocytic dysfunction may further exacerbate neuroinflammatory stress and neuronal vulnerability in *GBA1*-associated PD (Yarkova et al., 2024).

Modeling neuronopathic GD using hiPSCs

hiPSC-based models have emerged as a valuable tool for investigating the cellular and molecular pathology of neuronopathic GD. Neurons derived from GD patient hiPSCs consistently exhibit hallmark lysosomal defects, including reduced GCase activity, glucosylceramide accumulation, and impaired autophagic flux (Awad et al., 2015). These lysosomal abnormalities are associated with impaired biogenesis, which is attributed to the disrupted signaling of transcription factor EB (TFEB), a key regulator of lysosomal gene expression. Furthermore, neuronal progenitors derived from GD patient hiPSCs exhibit reduced differentiation potential, which is linked to the downregulation of canonical WNT/β-catenin signaling, indicating an early perturbation of neurodevelopmental programs (Awad et al., 2017).

Mechanistic studies using GD hiPSC models have revealed that hyperactivity of mTOR, a central regulator of cellular growth and metabolism, plays a central role in mediating lysosomal dysfunction. Inhibition of mTOR signaling was shown to restore lysosomal function and autophagic clearance in GD neurons (Brown et al., 2019). In GD patient-derived hiPSC neurons, acid ceramidase has been identified as a key mediator in the pathogenic cascade leading to α-synuclein accumulation in *GBA1*-mutant neurons. Pharmacological inhibition of this lysosomal enzyme has been shown to reduce α-synuclein burden and restore autophagic and lysosomal function, indicating therapeutic relevance in GD and GCase-related synucleinopathies (M. Kumar et al., 2023).

Beyond neuronal pathology, astrocytes derived from GD patient hiPSCs exhibit disease-relevant phenotypes, including intracellular lipid accumulation, lysosomal enlargement, and dysregulated cytokine secretion (Aflaki et al., 2020). These findings suggest that astrocytes play a role in contributing to the neuroinflammatory environment observed in Gaucher disease and associated synucleinopathies.

A recent study demonstrates a cell therapy strategy that employs hiPSC-derived neural precursor cells engineered to overexpress functional GCase. Following intravenous administration into a GD mouse model, these cells engrafted in the brain, where the secreted enzyme was taken up by adjacent GD neurons, improving enzymatic function, lowering substrate accumulation, and enhancing mitochondrial activity. These findings support the therapeutic potential of combined cell and gene correction strategies in *GBA1*-associated neurodegeneration (Peng et al., 2023).

While hiPSC-based studies have advanced the understanding of Gaucher disease (GD) pathogenesis, their reliance on neuron-centric monolayer cultures limits the investigation of intercellular dynamics and glial involvement, both which are increasingly recognized as integral to central nervous system pathology. Disease-relevant phenotypes observed in GD hiPSC-derived astrocytes, along with emerging evidence implicating microglial dysfunction, underscore the need for more physiologically representative models. Incorporating GD hiPSC lines into organoid models presents a promising approach for investigating disease mechanisms across neurodevelopmental stages and to identifying therapeutic targets within a physiologically relevant context.

Use of generative AI in thesis

This thesis has, in part, been prepared with the assistance of generative AI tools, including ChatGPT and Copilot. All generated content was carefully reviewed and edited, and responsibility for the final content rest with the author. A more detailed account of AI use is provided in the methodology section.

Aims of the thesis

The overarching objective of this thesis was to leverage the use of genome editing with pluripotent stem cells to develop robust *in vitro* models of rare pediatric CNS disorders. Through the combined use of targeted genetic modification and tailored differentiation strategies, these models enabled the investigation of disease-relevant mechanisms in neural cell types, including neurons and astrocytes. This approach facilitated a detailed analysis of cell-type-specific pathology and provided insights into the molecular mechanisms driving pediatric CNS disorders.

Specific aims:

- To decipher underlying disease mechanisms of AxD-causing *GFAP*-mutations at a cellular level (Paper I).
- To generate *MLC1*-mutated hPSC lines for *in vitro* modeling of megalencephalic leukoencephalopathy with subcortical cysts (Paper II).
- To generate hiPSC lines with tagged α-synuclein for visualization of endogenous protein in human cellular models of neurodegenerative disorders (Paper III).
- To generate a robust human *in vitro model* of Gaucher Disease and identify novel disease phenotypes and therapeutic targets (Paper IV).

Key results

Aberrant neurodevelopment in human iPS cell-derived models of Alexander disease (Paper I)

At the outset of this study, the contribution of human astrocytes to AxD pathogenesis remained largely unexplored. Most prior research had relied on animal models to study *GFAP* mutations and associated pathology. At the time, only a limited number of hiPSC-based studies had been conducted, highlighting the need for human-derived systems to better understand the disease. Given that AxD is a primary astrocytopathy and GFAP has been considered the canonical marker of astrocytes, we sought to gain a deeper understanding of the underlying disease mechanisms driving pathogenesis by examining the cellular and molecular mechanisms that AxD astrocytes exert on neurons.

For this study, we acquired a previously established hiPSC line derived from a patient with infantile-onset AxD, harboring the $GFAP^{R239C}$ mutation, alongside an isogenic control line (Battaglia et al., 2019). Using these hiPSC lines, we generated induced neurons (iNs) and induced astrocytes (iAs) and combined them into cocultures with different combinations of healthy and diseased cells (Fig. 4A), thereby allowing us to investigate both cell autonomous and cell non-autonomous effects of the GFAP mutation.

To investigate how the GFAP mutation affected the transcriptome of both neurons and astrocytes at a single-cell level, we performed single-cell RNA sequencing (scRNAseq) of mature cocultures. The sequencing results revealed four clusters each with variable abundance, for both the iN and iAs populations (Fig. 4B).

Clusters NEURO 1-3 consisted of CNS neurons of varying states of maturity (Fig. 4C). The NEURO 1 cluster is characterized by markers of neuronal precursors and was found to be enriched in the astroAxD/neuroAxD cocultures. NEURO 2 markers included genes related to neuropeptide secretion and processing, as well as a proteolipid membrane protein associated with neurodevelopment, indicating a more differentiated state. This cluster was more abundant in the astroC/neuroC cultures. The NEURO 3 cluster, which was enriched in the astroAxD/neuroC condition, expressed genes associated with hormone secretion while also sharing some of the genes from neuronal precursors seen in cluster NEURO 1, indicating a transcriptionally distinct population separate from both NEURO 1 and NEURO 2. The overrepresentation of this cluster in the cultures containing AxD astrocytes and WT neurons strongly suggests that diseased astrocyte environment is sufficient to alter the transcriptional profile of otherwise healthy neurons, causing them to adopt an aberrant maturation trajectory. This shift in neuronal state supports the hypothesis that AxD astrocytes exert a non-cell-autonomous effect contributing to disease pathology.

The final neuronal subpopulation, NEURO 4, is found in similar numbers across the different conditions (Fig. 4E) and consists of peripheral neurons, now known to arise from NGN2-based iN generation protocols. (H.-C. Lin et al., 2021).

The four astrocyte clusters included two subpopulations, ASTRO 1 and ASTRO 4, with transcriptomic signatures indicating a lower level of maturity, with the ASTRO 4 cluster expressing additional genes related to cell proliferation. The ASTRO 3 cluster displayed increased levels of collagen expression, frequently observed in *in vitro* astrocyte cultures. The cells of the ASTRO 2 subpopulation were the most mature, indicated by the expression of S100B and GFAP (Fig. 4C and 4F). This cluster was highly enriched in the astroC/neuroC condition.

In addition to the iN and iAs populations, the scRNAseq analysis revealed a diverse non-neural cluster of cells (termed the AxD cluster, Fig. 4B) coming from AxD cells treated with the TFs to generate iAs. This cluster displayed markers of surface ectoderm along with astrocytic and neural markers (Fig. 4C and Fig. 4G). The mixed identity of this cluster suggests an impaired lineage commitment and differentiation in AxD astrocytes. These findings further indicate that AxD astrocytes exhibit intrinsic deficits in differentiation fidelity, which may contribute to broader cellular dysregulation in the context of Alexander disease.

Collectively, the scRNAseq analysis of cell populations revealed a higher proportion of less mature cells in cocultures containing AxD astrocytes (Fig. 4E) and the existence of a distinct AxD cluster with non-neural gene expression signatures.

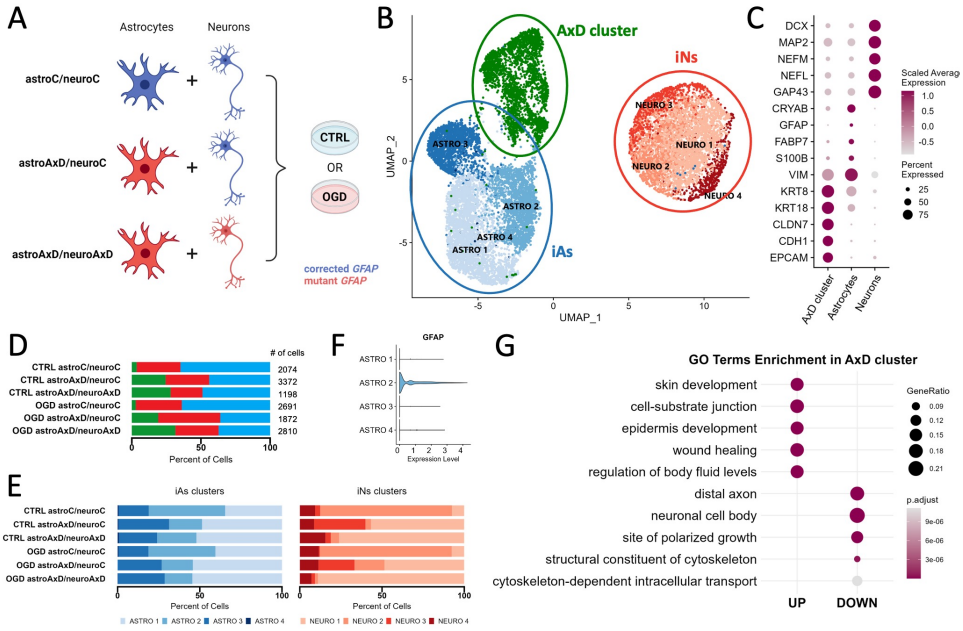


Figure 4: Single-cell transcriptomic profiling of astrocyte-neuron cocultures reveals cell-type diversity and disease-associated alterations.

(A) Schematic of coculture conditions used for the scRNAseq experiments. (B) UMAP plot showing clustering of three major cell populations across all conditions. (C) Dot plot of selected marker genes for AxD cluster, iAs, and iNs. (D-E) Proportional bar plots showing increased abundance of less mature cells and the AxD cluster in conditions with AxD astrocytes. (F) Violin plot showing GFAP expression mapped to astrocytic clusters, predominantly expressed in ASTRO 2. (G) Overrepresentation analysis results showing the top five most up- and downregulated gene ontology (GO) terms in the AxD cluster compared to iAs and iNs.

Given our aim to investigate the role of GFAP-mutated astrocytes in AxD pathology and prompted by the transcriptomic analysis that revealed less mature neuronal and astrocytic populations in the astroAxD/neuroC condition, we sought to further characterize these immature cell states through immunocytochemistry (ICC).

We performed immunocytochemical analysis of MAP2-stained neurons and vimentin-stained astrocytes (Fig. 5A), which revealed comparable numbers of both cell types to the control condition (Fig. 5B). However, a significantly higher proportion of undifferentiated cells could be identified in the condition with AxD astrocytes. These undifferentiated cells displayed a large, spherical morphology and lacked discernible cellular processes (Fig. 5A), consistent with an immature or developmentally stalled phenotype.

Quantification of GFAP$^+$ cells showed no significant difference between the two conditions (Fig. 5D). However, given that GFAP is also expressed in radial glia, we proceeded with quantifying the morphology of the GFAP$^+$ cells (Fig. 5C and 5D).

The morphological analysis revealed that cocultures with AxD astrocytes contain GFAP$^+$ cells with reduced circularity and increased perimeter, characteristics associated with immature astrocytes and radial glia. These structural alterations suggest impaired astrocyte differentiation in the AxD condition. Taken together, this coculture analysis further supports the transcriptomic findings and highlights a reduced degree of cellular maturation in the presence of AxD astrocytes.

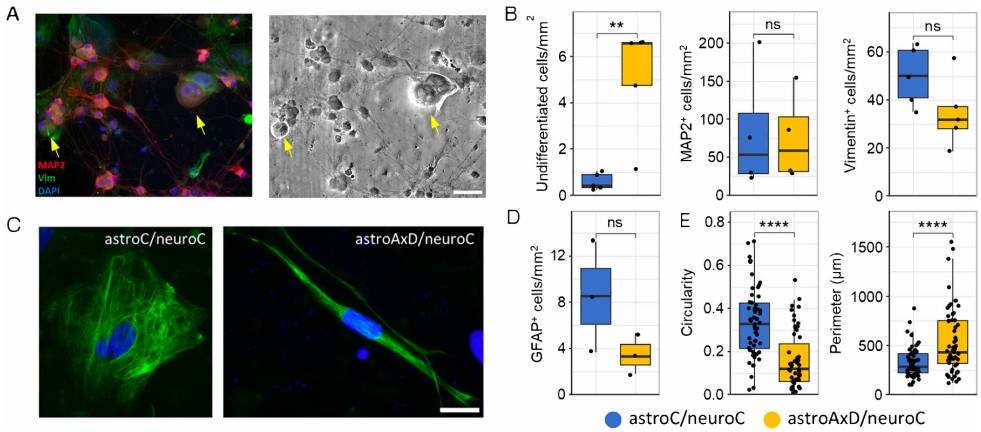


Figure 5: Immunocytochemical analysis reveals reduced cellular differentiation in astroAxD/neuroC cocultures.

(A) Representative images of cocultures labeled with MAP2 (neurons, red), vimentin (astrocytes, green), and DAPI (nuclei, blue); arrows indicate undifferentiated cells. Scale bar: 50 µm. (B) Quantification of undifferentiated, MAP2$^+$, and Vimentin$^+$ cells across astroC/neuroC and astroAxD/neuroC conditions (n= 4-5). (C) Representative image of GFAP$^+$ cells (green) with DAPI (blue). Scale bar: 20 µm. (D) Quantification of GFAP$^+$ cells per condition (n=3). (E) Quantifications of circularity and perimeter of individual GFAP$^+$ cells (n=55 per group).

To further investigate how the GFAP^{R239C} mutation influences cellular transcription states, we performed differential expression analysis (DEA) across coculture conditions. Specifically, we compared both AxD astrocyte conditions (astroAxD/neuroC and astroAxD/neuroAxD) against the isogenic control condition (astroC/neuroC) in two separate comparisons. This revealed 26 downregulated and 21 upregulated genes shared across the AxD conditions (Fig. 6A). These shared transcriptional changes, regardless of neuronal genotype, indicate a molecular signature driven by AxD astrocytes.

Within AxD astrocytes, we observed significant downregulation of *S100B*, *GFAP*, *PTRZ1,* and *ACAN* (Fig. 6B), markers typically associated with astrocyte identity and maturation, suggesting disrupted differentiation. When examining differentially expressed genes (DEG) in neurons, we found that exposure to AxD astrocytes altered neuronal gene expression regardless of neuronal genotype. Upregulated genes were associated with pathways involved in mitochondrial function, ion channel activity, protein aggregation, and oxidative stress response, indicating that mutant astrocytes influence neuronal homeostasis through indirect mechanisms.

Given the transcriptional indications of altered intercellular signaling in AxD cocultures, we further investigated the cell-cell communication using CellChat analysis, focusing on the astroAxD/neuroC cocultures (Fig. 6C).

This analysis revealed an increase in both strength and number of interactions, especially involving the AxD cluster, compared to the astroC/neuroC condition. As CellChat accounts for differences in cluster size, this suggests that the AxD cluster displays intrinsically elevated signaling activity. In contrast, the mature astrocyte cluster (ASTRO 2) exhibited diminished engagement in cell communication, and the ASTRO 4 cluster was too sparsely populated to support confident detection of ligand-receptor interaction.

Several signaling mechanisms involved in neurodevelopment and gliogenesis were dysregulated in the astroAxD/neuroC condition (Fig. 6E). For example, *RELN* and *EGF* signaling were both elevated. *RELN* is linked to neuronal migration, while *EGF* is critical for promoting and sustaining progenitor states during gliogenesis. These changes suggest that isogenic control neurons signal to less differentiated, radial glia-like astrocytes in astroAxD/neuroC cocultures, an interaction that is not observed in the other two conditions. Meanwhile, other interactions that were typically active in healthy cocultures, such as FGF, TGFβ, and LIFR signaling, were reduced or absent in astroAxD/neuroC cocultures. These pathways are crucial for regulating astrocyte development and neuron-glia communication. FGF signaling supports astrocyte proliferation and maturation, TGFβ supports astrocyte differentiation and reinforces astrocytic identity, and LIFR signaling is involved in astrocyte survival and response to injury. These signaling pathways were instead prominent in astroC/neuroC, where both mature and immature astrocyte clusters, along with neuronal populations, contributed substantially to intercellular communication.

The AxD cluster also showed increased responsiveness to additional developmental signals such as non-canonical WNT and Notch, which are associated with progenitor maintenance, cytoskeletal remodeling, and early patterning processes. Together, these findings reinforce a shift toward immature, progenitor-like signaling behavior in AxD astrocytes, consistent with disrupted gliogenesis and altered neuron-astrocyte communication.

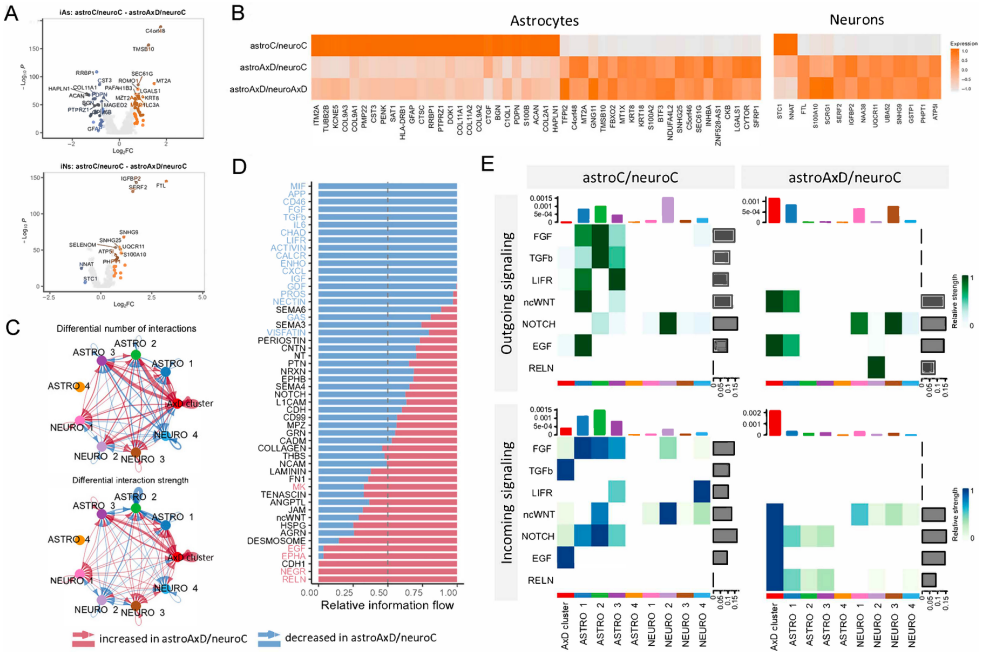


Figure 6: Transcriptional and intercellular signaling alterations in cocultures with AxD astrocytes. (A) Vulcano plots displaying DEGs in astrocytes and neurons from astroAxD/neuroC compared to astroC/neuroC cocultures. (B) Heatmap of DEGs shared between astroC/neuroC vs. astroAxD/neuroC and astroC/neuroC vs. astroAxD/neuroAxD comparisons. (C) Plots summarizing the differential number (top) and strength (bottom) of cell-cell interactions defined by CellChat analysis. Red indicates increased interactions and blue indicates decreased interactions in the astroAxD/neuroC condition. (D) Information flow chart showing dysregulated pathways identified by CellChat analysis. Significantly affected pathways in the astroAxD/neuroC condition are color-coded according to directionality of change (red = increase, blue = decrease). (E) Heatmap of outgoing (ligand-expressing) and incoming (receptor-expressing) signaling patterns, restricted to pathways involved in neurodevelopment and astrogenesis. Panels display altered singling dynamics in astroAxD/neuroC relative to control.

To better understand how the AxD-causing GFAP^{R239C} mutation impacts neural lineage specification during early brain development, we turned to a model system more suitable for developmental analysis. While the coculture platform revealed impaired astrocyte maturation and altered neuron-glia signaling, it lacked the spatial and temporal resolution to capture morphogenic cues and intrinsic patterning. We therefore employed unguided neural organoids, which recapitulate features of embryonic brain development in 3D, allowing us to examine the effect of the AxD mutation on early fate decisions. We generated these organoids from the same control and AxD hiPSCs and cultured them for 165 days before performing scRNAseq.

Single-cell transcriptomic profiling revealed 20 distinct cell populations (Fig. 7A and 7B). These included a range of neural subtypes, such as excitatory neurons, intermediate progenitors, radial glia, astrocytes, and choroid plexus cells, alongside non-neural populations of mesodermal and endodermal origin, including epithelial and pancreatic-like acinar cells. Relative to control organoids, AxD models exhibited a pronounced depletion of astrocytes, choroid plexus cells, pre-oligodendrocyte progenitor cells (pre-OPCs), and peripheral neurons. Conversely, there was an enrichment of mesenchymal-like and acinar cell populations, suggesting a deviation from neuroectodermal lineage commitment toward non-neural differentiation pathways. To investigate the basis of this, we examined astrocytic and neuronal progenitors (Fig. 7A, magenta rectangle and green rectangle, respectively) using DEA (Fig. 7C). In the radial glia cluster, upregulated genes included radial glia markers such as *HOPX* and *MOXD1*, as well as *CLPS* and *REG3G*, which are typically associated with pancreatic lineages. Gene ontology (GO) enrichment analysis revealed increased expression of processes related to gliogenesis, epithelial proliferation, integrin binding, and inflammatory responses. These features are consistent with disrupted or misdirected glial differentiation.

In neuronal progenitors, the expression of neuronal commitment markers, such as *STMN2* and *GAP43,* was reduced. Several protocadherin genes implicated in neural circuit formation were found to be dysregulated. REG3G and CPLS were also upregulated within this population, indicating a transcriptional bias toward non-neural lineage specification. Consistent with these changes, GO terms associated with neurogenesis were downregulated in AxD-derived progenitor clusters.

To validate these findings at the protein level, we stained mature organoids for the astrocytic markers GFAP and S100B (Fig. 7D). AxD organoids displayed a pronounced reduction in $GFAP^{+}$ cells, supporting the conclusion that the $GFAP^{R239C}$ mutation impairs neuroectodermal differentiation and promotes endodermal and mesodermal fate selection in unguided neural organoids.

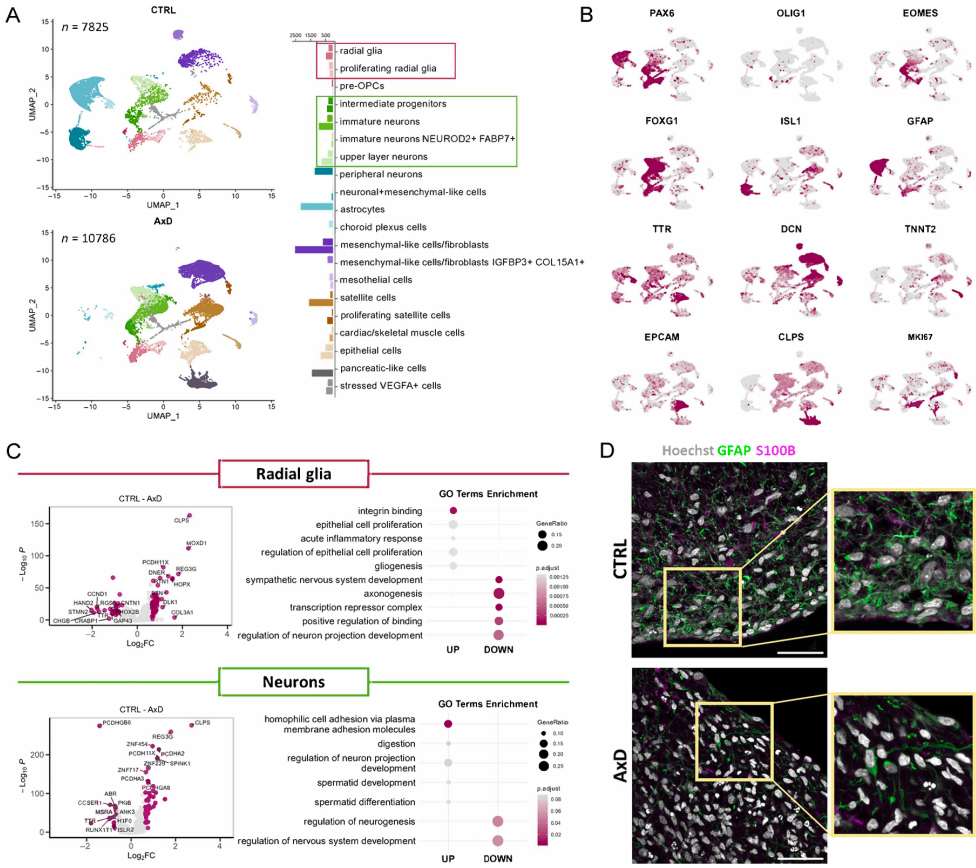


Figure 7: AxD neural organoids display impaired neuroectodermal commitment and lack of astrocytes.

(A) UMAP projections of annotated cell populations from scRNAseq analysis of day 165 unguided neural organoids. Split by condition (Control vs AxD). Legend includes bar plot showing absolute cell counts across clusters in each condition (top: Control, bottom: AxD). (B) Expression patterns of selected marker genes highlighting neuroectodermal clusters, as well as off-target populations that were overrepresented in AxD organoids. (C) DEA comparing clusters of radial glia (magenta) and neuronal progenitors (green) between control and AxD conditions. GO overrepresentation analysis showing the most up- and downregulated gene ontology (GO) terms from these clusters in the AxD organoids. (D) Immunofluorescent images of day 165 control and AxD unguided neural organoids stained for GFAP (green), S100B (magenta) and Hoechst (gray). Scalebar = 50 µm.

Given the broad lineage potential and heterogeneity of unguided neural organoids, we next investigated whether a more directed differentiation approach could enhance the neuroectodermal commitment in AxD-organoids. Dual SMAD inhibition is a well-established method for promoting neuroectodermal induction and is routinely employed in the generation of cortical organoids. We therefore tested whether this approach could rescue the lineage specification defect observed in previous experiments.

Cortical organoids were cultured for 165 days prior to scRNAseq to assess the impact of directed differentiation. Based on marker gene expression, we annotated 12 transcriptionally distinct populations of cells (Fig. 8A and 8B), indicating reduced cellular diversity compared to unguided neural organoids. These included neuronal and glial populations spanning multiple stages of maturation. AxD cortical organoids displayed heavily diminished neuronal populations, and the remaining neuronal population lacked *FOXG1* expression, a key forebrain marker (Fig. 8C). In addition, AxD organoids did not generate pre-OPCs and instead displayed an enrichment of radial glia.

We also observed increased representation of non-neuronal lineages in AxD cortical organoids. In contrast to controls, the AxD cortical organoids contained large populations of muscle cells and a greater abundance of mesenchymal-like cells, mirroring the off-target differentiation previously observed in the unguided organoid model.

To further examine astrocyte development under dual SMAD inhibition, we interrogated the AxD and control outer radial glia/astrocyte populations through DEA (Fig. 8D). The transcription factors *SOX9* and *NFIB*, which are critical for gliogenesis, were significantly downregulated in AxD organoids, along with astrocytic markers *AQP4*, *FABP7*, *BCAN,* and *NCAN*. Downregulated genes also included the forebrain marker *FOXG1*, the neurogenesis-associated gene *NNAT*, and the synaptic protein *NRXN1*.

Upregulated genes included *CHL1*, a neural cell adhesion protein; *SPARCL1* and *FN1*, which contribute to ECM architecture; and *CRABP2*, a retinol-binding protein. Upregulated GO terms included axonogenesis, collagen-containing extracellular matrix, and components of the endoplasmic reticulum lumen, while the GO enrichment analysis of downregulated genes involved cell-fate commitment, gliogenesis, and forebrain development.

Finally, to validate these transcriptional changes, we performed immunocytochemical analysis on day 165 cortical organoids, staining for SOX9 as astrocytes/outer radial glia marker, DCX as an early neuronal marker, and FOXG1 as a forebrain marker (Fig. 8E). Quantifications revealed a prominent reduction of both $SOX9^{+}$ and DCX^{+} cells, and a dramatic decrease of $FOXG1^{+}$ cell in the AxD condition (Fig. 8F), consistent with the scRNAseq data.

These findings indicate that, even under directed differentiation, AxD organoids fail to generate essential neural and astroglial populations, providing mechanistic evidence that the *GFAP*^{R239C} mutation disrupts neuroectodermal commitment during early developmental stages.

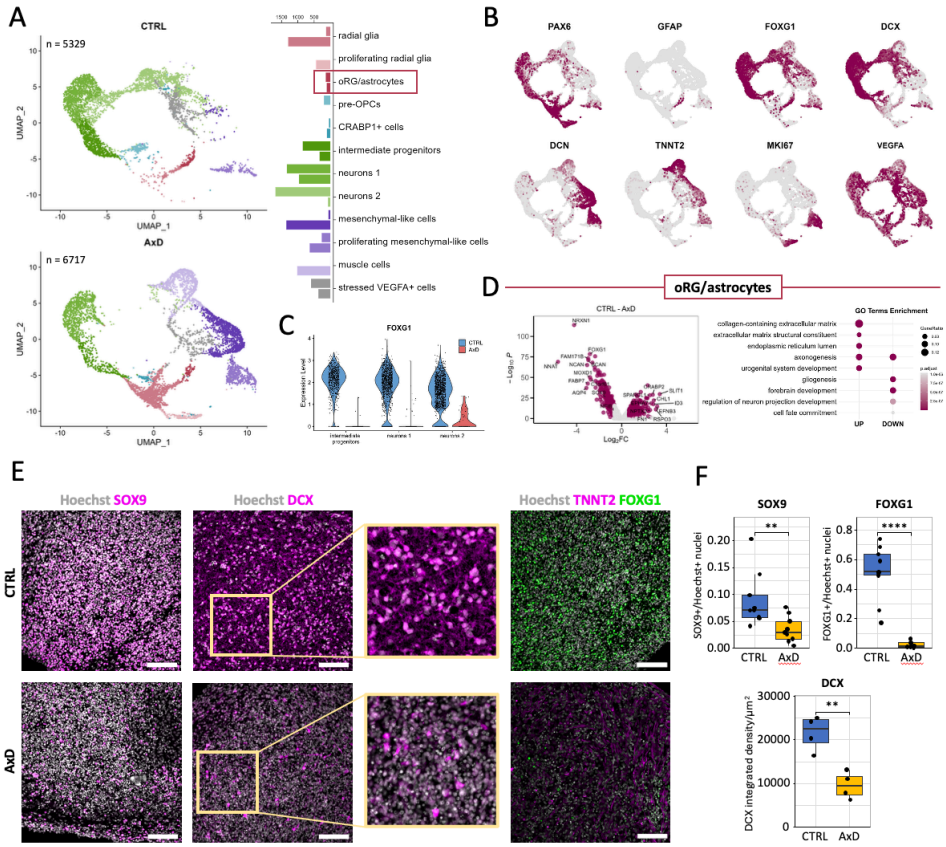


Figure 8: Directed Differentiation Fails to Rescue Neural and Astroglial Specification Deficits in AxD Cortical Organoids.

(A) UMAP projections of annotated cell populations from scRNAseq analysis of day 165 cortical organoids differentiated under dual SMAD conditions. Split by condition (Control vs AxD). Legend includes bar plot displaying absolute cell counts across annotated clusters in each condition (top: Control, bottom: AxD). (B) Expression of lineage-specific marker genes highlighting neuronal and glial populations, along with mesoderm-associated clusters enriched in AxD organoids. (C) Feature plot showing FOXG1 expression across neuronal clusters, with reduced levels observed in AxD-derived cells. (D) Differential gene expression analysis performed on outer radial glia/astrocyte clusters (magenta), comparing control and AxD organoids. Dot plot summarizes GO terms with the greatest enrichment among upregulated and downregulated genes in AxD samples. (E) Immunofluorescence images of day 165 cortical organoids stained for SOX9 (outer radial glia/astrocytic marker, magenta), DCX (immature neuronal marker, magenta), FOXG1 (forebrain identity, green), and TNNT2 (mesodermal marker, magenta), shown for control and AxD conditions. Nuclei counterstained with Hoechst. Scale bar = 100 µm. (F) Quantification of immunofluorescent signal intensity for SOX9 and FOXG1, normalized to Hoechst nuclear signal. Data points represent three fields of view from independent organoids per condition. DCX signal was quantified as integrated density per µm² across four organoids per group. No significant difference observed in total Hoechst staining.

Neural development requires precise coordination of lineage specification and cellular maturation; even subtle deviations from this program can have profound consequences. Our study demonstrates that the *GFAP*^{R239C} mutation, associated with AxD, impairs the acquisition of neural identity and astrocyte differentiation across multiple human *in vitro* models. In astrocyte cocultures, AxD astrocytes exhibited immature molecular profiles and disrupted signaling behavior, which led to non-cell-autonomous transcriptional changes in adjacent neurons, including the suppression of neurogenic and synaptic pathways, underscoring the interdependence of glial and neuronal development. Unguided neural AxD organoids exhibited impaired generation of astrocytes and other neural cell types, accompanied by a relative expansion of mesodermal and endodermal lineages. Notably, cortical organoids produced via dual SMAD inhibition failed to rescue neural differentiation potential, indicating a persistent disruption of neuroectodermal specification. Despite directed neuroectodermal patterning, AxD organoids continued to exhibit a depletion of neuronal and glial populations, coupled with elevated expression of non-neural lineage markers. These converging phenotypes suggest an early developmental defect driven by the *GFAP*^{R239C} mutation, which compromises both astroglial and neuronal trajectories.

Generation of MLC1-mutated human pluripotent stem cells (Paper II)

Both Alexander disease and Megalencephalic Leukoencephalopathy with subcortical Cysts (MLC) are rare CNS disorders involving primary astrocyte dysfunction. Still, they arise from distinct molecular mechanisms and lead to divergent pathological outcomes. Despite these differences, their shared classification as astrocytopathies highlights a broader vulnerability of the developing brain to glial disruption. To explore disease mechanisms in MLC and expand our stem cell-based modeling efforts, we generated human pluripotent stem cell lines carrying pathogenic mutations in *MLC1*. Given the limited availability of physiologically relevant human models for this disorder, the engineered cell lines developed in this study provide a platform for examining the impact of *MLC1* mutations on astrocyte function and their contribution to central nervous system dysregulation.

To create robust hPSC-based disease models of MLC, we designed genome editing strategies to generate human embryonic stem cell (hESC) lines with homozygous, recessive mutations in *MLC1*. The selected variants, S93L in exon 4 and S280L in exon 10, are both pathogenic mutations identified in patients with the disease. S93L is associated with a milder phenotype, while S280L represents a more severe and less common variant of the disorder (Ilja Boor et al., 2006). Genome editing was performed using both the Cas9 and Cas9 nickase systems, with two sgRNAs targeting each locus. Screening of edited clones yielded three homozygous lines for S93L, and two homozygous and one heterozygous line for S280L.

Following clonal expansion, we assessed whether any on-target genomic disruptions had occurred, as such changes frequently go undetected in standard quality control (QC) workflows (Weisheit et al., 2021). To this end, we developed custom quantitative genomic PCR (qgPCR) assays for both loci. This analysis revealed loss-of-heterozygosity (LOH) in one of the S93L clones (Fig. 9A) and all three S280L clones (Fig. 9B). Based on these findings, we selected the two verified S93L clones, A8 and A10, for further analysis.

In addition to on-target effects, CRISPR-based genome editing is known to introduce off-target mutations due to partial homology between sgRNAs and unintended genomic regions. To assess this, we screened the top five predicted off-target sites for the sgRNA used to generate the S93L clones, and no unintended edits were detected.

We then proceeded with standard quality control measures to validate the identity and properties of the edited hESC lines. We analyzed the expression of markers of undifferentiated state (Fig. 9C), which showed that SOX2, NANOG, and OCT3/4 were expressed at levels comparable to those of the parental hESC line. To confirm functional pluripotency, we performed trilineage differentiation followed by immunocytochemical analysis, which validated that the clones could differentiate into all three germ layers.

Short Tandem Repeat (STR) profiling ruled out cross-contamination and confirmed that the edited lines originated from the parental hESC clone. Mycoplasma testing performed by an independent service confirmed that the cultures were pathogen-free.

Given the potential for chromosomal abnormalities during prolonged culture or genome editing, we performed molecular karyotyping on both S93L lines and the parental hESCs. While no editing-induced abnormalities were identified, genomic analysis revealed pre-existing structural variants in the parental line that were also present in the derived clones (Fig. 9D).

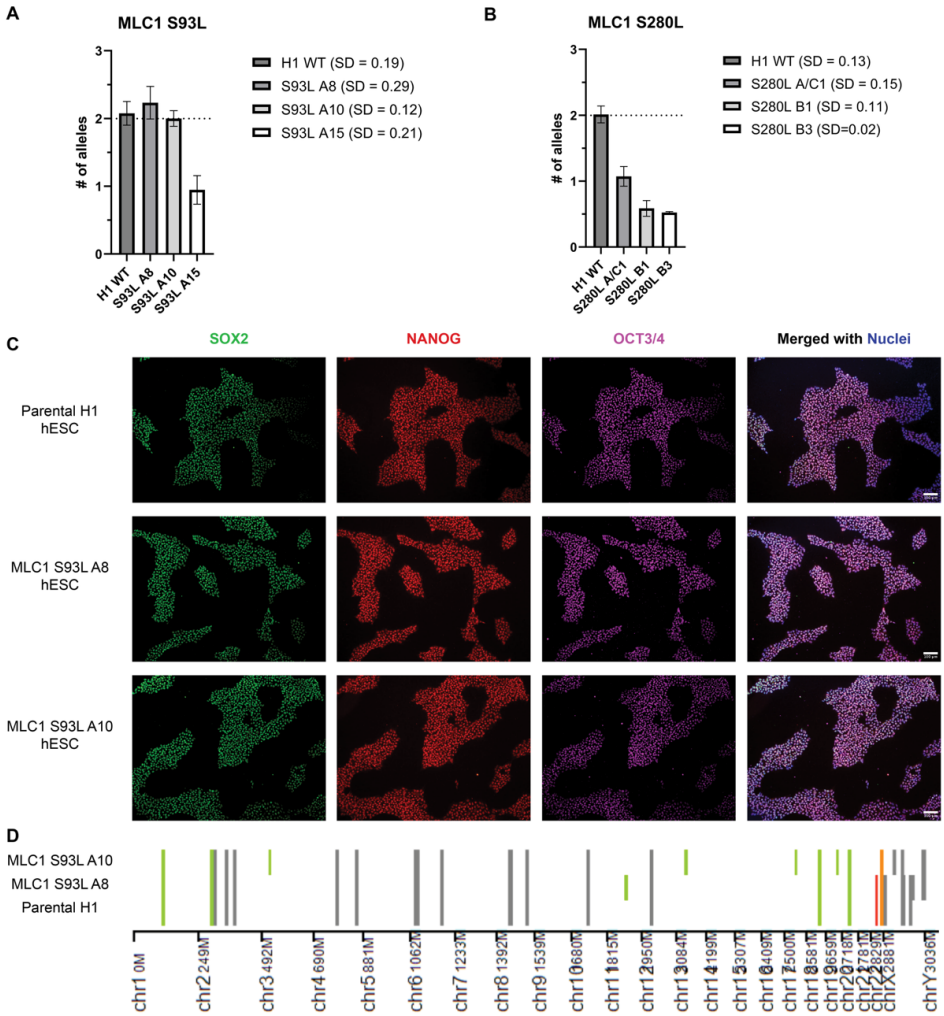


Figure 9: Characterization of *MLC1*-edited hESC lines.
(A) Quantitative genomic PCR results for the S93L locus, displaying the number of detected alleles per clone. (B) Quantitative genomic PCR results for the S280L locus, displaying the number of detected alleles per clone. (C) Immunocytochemical analysis of undifferentiated state markers in the parental H1 hESC line and two MLC1^{S93L} clones (A8 and A10). Cells were stained for SOX2, NANOG, and OCT3/4. Scale bar = 100 µm. (D) Molecular karyotyping results showing identified genetic variants in the parental line and derived S93L clones.

While the two MLC1^{S93L} clones passed key QC criteria and could be used for disease modeling, the presence of pre-existing genetic variants inherited from the parental hESC line limits their application. To overcome these constraints and avoid confounding effects, the genome editing strategy utilized in this paper can be applied to karyotypically stable PSC lines to generate *MLC1*-mutant lines suited for isogenic comparisons and mechanistic studies.

Generation of reporter hiPSC lines for visualization of endogenous α-synuclein in disease models of neurodegenerative disorders (Paper III)

In certain lysosomal storage disorders, such as Gaucher disease (GD), the accumulation of undegraded substrates within lysosomes subsequently results in secondary aggregation of α-syn. Since α-synuclein is genetically encoded, it can be endogenously tagged by genome engineering, allowing for the visualization of protein aggregation and lysosomal accumulation.

Using fibroblasts from a healthy control (HC), we employed mRNA-based reprogramming to generate an HC hiPSC line. Our goal was to generate an α-synuclein reporter line that preserved both the functional integrity and native expression profile of the protein. Therefore, we designed three different genome editing approaches to tag the *SNCA* gene, which encodes α-syn, using CRISPR/Cas9 (Fig. 10A).

Given the amphipathic nature and synaptic localization of the protein, we inserted a short HA-tag at either the N- or C-terminus, and integrated mCherry exclusively at the C-terminus (Fig. 10B). Sanger sequencing was used to confirm the integration of the tags into *SNCA* (Fig. 10C). Next, we performed a thorough characterization, assessing the undifferentiated state, G-band karyotype, and off-target effects of the tagged hiPSC lines in order to confirm that the genome editing had not conferred any unwanted effects.

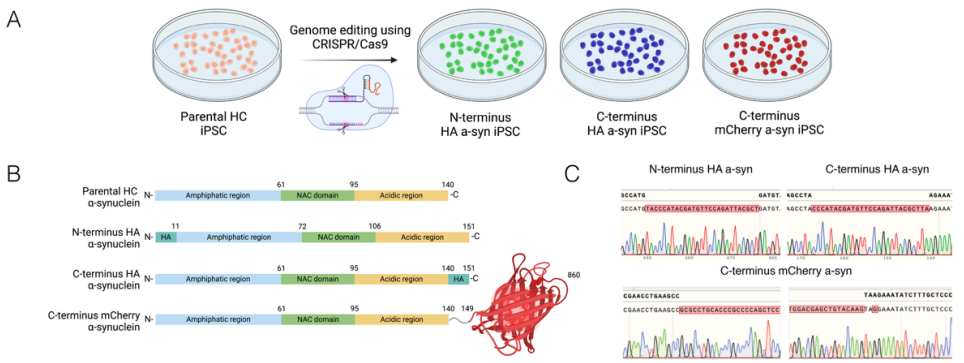


Figure 10: Generation of *SNCA*-tagged iPSC lines.
(A) Overview of CRISPR/Cas9-based genome editing strategies used to insert HA-tag or mCherry into *SNCA*. (B) Schematic of α-synuclein structure showing the amphipathic region, NAC domain, and acidic tail, with tag placement at the N- or C-terminus. (C) Representative Sanger sequencing chromatograms confirming tag integration at the *SNCA* locus.

To verify that the tagged proteins were of the correct molecular weight, we differentiated iNs in monoculture for 21 days, followed by protein extraction and Western blot analysis against both tagged protein and the introduced tags (Fig. 11C). In the N-terminal HA-tagged line, α-synuclein expression was markedly reduced compared to the parental line, and no detectable HA signal was observed, indicating that N-terminal tagging may interfere with protein stability, compromise epitope accessibility, or promote construct degradation. By contrast, the C-terminus HA-tagged line exhibited α-synuclein expression comparable to the parental line, accompanied by a strong HA signal, approximately 1 kDa heavier than the untagged protein, consistent with the expected mass of the tagged construct.

For the mCherry-tagged line, the C-terminus fusion was predicted to increase the protein size by ~31 kDa. Western blot analysis revealed a corresponding band detected by both α-synuclein and mCherry antibodies (Fig. 11D, red arrow), along with additional lower-molecular-weight bands (Fig. 11D, blue arrows) recognized by mCherry alone or by both antibodies. These bands are likely the result of proteolytic cleavage, yielding truncated protein fragments that retain mCherry and, in some cases, α-synuclein immunoreactivity.

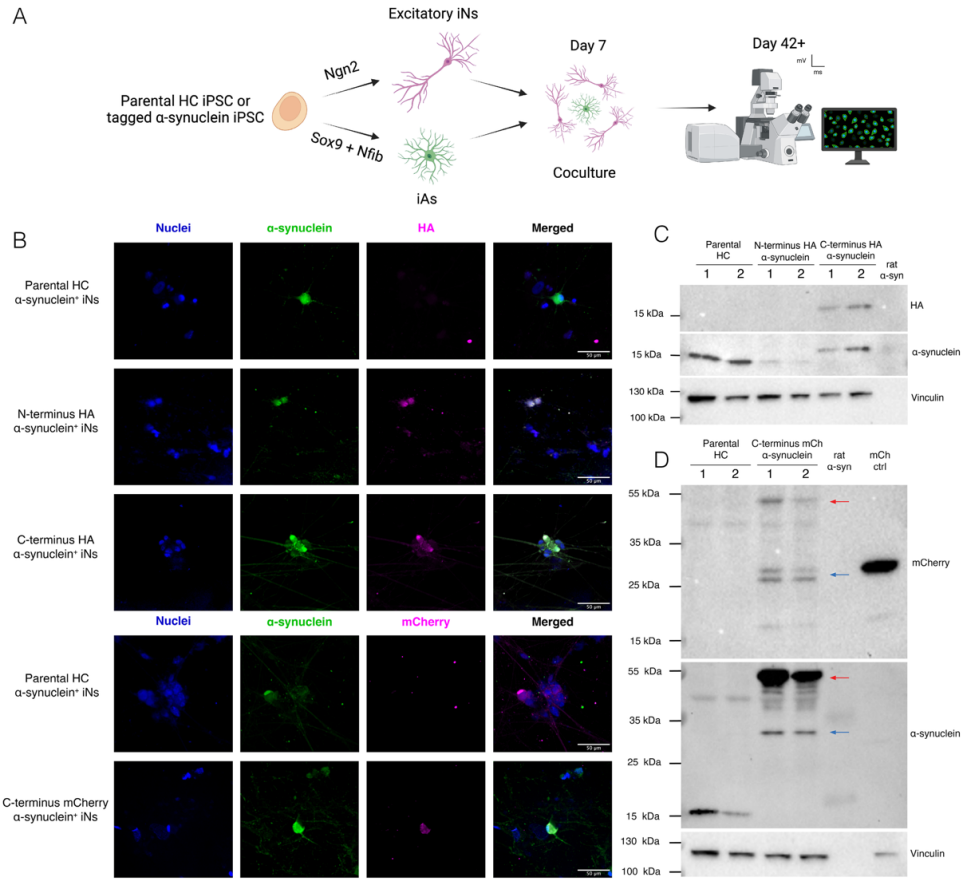


Figure 11: N-terminus HA-tag affects protein expression and C-terminus mCherry-tag causes cleavage of fusion protein.
(A) Schematic of the forward programming strategy used to generate cocultures of iNs and iAs for imaging experiments. (B) Representative images of mature cocultures stained for α-synuclein (green) and the respective tag (HA or mCherry, magenta). Scale bar = 50 µm. (C) Western blot analysis of protein extracts from iNs derived from the parental and HA-tagged lines stained for α-synuclein and HA. Rat brain lysate overexpressing α-synuclein served as positive control. Vinculin was included as loading control. (D) Western blot of iNs from the parental and C-terminus mCherry-tagged line stained for α-synuclein and mCherry. Controls included rat brain lysate overexpressing α-synuclein and 293T cells expressing mCherry. Vinculin was used as loading control.

To assess whether tagging of α-synuclein alters neuronal function, we generated mature cocultures of iNs and iAs from each line and performed whole-cell patch-clamp recordings to characterize both passive and active electrophysiological properties. The resting membrane potential did not differ significantly across the parental and three tagged lines (Fig. 12A), consistent with earlier reports using this culture system (Canals et al., 2023), indicative of functional neuronal maturity.

Next, we quantified the proportion of neurons capable of eliciting spontaneous action potentials (APs) and exhibiting spontaneous excitatory postsynaptic currents (EPSCs) (Fig. 12B). Neurons derived from the parental HC and both C-terminus tagged lines displayed 80-90% responsiveness for both APs and EPSCs. In contrast. those from the N-terminus HA-tagged line exhibited reduced activity, with only ~50% of cells functionally active for both parameters. This diminished excitability and synaptic response suggest that N-terminus tagging of α-synuclein impairs the protein´s synaptic function. Given this deficit, the N-terminus HA-tagged line was excluded from further electrophysiological analysis.

We then compared passive neuronal properties between the parental HC and the C-terminus-tagged lines. No significant differences were observed in input resistance (RI, Fig. 12C) or membrane capacitance (Cm, Fig. 12D), supporting preservation of baseline electrical characteristics. Similarly, analysis of action potential (AP) threshold (Fig. 12E) and maximum AP amplitude (Fig. 12F) revealed no significant differences between the C-terminally tagged and parental lines, indicating that neuronal firing properties were preserved following C-terminal tagging. Finally, analysis of synaptic transmission revealed that both spontaneous EPSC amplitudes (Fig. 12G) and EPSC frequency (Fig. 12H) were comparable between the parental HC and C-terminus-tagged lines, indicating that synaptic connectivity was not compromised.

Altogether, these data demonstrate that placement of an HA epitope or mCherry tag at the C-terminus of α-synuclein does not interfere with the functional properties of hiPSC-derived induced neurons. In contrast, N-terminal HA-tagging impaired neuronal excitability and synaptic transmission, highlighting the structural sensitivity of the amphipathic domain and its potential incompatibility with genetic tagging strategies.

After observing that the C-terminus mCherry tag leads to fusion protein cleavage and the N-terminus HA tag compromises neuronal function, we concluded that the C-terminus HA-tagged hiPSC line was the most suitable model for visualizing endogenous protein accumulation. To demonstrate its utility in disease modeling, we used this line to study α-synuclein distribution and aggregation. We generated cocultures of iNs and iAs and treated them with BFA1, an inhibitor of cellular autophagy, to promote intracellular accumulation of α-syn. Using confocal microscopy and immunocytochemistry, we detected a strong and well-defined HA signal with a distribution pattern similar to α-synuclein staining (Fig. 12I). Quantification of HA intensity in untreated and BFA1-treated cocultures revealed a significant increase following BFA1 treatment (Fig. 12J), validating the usefulness of this line for tracking α-syn accumulation in a neuronal context.

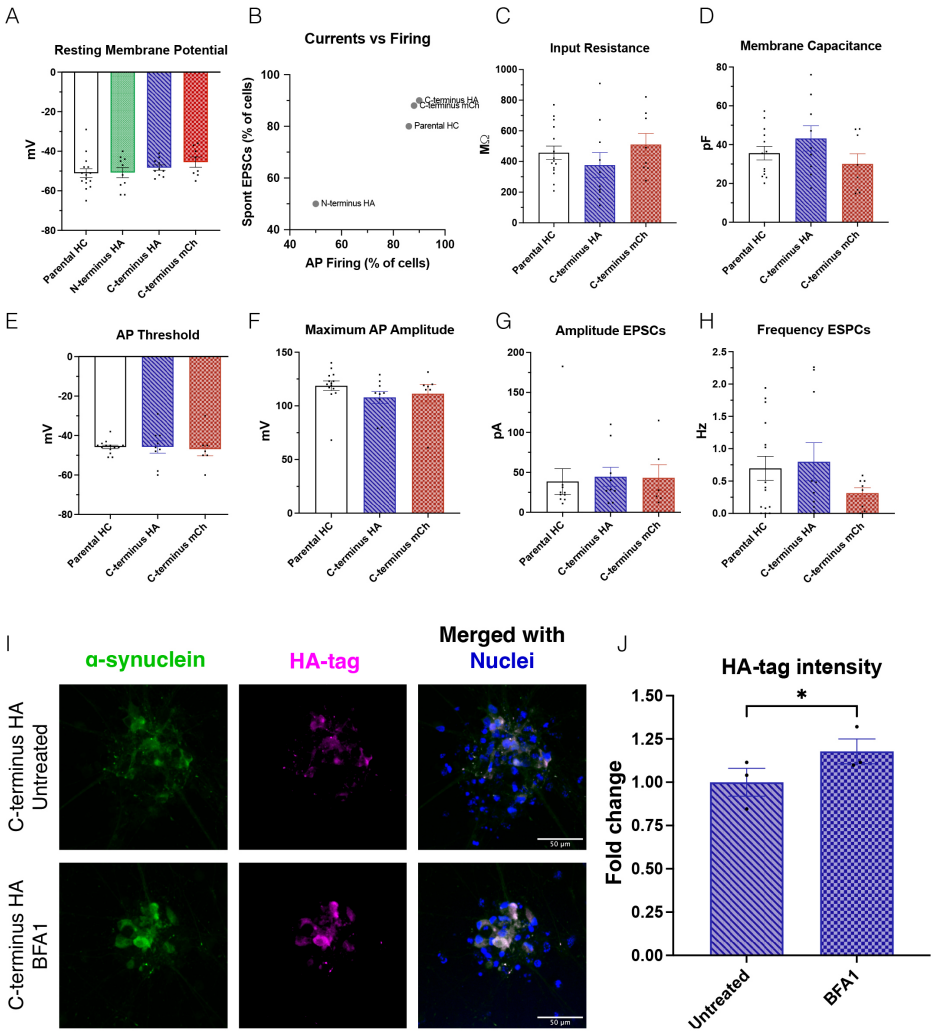


Figure 12: C-terminus tagging of α-synuclein preserves neuronal function and C-terminus HA iNs enables visualization of protein accumulation.

(A) Resting membrane potential of iNs from the different iPSC lines measured in mV. (B) Proportions of patched iNs capable of firing spontaneous action potentials (X-axis) and exhibiting spontaneous excitatory postsynaptic currents (Y-axis), compared across all lines. (C) Input resistance of iNs from the different iPSC lines measured in MΩ. (D) Membrane capacitance of iNs from the different iPSC lines measured in pF. (E) Action potential threshold of iNs from the different iPSC lines measured in mV. (F) Maximal action potential amplitude of iNs from the different iPSC lines measured in mV. (G) Amplitude of the excitatory postsynaptic currents of iNs from the different iPSC lines measured in pA. (H) Frequency of the excitatory postsynaptic currents of iNs from the different iPSC lines measured in Hz. (I) Representative immunocytochemistry images of mature cocultures of iN and iAs from the C-terminus HA-tagged iPSC line with and without BFA1 treatment. Scale bar = 50 µm. (J) Fold change of HA intensity in cocultures derived from the C-terminus HA-tagged iPSC line, comparing BFA1 treated to untreated cells, and normalized within each independent experiment. Bar graph shows mean ± s.e.m. of 3 independent experiments.

In conclusion, this proof-of-concept experiment demonstrates that the C-terminus HA-tagged hiPSC line permits reliable and specific detection of endogenous α-synuclein via the introduced HA-tag, without relying on overexpression paradigms. As such, it represents a valuable tool for visualizing α-synuclein accumulation under lysosomal stress and for modeling synucleinopathies in human stem cell-derived systems.

Patient hiPSC-derived brain organoids of neuronopathic Gaucher disease identify imbalances in neural lineages and deficits in interneuron function (Paper IV)

Neuronopathic forms of Gaucher disease are characterized by profound neurodegeneration, although the underlying pathogenic mechanisms remain unclear. Neuropathological analyses have identified region-specific neuronal loss, particularly within cortical layers III and V, indicating selective vulnerability among distinct neuronal subtypes. To investigate this relationship, we developed a human *in vitro* model that closely mimics cortical development using regionally patterned forebrain organoids derived from patient-derived and CRISPR-edited hiPSC lines. Using this model, we aim to uncover novel cellular phenotypes and identify therapeutic targets arising during early cortical maturation.

To establish a reliable and disease-relevant *in vitro* model of Gaucher Disease, we first generated hiPSCs through mRNA-based reprogramming of fibroblasts from an acute neuronopathic GD patient (GD) and a healthy control (HC). Both lines underwent comprehensive characterization and quality control. This included assessment of stem cell morphology, expression of markers of undifferentiated state at both protein and mRNA-levels, as well as karyotyping, and evaluation of trilineage differentiation capacity. Unexpectedly, genetic analysis revealed that the HC carries a *GBA1*^{N370S} mutation, which is associated with non-neuronopathic GD and linked to increased risk of Parkinson Disease.

To generate isogenic controls, we employed targeted genome editing strategies. CRISPR/Cas9-based editing was used to disrupt *GBA1* in the HC hiPSCs, generating an isogenic *GBA1* knockout (HC-KO) line as well as to generate an isogenic control line to GD. However, due to the presence of a highly homologous pseudogene (*psGBA*) in close proximity to *GBA1*, editing specificity was a concern. However, we identified a unique 55-bp sequence within exon 9 that is absent in *psGBA*, allowing for precise targeting of *GBA1*. The *GBA1*^{P415R} mutation in the GD line is located near this region, allowing efficient correction using CRISPR/Cas9 while minimizing off-target editing of the pseudogene.

Correction of the second mutation in the GD line, *GBA1*^{L444P}, posed a greater challenge. It was located in exon 10 where there are no sequence mismatches between *GBA1* and *psGBA*. As a result, we adopted an alternative approach based on prime editing, which offers higher specificity for single-nucleotide changes. Using this strategy, we successfully corrected the *GBA1*^{L444P} mutation and generated the isogenic control line (GD-iso) to the GD hiPSCs.

Following genome editing, we performed a thorough characterization of the isogenic hiPSC lines to confirm that no unintended effects were introduced during the editing procedure. As a final validation step, all four hiPSC lines were differentiated into iNs for seven days, followed by a GCase activity assay to assess the enzymatic function of the *GBA1* gene product. This analysis confirmed successful rescue of enzymatic activity in the GD-iso line and a marked reduction in the HC-KO line, which exhibited lower GCase activity than the GD line. These results validate that we generated a patient-derived GD hiPSC line and an apparently healthy *GBA1* carrier (HC), and that we successfully corrected the GD phenotype in GD-iso while knocking out *GBA1* in HC-KO. Together, this set of genetically defined hiPSC lines models a spectrum of *GBA1*-associated disease states, ranging from fully healthy to complete enzyme loss, and represents a valuable resource for investigating the underlying mechanisms of neuronopathic Gaucher Disease.

Neuropathological studies of Gaucher disease have identified cortical degeneration and gliosis as prominent features in affected individuals (Furderer et al., 2022). The frequent occurrence of myoclonic seizures in neuronopathic forms further implicates dysfunction of cortical circuits in the underlying disease process (Nalysnyk et al., 2017). To model these aspects, we differentiated the four hiPSC lines into human forebrain cortical organoids (hCO) and human subpallial organoids (hSO), maintaining them in culture up to 150 days *in vitro* (DIV150).

To examine how GD influences lineage commitment and cell-type composition within hCO and hSO models, we performed scRNAseq on DIV150 organoids derived from the four hiPSC lines. Transcriptomic analysis revealed 21 distinct cell populations in the hCO (Fig. 13A) and 18 populations in the hSO (Fig. 13E), representing major neural lineages across progenitor, excitatory, inhibitory, and glial compartments (Fig. 13D and 13H).

In GD-derived hCOs, we observed increased proportions of excitatory neurons, Reelin-positive migrating neurons, mature interneurons, and neurons enriched for mitochondrial gene expression. These changes were accompanied by a reduction in proliferating progenitors, interneuron progenitors, and radial glia compared to both isogenic and healthy control lines (Fig. 13B and 13C). This pattern suggests a shift toward neuronal maturation at the expense of progenitor maintenance, potentially reflecting disrupted lineage trajectories or premature differentiation.

In GD hSOs, we identified elevated levels of migratory interneurons originating from the lateral ganglionic eminence and decreased representation of medium spiny neurons (Fig. 13F and 13G). These alterations suggest a selective bias in subpallial differentiation, implicating GD-associated perturbations in cell fate decisions relevant to inhibitory circuitry formation.

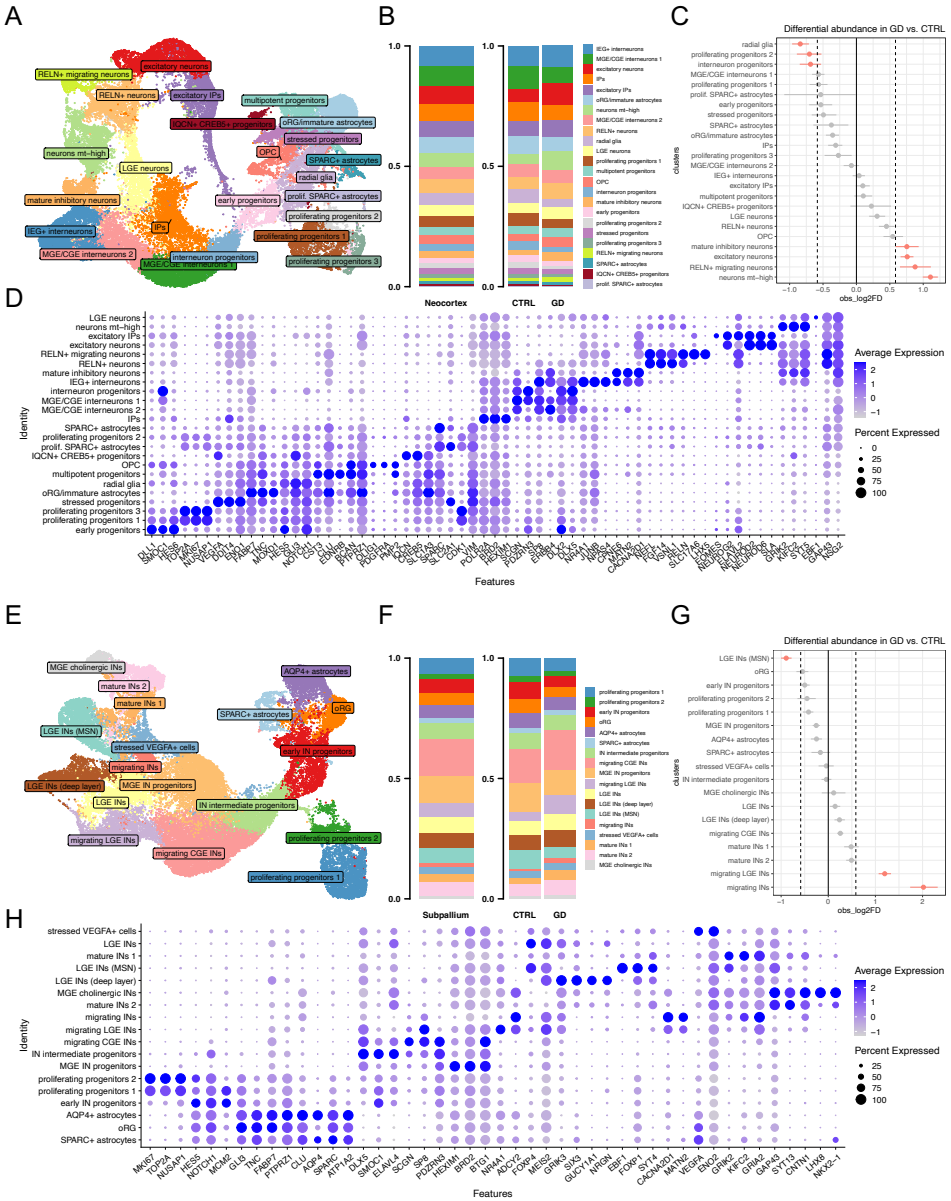


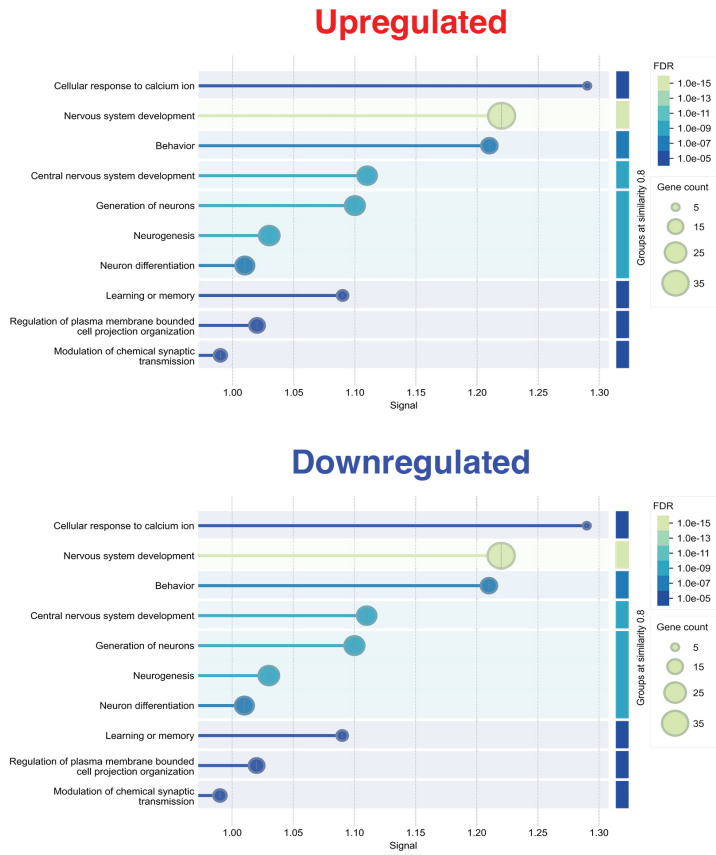
Figure 13: Single-cell transcriptomic analysis of cortical and subpallial organoids at DIV150.
(A) UMAP plot showing all cell populations identified in hCOs. (B) Bar plot depicting absolute cell counts of hCO populations for each condition (CTRL and GD). (C) Differential abundance of hCO cell populations comparing GD and CTRL conditions. (D) Dot plot showing expression of cell type-specific markers used to define hCO populations, with dot size indicating percentage of expressing cells and color representing average expression level. (E) UMAP plot showing all cell populations identified in hSOs. (F) Bar plot depicting absolute cell counts of hSO populations for each condition (CTRL and GD). (G) Differential abundance of hSO cell populations comparing GD and CTRL conditions. (H) Dot plot showing expression of cell type-specific markers used to define hSO populations, with dot size indicating percentage of expressing cells and color representing average expression level.

We performed gene ontology (GO) enrichment analysis on DIV150 hCO organoids to compare biological processes between GD and control conditions. Among the most prominent differences were pathways linked to inhibitory neuron development (Fig. 14A). Given the presence of both excitatory and inhibitory neurons in hCOs, this finding suggests increased susceptibility of inhibitory lineages in the context of GD. To investigate this further, we analyzed differentially expressed genes (DEGs) in GD hCOs to identify lineage-specific changes. Genes upregulated in Gaucher disease were closely linked to excitatory neuron development and predominantly expressed within excitatory neuronal populations (Fig. 14B). In contrast, downregulated genes were associated with inhibitory neurogenesis and localized to interneurons and their progenitors, suggesting a shift in lineage specification favoring excitatory over inhibitory neuronal fates. To validate these findings, we examined expression of *DLX* genes, key regulators of interneuron development, across hCOs from all four hiPSC lines. *DLX* expression was consistently reduced in both GD and HC-KO lines (Fig. 14C), strongly indicating an interneuron development deficit in our GD organoid models.

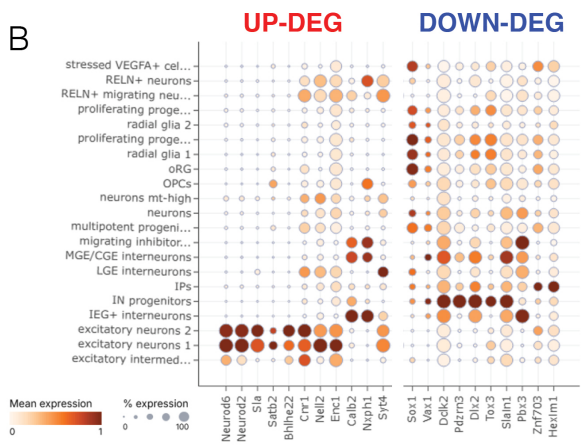
Day 150 Cortical DEGs

Biological processes (GO) enrichment

A



B



C

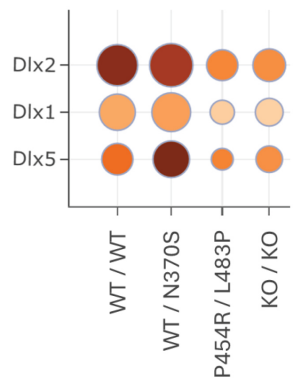
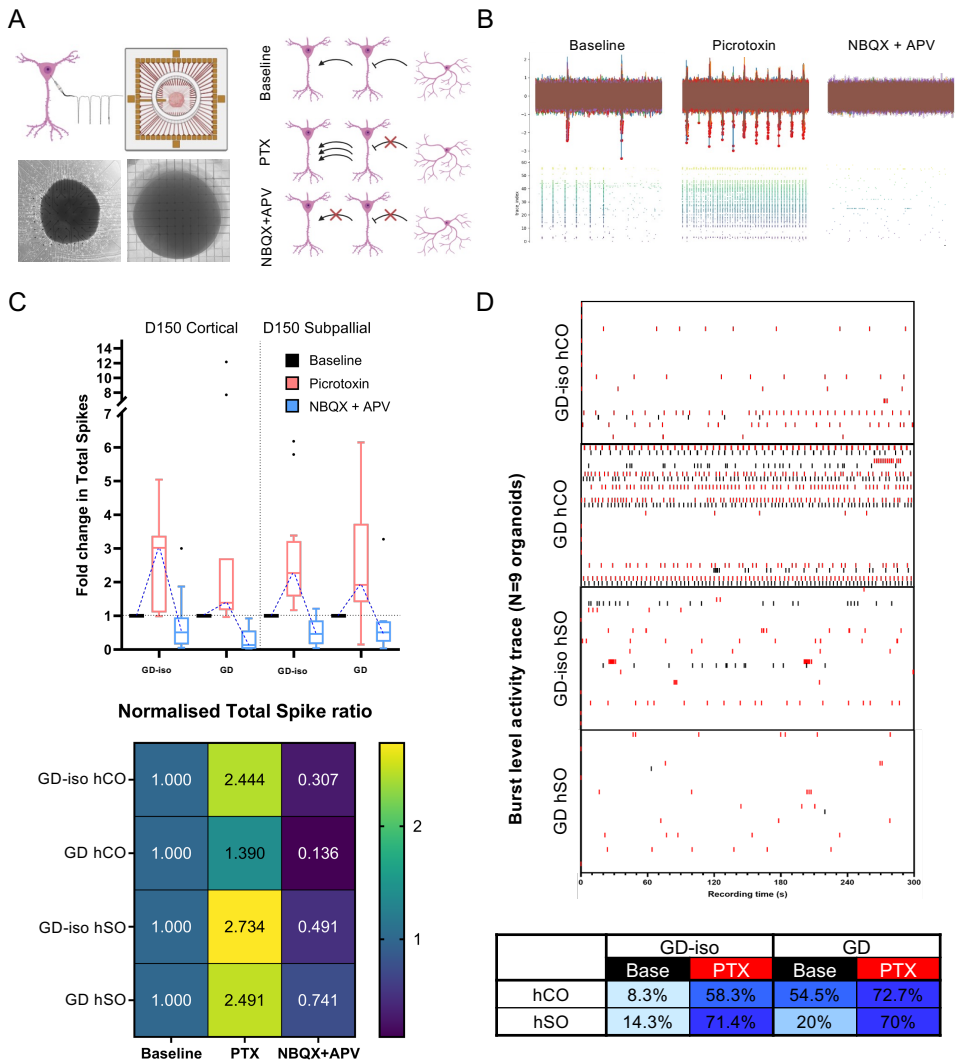


Figure 14: DEG analysis reveals inhibitory lineage disruption and calcium-associated gene dysregulation in GD-derived cortical organoids.

(A) Top 10 enriched biological processes from GO analysis of upregulated and downregulated genes in hCOs at DIV150. (B) Dot plot displaying expression patterns of selected DEGs across hCO populations, with dot size indicating percentage of expressing cells and color intensity representing average expression level. (C) Dot plot showing expression levels of Dlx family genes across hCOs derived from each hiPSC line, visualized by percentage of expressing cells (dot size) and average transcript abundance (color intensity).

Given the transcriptomic data pointing to impaired inhibitory neuron development, we examined functional deficits using Multi-Electrode Array (MEA) recordings of DIV150 hCO and hSO organoids. We focused on the GD and GD-iso lines to allow for direct comparison between a patient-derived model and its isogenic control. We designed an experimental paradigm to investigate inhibitory neuron deficits, first recording the basal activity, followed by a treatment to block inhibitory signaling, then proceeding with a block of inhibitory GABAergic signaling (Fig. 15A and 15B). MEA recordings revealed that blockade of inhibitory input led to a substantially greater increase in excitatory activity in GD-iso hCOs compared to GD hCOs. While GD organoids exhibited only a 1.39-fold increase, the isogenic control line showed a 2.44-fold increase in excitation (Fig. 15C). Basal activity levels were also markedly higher in GD hCOs relative to GD-iso counterparts (Fig. 15D). Together, these results support the presence of functional inhibitory deficits in GD organoids, consistent with transcriptional signatures of interneuron loss and altered lineage specification.

Taken together, these results indicate that GD hCOs exhibit reduced inhibitory responsiveness, leading to elevated basal activity and limited excitation gain following GABAergic blockade. These features reflect an imbalance in network-level excitatory and inhibitory dynamics, suggesting a shift toward heightened neural excitability, consistent with the presence of myoclonic seizures in neuronopathic Gaucher Disease.



	GD-iso		GD	
	Base	PTX	Base	PTX
hCO	8.3%	58.3%	54.5%	72.7%
hSO	14.3%	71.4%	20%	70%

Figure 15: MEA recordings reveal impaired inhibitory function and heightened network excitability in GD cortical organoids.

(A) Schematic of the MEA setup, experimental paradigm, and representative images of hCOs and hSOs positioned on electrode arrays. (B) Representative activity traces (top) and spike raster plots (bottom) recorded under basal conditions, following PTX treatment (GABAergic blockade), and after NBQX + APV application (glutamatergic blockade). (C) Fold change in total spike count (top) and corresponding quantification (bottom) comparing basal, PTX, and NBQX + APV conditions in GD and GD-iso organoids. (D) Raster plots showing burst activity under basal and PTX conditions (top), and table summarizing the proportion of organoids from GD and GD-iso lines displaying bursts across conditions (bottom).

Discussion

Bridging the gap in rare CNS disease research

For individuals affected by rare CNS disorders, the journey from diagnosis to care is often marked by prolonged uncertainty, delayed recognition, and limited therapeutic options. These disorders, although individually uncommon, collectively represent a significant clinical and scientific challenge, placing a considerable burden on patients, their families, and the healthcare system. Early-onset, rapid disease progression, and severe neurological manifestations, as those seen in Alexander disease, Megalencephalic leukoencephalopathy with subcortical cysts, and neuronopathic Gaucher disease, add further to the complexity of care.

Despite the growing availability of genetic diagnostics, which increasingly enable precise identification of pathogenic variants, bridging the gap between genotype and phenotype remains a significant challenge. More rare CNS disease-causing variants are being identified than ever before (Firdaus & Li, 2024), yet their functional impact often remains poorly understood. Effective translation of molecular findings into mechanistic insights and targeted interventions requires experimental systems that faithfully recapitulate human brain development, cellular diversity, and disease context.

Until recently, such models have been limited, particularly for capturing early neurodevelopmental pathology in pediatric CNS disorders. The emergence of human induced pluripotent stem cell (hiPSC)-derived models has transformed this landscape, offering a robust platform for investigating mechanisms underlying rare CNS disorders. Patient-derived hiPSC lines capture individual genetic backgrounds and disease-relevant mutations, allowing interrogation of mutation-driven functional impairments. To control for genetic variability, genome editing enables the generation of isogenic lines that differ only at the mutation of interest, allowing for controlled interrogation of mutation-specific effects. Forward programming protocols support efficient generation of neurons and astrocytes, enabling precise analysis of cellular phenotypes and transcriptional alterations. Three-dimensional organoid systems enhance physiological relevance by introducing spatial architecture and lineage diversity, making them particularly suited for modeling neurodevelopmental abnormalities.

When applied in combination, these complementary strategies enable reconstruction of early CNS development and disease-associated processes using genetically defined human cells, addressing longstanding challenges in modeling and supporting reproducible analysis of neurodevelopmental and glial contributions to CNS pathology.

Building upon these advances, the purpose of this thesis was to leverage genome editing with pluripotent stem cell technology to establish robust *in vitro* models of pediatric CNS disorders that enable targeted investigation of disease mechanisms at cellular and tissue levels. Specifically, to facilitate detailed investigation of disease-relevant mechanisms in neurons and astrocytes, two cell types integral to the pathophysiology of many neurodevelopmental and neurodegenerative conditions.

The work was structured around four key aims: (1) to decipher underlying disease mechanisms of AxD-causing *GFAP* mutations at a cellular level, using patient-derived hiPSCs differentiated into astrocyte-neuron cocultures and neural organoids to investigate transcriptional dysregulation, stress vulnerability, and lineage commitment; (2) to generate *MLC1*-mutated stem cell lines for *in vitro* modeling of MLC pathophysiology, by introducing S93L and S280L mutations into hESC through CRISPR/Cas9 genome editing; (3) to generate α-synuclein reporter hiPSC lines by introducing an HA-tag at the C-terminus of SNCA, enabling sensitive visualization of endogenous protein localization and accumulation in human neuronal models under lysosomal stress conditions; (4) to generate a robust human *in vitro* model of Gaucher Disease and identify novel disease phenotypes and therapeutic targets, using both patient-derived and CRISPR-edited hiPSC lines to create regionally patterned forebrain organoids.

Tailoring Human CNS model complexity to biological question

A central premise throughout this thesis is that model design should be tailored to the resolution required to address specific biological questions. This idea aligns with the aphorism: *all models are wrong, but some are useful*; their utility lies in how effectively they capture the features relevant to the question at hand. Rather than selecting models based solely on their complexity, each *in vitro* model was chosen to match the scope and depth of the biological question being asked. By combining both 2D coculture systems and 3D organoid platforms, the studies dissected CNS pathogenesis across multiple levels, from cell-type-specific disruptions to emergent developmental phenotypes within spatially organized tissue models. This adaptable, modular strategy facilitated targeted investigation of rare disorders, each defined by distinct cellular vulnerabilities and developmental trajectories.

The 2D coculture systems effectively revealed impaired astrocyte differentiation and disrupted neuron-astrocyte communication. In Paper I, cocultures of neurons and astrocytes demonstrated that *GFAP* mutations compromise astrocytic lineage fidelity and induce transcriptional changes in neighboring neurons. These results highlight the utility of controlled coculture models in identifying non-cell-autonomous effects, particularly the influence of astrocytes on neuronal identity and function.

3D organoid platforms provided enhanced spatial and developmental resolution compared to coculture systems. In the AxD unguided and cortical organoid models, the *GFAP*^{R239C} mutation disrupted neuroectodermal specification, leading to a reduced generation of astrocytes, neurons, and pre-OPCs, accompanied by an enrichment of mesodermal and endodermal lineages. Even under directed conditions using dual SMAD inhibition, AxD cortical organoids failed to restore forebrain fate and exhibited persistent misallocation of cell identities. Compared to the 2D cocultures, the organoids captured a broader range of developmental dysfunction, indicating that *GFAP* mutations influence not only astrocytic identity but also regional fate decisions, neuronal specification, and the coordinated assembly of forebrain circuits. These insights were only accessible through the spatial organization and temporal resolution provided by the 3D model system.

Paper IV used regionally patterned cortical and subpallial organoids with biallelic *GBA1* mutations to model Gaucher disease (GD). This system revealed early forebrain vulnerabilities, including progenitor depletion, interneuron differentiation defects, and network hyperexcitability. These features were identified using single-cell transcriptomics and MEA recordings, which together captured changes in both cell identity and neural activity. Neurodevelopmental abnormalities associated with *GBA1* mutations have also been observed in organoid models of Parkinson disease, including disrupted neuronal differentiation and dysregulated cell cycle progression (Rosety et al., 2023). Although the clinical presentations differ, this study supports the notion that *GBA1* mutations can disrupt cortical development and neural circuit formation across various disease contexts.

Looking forward, expanding CNS models to include additional cell types, such as microglia and oligodendrocytes, will be crucial for capturing complex multicellular disease mechanisms. In the Gaucher disease organoid system, incorporating microglia may uncover inflammatory features not captured by neuroectodermal models. This is particularly important given the growing evidence of neuroinflammatory involvement in neuronopathic GD. Methods for deriving microglia-like cells from hiPSCs now make it feasible to explore immune-neural crosstalk using triculture or fusion strategies with organoids (S.-W. Chen & Wong, 2023).

For Alexander disease, incorporating oligodendrocytes (García-León et al., 2018) alongside neurons and astrocytes in a triculture system would enable direct investigation of neuron-astrocyte-oligodendrocyte interactions, an area not addressed in existing models. While cocultures of astrocytes and oligodendrocytes have been explored previously (L. Li et al., 2018), this expanded setup would allow a mechanistic dissection of cell-intrinsic and non-cell-autonomous effects of *GFAP* mutations across all three major glial and neuronal lineages. These configurations may offer more profound insights into processes such as myelination, metabolic coupling, and glial support functions under pathological conditions.

Taken together, these studies illustrate that the usefulness of a model lies not in its complexity alone, but in its ability to illuminate disease-relevant biology. This thesis presents a modeling approach that adjusts system complexity to biological context, allowing clear interpretation of central nervous system pathology across molecular and cellular levels.

Disease mechanisms in rare CNS disorders

Although rare CNS disorders are defined by distinct genetic causes and clinical manifestations, the models explored in this thesis reveal convergent disruptions in neurodevelopmental trajectories. In Alexander disease, astrocyte-intrinsic dysfunction driven by *GFAP* mutations impairs glial maturation and alters neuronal identity through non-cell-autonomous mechanisms, as demonstrated in coculture systems and organoid models. In contrast, neuronopathic Gaucher disease involves lysosomal and metabolic stress that compromises progenitor maintenance and interneuron specification, as shown in regionally patterned organoids. Despite different molecular origins, both disorders exhibit disrupted lineage specification and signaling coordination during early forebrain development.

Previous studies have demonstrated that *GFAP* mutations compromise maturation and structural integrity of astrocytes. Kondo et al. (2016) described patient-derived AxD astrocytes with persistent Nestin expression, disrupted filament architecture, and impaired neuronal support. Battaglia et al. (2019) further elucidated the molecular mechanisms underlying these abnormalities, demonstrating that *GFAP* is subject to aberrant phosphorylation and caspase-mediated cleavage in AxD cells, leading to filament aggregation and cytoskeletal collapse. In our 2D coculture model, these defects were reflected morphologically by the emergence of astrocytes with elongated, polarized processes and hypertrophic somas, consistent with a radial glia-like morphology. These cells exhibited reduced circularity, increased perimeter, and limited expression of mature astrocytic markers, accompanied by a transcriptionally distinct cluster with ectodermal and immature glial gene signatures indicative of fate misdirection and differentiation arrest.

In parallel, these astrocyte abnormalities were associated with altered neurogenic support and non-cell-autonomous disruption. Li et al. (2018) reported that AxD astrocytes impair oligodendrocyte lineage progression and disrupt myelination, even in the absence of direct genetic defects in those cells. Extending this concept, our coculture system revealed comparable effects on neurons, which displayed downregulation of differentiation and adhesion-related genes, including *DCX* and selected protocadherins, suggestive of impaired maturation and migratory behavior. CellChat analysis revealed heightened signaling from the AxD cluster via RELN and EGF pathways, linked to progenitor retention, spatial guidance, and concurrent suppression of canonical neuro-glial cues such as TGFβ, FGF, and LIFR. These findings suggest that AxD astrocytes are unable to coordinate appropriate developmental signals, resulting in disrupted signaling dynamics and impaired cortical assembly.

Yi et al. (2025) reported that AxD astrocytes exhibit reduced laminin expression and basement membrane instability, contributing to cortical lamination defects and impaired cellular adhesion. Our transcriptomic data support and extend these findings, showing downregulation of key ECM and adhesion-related genes, including *BCAN*, *NCAN*, and *SPARCL1*. This disruption of structural scaffolding and spatial patterning may impair cortical layering and connectivity, reinforcing neurodevelopmental deficits initiated by signaling perturbation.

Although not phenotypically evaluated in this thesis, MLC exemplifies another astrocyte-driven leukodystrophy, linked to mutations in *MLC1*, *HEPACAM*, *GPRC5B*, and *AQP4*, which converge on ion and water regulation at the gliovascular interface (Passchier et al., 2024). Brignone et al. (2024) utilized primary human fetal astrocyte cultures to demonstrate that *MLC1* responds to calcium signaling and interacts with the chloride channel ClC-2 to mediate volume recovery. Disease-associated mutations impaired MLC1 membrane localization and assembly, disrupting its interaction with ClC-2 and leading to astrocytic swelling. These results provide mechanistic insights into glial osmoregulation; however, without stem cell-based models, it remains challenging to investigate how these defects affect early neural development. Compared with AxD and GD, which were studied using human pluripotent systems, MLC is less well characterized in early neurodevelopmental contexts. This highlights the need for robust *in vitro* platforms to clarify how astrocyte dysfunction contributes to leukodystrophy pathogenesis. The *MLC1* hPSC lines described in Paper II were not suitable for disease modeling due to inherited genetic variants present in the parental hESC line. To overcome this limitation, new genome-edited stem cell lines can be generated using genetically stable hPSCs with the genome editing strategy from this study. These refined models could be used to study how *MLC1*-related defects in ion and water regulation affect gliovascular interactions during early neural development, and how impaired volume control alters astrocyte function in MLC.

Building on insights from astrocyte-centered disorders, neuronopathic Gaucher disease presents a distinct model of early neurodevelopmental dysregulation. Although classified as a lysosomal storage disorder, GD exhibits early perturbations in progenitor dynamics and lysosomal function in pluripotent stem cell-based systems. Two key iPSC-based studies, Tiscornia et al. (2013) and Srikanth et al. (2021), have highlighted these pathological features. Tiscornia et al. reported premature neuronal differentiation and aberrant rosette formation in GD neural precursors, while Srikanth et al. identified lipid-mediated mTORC1 activation and autophagic defects. Our scRNAseq data similarly revealed progenitor depletion, accelerated excitatory neurogenesis, and reduced *DLX* gene expression, accompanied by deficits in GABAergic lineage maturation. Consistent with this, our MEA recordings indicated impaired inhibitory signaling in GD organoids, reflected in elevated baseline activity and reduced excitatory gain following GABAergic blockade. Collectively, these results suggest that neural progenitors in GD exhibit reduced proliferative potential and bias toward early excitatory differentiation, leading to impaired interneuron formation and disrupted cortical organization. The reduction in inhibitory signaling contributes to hyperexcitability in cortical organoids, reflecting neural dynamics similar to those underlying myoclonic seizures in neuronopathic Gaucher disease. Similarly, Srikanth and Feldman (2020) identified elevated Dkk1 expression and suppressed Wnt signaling in GD-derived neural progenitor cells, resulting in loss of lysosomal integrity and impaired neurogenesis. Rescue of Wnt signaling with Wnt3 reversed these deficits, highlighting a regulatory axis through which lipid dysregulation may influence progenitor maintenance and glial-neuronal cross-talk. While our study did not directly interrogate Wnt activity, the depletion of interneuron progenitors and reduction of regional patterning genes suggest upstream disruption in morphogen gradients, potentially implicating Wnt and other developmental pathways in GD-associated cell fate imbalance.

Mitochondrial dysfunction has long been proposed in GD, but evidence from human models remains limited. Osellame et al. (2013) demonstrated fragmented mitochondria and defective mitophagy in a GBA1 knockout mouse, attributed to lysosomal and proteostasis defects. While mechanistically informative, full knockouts differ from the genotypes found in patients, potentially amplifying pathology and bypassing compensatory mechanisms. This limitation also applies to our GD-KO iPSC line, which, despite its value for studying *GBA1*-dependent effects, does not fully capture patient-specific disease features. Our GD organoids recapitulate features of this mitochondrial defect, with upregulation of mitochondrial genes in migratory interneurons and enriched signatures related to oxidative phosphorylation. These cells may represent metabolically stressed subpopulations vulnerable to degeneration or impaired migration. While further mechanistic clarification is needed, our findings point to a link between defective lipid catabolism and disrupted mitochondrial function, potentially mediated by compromised lysosomal activity.

While previous studies have begun to examine astrocyte contributions in Gaucher disease, our organoid model did not specifically assess astrocyte pathology. Aflaki et al. (2020) and Yarkova et al. (2024) reported lysosomal stress and mitochondrial fragmentation in GD and *GBA1*-PD astrocytes, pointing to potential glial involvement in proteostatic and inflammatory responses. In our model, transcriptomic profiles and structural markers indicate deviations in glial lineage specification; however, further analysis will be necessary to clarify the extent and cell-type specificity of astrocyte dysfunction.

Together, the findings presented in this thesis underscore neurodevelopmental disruption as a central feature of rare CNS disorders, shaped by distinct yet converging pathogenic mechanisms. In Alexander disease, intrinsic astrocyte dysfunction disrupts signaling and extracellular organization, altering the developmental environment of neighboring neural populations. In Gaucher disease, lysosomal and metabolic stress impair progenitor maintenance and promote early excitatory differentiation at the expense of interneuron specification, resulting in disrupted inhibitory signaling and altered network excitability. Through the integration of 2D coculture systems and 3D organoid models, this thesis examines the timing, spatial organization, and cellular interactions associated with disease onset. These findings provide a basis for future therapeutic strategies aimed at correcting glial dysfunction, alleviating metabolic stress, and restoring cell signaling during early disease progression.

Resources for modeling of α-synuclein dynamics

To further explore how lysosomal dysfunction contributes to disease mechanisms, the HA-tagged *SNCA* hiPSC line generated in this thesis provides a targeted platform for studying α-synuclein accumulation under lysosomal stress. By enabling the specific detection of endogenous α-synuclein via epitope tagging at the *SNCA* C-terminus, this resource supports the visualization of its accumulation and facilitates the study of α-synuclein-associated mechanisms in hPSC-derived systems that model lysosomal storage disorders and synucleinopathies.

Alternative *in vivo* models have been developed to address limitations in capturing long-term protein dynamics and systemic aspects of α-synuclein pathology. The α-synuclein-GFP transgenic mice described by Hansen et al. (2013) were generated using a bacterial artificial chromosome (BAC) construct, which expresses human wildtype α-synuclein fused to GFP under the control of the mouse α-synuclein promoter. This model enables tracking of tagged protein aggregates and demonstrates age-dependent progression of pathology relevant to PD (Hansen et al., 2013). Similarly, the zebrafish model developed by Zini et al. expresses mCherry-tagged human α-synuclein under neuronal promoters, allowing for targeted

expression within the nervous system and replicating key features of Lewy body disorders, including aggregation and neurodegeneration (Zini et al., 2025). Both models use transgenic constructs that bypass endogenous SNCA regulation, which may alter α-synuclein expression and limit their suitability for modeling disease-relevant regulatory mechanisms.

For transcriptional-level interrogation, Basu et al. introduced a CRISPR/Cas9-based NanoLuciferase reporter at the 3′ end of the endogenous *SNCA* gene in HEK293T cells. This approach enables real-time monitoring of *SNCA* expression within its native regulatory context, complements protein-level analyses by capturing transcriptional responses (Basu et al., 2017). Although it may be less applicable to lysosomal storage disorder models, where α-synuclein accumulates due to secondary aggregation, it remains valuable for investigating transcriptional mechanisms implicated in α-synuclein-related pathologies.

A further contribution to transcriptional screening comes from Stahl et al., who developed a SNCA-GFP-luciferase reporter cell line to identify modulators of α-synuclein expression through high-throughput compound screening (Stahl et al., 2021). Although focused on transcriptional regulation, this approach underscores the utility of reporter systems for compound screening.

The HA-tagged *SNCA* hiPSC resource established in this thesis, when further engineered to include disease-relevant mutations resulting in α-synuclein accumulation, may be used to explore factors influencing α-synuclein localization and aggregation, complementing transcriptional screening approaches such as those demonstrated by Basu et al and Stahl et al. Unlike transgenic models, this system enables endogenous protein expression in human neurons, providing a physiologically relevant context for studying stress-related dynamics and disease mechanisms. In doing so, it extends the experimental framework developed in this work for investigating lysosome-related pathways.

Conclusion and future directions

The integrated 2D coculture and 3D organoid platforms established in this thesis provide a versatile and physiologically relevant framework for dissecting cellular and molecular mechanisms underlying rare CNS disorders, with a particular focus on Alexander disease and neuronopathic Gaucher disease. These models enable systematic investigation of disease mechanisms, spanning cell-intrinsic dysfunction and broader alterations in tissue organization and developmental patterning.

By leveraging patient-derived and genome-edited pluripotent stem cell lines, this thesis established direct comparisons between disease and isogenic control models, reducing genetic variability and enabling focused investigation of mutation-specific effects. The 2D coculture system enabled investigation of cell-autonomous abnormalities and non-cell-autonomous influences specific to glial-neuronal signaling. This model effectively demonstrated that *GFAP* mutations in AxD astrocytes perturb neuronal identity and transcriptional regulation, highlighting the role of intercellular signaling in guiding neurodevelopmental processes. Complementing these findings, the 3D organoid system provided insight into spatial patterning, interneuron migration, and metabolic adaptation across neural lineages. In both Alexander disease and neuronopathic Gaucher disease models, organoids revealed disruptions in progenitor maintenance, lineage specification, and regional identity, demonstrating the utility of spatially organized systems for modeling early neurodevelopmental pathology. Used in combination, the 2D and 3D models deepen mechanistic understanding of how glial dysfunction and lysosomal stress perturb neurodevelopmental processes and contribute to neurodegenerative progression. Although Alexander disease and neuronopathic Gaucher disease arise from distinct genetic mutations, both exhibit overlapping features, including disrupted cell communication, metabolic imbalance, and abnormal lineage development, all of which contribute to their neurological symptoms.

The HA-tagged SNCA hiPSC line generated in this thesis serves as a valuable resource for examining α-synuclein dynamics in human neurons. By tagging the endogenous protein, it enables reliable detection under physiological conditions, avoiding the confounding effects of overexpression paradigms. This system enables the tracking of endogenous α-synuclein accumulation under lysosomal stress, supporting the investigation of disease mechanisms in both lysosomal storage disorders and synucleinopathies.

Future studies will benefit from the inclusion of additional cell types, such as microglia and oligodendrocytes, to capture multicellular interactions and inflammatory responses. Microglia may help clarify how immune-neural signaling contributes to neuroinflammation and disease progression, particularly in neuronopathic Gaucher disease. Likewise, incorporating oligodendrocytes into the 2D Alexander disease model may shed light on cellular processes driving white matter degeneration. Expanding model complexity in a biologically informed and modular manner will be crucial for deepening mechanistic insights and advancing translational efforts. The experimental resources and findings presented in this thesis contribute to a framework for investigating potential therapeutic strategies in rare CNS disorders.

Key methods

Human pluripotent stem cells

Generation of human induced pluripotent stem cells

The parental hiPSC lines used in Papers III and IV of this thesis were generated by the Cell and Gene Therapy Core Facility at Lund University through mRNA-based reprogramming of fibroblasts, in accordance with the Stemgent StemRNA 3rd Gen Reprogramming Kit protocol from Reprocell (see Table 2). Clonal iPSC lines were established by manual selection of human ESC-like colonies. They were characterized per the recommended Standards for Human Stem Cell Use in Research from the International Society for Stem Cell Research (ISSCR) at the time of generation (Ludwig et al., 2023).

When generating hiPSC for disease modeling, the use of transient and non-integrating reprogramming factors is essential to ensure an accurate representation of the disease phenotype and to preserve the genetic integrity of the cell line. This leaves three main approaches: mRNA, Episomal, or Sendai Virus-based reprogramming.

mRNA-based reprogramming offers high reprogramming efficiency and a low risk of genetic alterations. As the technique relies on transient mRNA, there is no risk of integration of the reprogramming factors. However, this also makes the reprogramming more technically demanding and labor-intensive than its counterparts, as it requires daily transfections, expensive reagents, and rigorous handling of RNA. There are also limitations to what cells can be efficiently reprogrammed using an mRNA-based approach, as blood cells have proved to be resilient against efforts to generate hiPSCs utilizing this technique.

Episomal reprogramming relies on plasmid vectors to deliver the reprogramming factors into the somatic cells of choice, following transfection of the plasmids. These generally do not integrate into the genome and are typically lost after several passages. Episomal vectors are a cost-effective and easy-to-use alternative to mRNA-based reprogramming. It's widely used in basic research but suffers from low efficiency compared to alternative methods. Residual DNA may linger in early passages, and the technique thereby requires additional QC and verification to

confirm the clearance completely. Additionally, although the risk is low, the vector may integrate to some extent, leaving a genomic footprint.

Sendai virus reprogramming utilizes RNA virus to deliver the factors, offering high efficiency of reprogramming with no risk of genetic integration and minimal cytotoxicity. The Sendai virus RNA is gradually lost following cell division; however, residual virus RNA can persist, and this requires validation. Additionally, the technique is relatively expensive and requires a Biosafety Level 2 laboratory.

All aspects considered, mRNA-based reprogramming is the most suitable approach for our needs to generate hiPSCs at a small scale for disease modeling in an academic research setting.

Stem cell culturing

Pluripotent stem cells, hiPSC and hESC alike, were cultured on hESC-qualified Matrigel coating. The cells were maintained at 37°C in humidified air with 5% CO2, and media was changed daily with mTeSR1 or mTeSR+ media from STEMCELL Technologies. For splits, cells were dissociated using StemPro Accutase Cell Dissociation Reagent, pelleted by centrifugation, and seeded in media containing Rock Inhibitor to promote cell survival.

Among commercially available feeder-free media, mTeSR and mTeSR+ are widely used for maintaining hPSC, supporting robust pluripotency and consistent colony morphology across a range of cell lines. These formulations are chemically defined but contain BSA, which limits clinical applications.

Compared to Essential 8, which is fully chemically defined and optimized to reduce variability, mTeSR media offers greater buffering capacity and resilience to environmental fluctuations. This is especially true for mTeSR+, which incorporates stabilized FGF2. The stabilized formulation extends the growth factor activity and allows for less frequent feeding without compromising quality. NutriStem is a xeno-free and GMP-compliant medium with lower levels of growth factors such as FGF2 and TGF-β, which may reduce differentiation bias. Its defined formulation suits therapeutic workflows and clinical translations. StemFlex is optimized for high-stress conditions such as clonal expansion and gene editing. It includes enhanced survival factors and allows for flexible feeding schedules. However, the presence of animal-derived components may limit its applications.

In this context, mTeSR formulations strike a helpful balance between defined conditions, cellular robustness, and ease of use. This makes them well-suited for routine culture and experimental reproducibility.

Genome editing

CRISPR/Cas9-based genome editing

CRISPR/Cas9-based genome editing was used throughout Papers II-IV to introduce disease mutations, generate reporter lines, correct disease mutations, and create disease KO lines. For this purpose, sgRNAs were designed using readily available online software (such as Benchling) to target the region of interest for genome editing, and cloned into Cas9 vectors: Cas9, pSpCas9(BB)-2A-Puro (pX459) V2.0 (Addgene #62988), and Cas9 nickase, pSpCas9n(BB)-2A-Puro (pX462) V2.0 (Addgene #62987), based on the findings of Hsu et al. (2013) and Ran et al (2013).

The donor DNAs were designed with flanking homology arms on each side of the target sgRNA sequence to facilitate HDR, and the size of these arms varied depending on the size of the sequence to be integrated. For smaller edits, single-strand donor oligonucleotides (ssODNs) were used. They comprised of homology arms of 30-50 nt, the desired edit, and a silent PAM mutation to avoid the same loci from being retargeted by the Cas9. At both extremes, the ssODNs were designed to have phosphorothioate bonds, which protect the ssODN from exonuclease activity. For the generation of the mCherry reporter line, the donor DNA consisted of the mCherry sequence (711 nt), a linker sequence upstream of the mCherry cDNA, and homology arms of 500 nt on each side, followed by the target sequence for the sgRNA used to target the SNCA C-terminus. Due to the size of the donor sequence, the donor DNA was used in the form of a plasmid instead of ssODN. The sgRNA target sequence at the end of each homology arm was added to the donor vector to facilitate linearization within the hiPSCs, thereby increasing the availability for HDR. All CRISPR/Cas9-based genome editing constructs were introduced to the cells by transfection, using Lipofectamine Stem Transfection Reagent.

Prime editing

Prime editing was used in the generation of the GD isogenic control (GD-iso) hiPSC line in Paper IV, where regular CRISPR-Cas9 genome editing was not suitable due to the presence of the *GBA1* pseudogene *psGBA*. Prime editing constructs (pegRNA and ngRNA) were designed using PrimeDesign software. The pegRNAs were selected based on the retrotranscriptase template and primer binding site length and cloned into the pU6-pegRNA-GG-Vector acceptor plasmid (Addgene #132777. The ngRNA was designed to correct the mutation and introduce a silent PAM mutation and cloned into the BPK1520 plasmid (Addgene #65777). The prime editing constructs were introduced into the hiPSCs by nucleofection using a Lonza 4D Nucleofector. Based on the findings of Anzalone et al. (2019), for a more detailed description, see Materials and Methods in Paper IV.

Approach to genome editing

As demonstrated here, the choice of genome editing tool depends mainly on the specific needs of the project where it's used. CRISPR-Cas9 is the most widely used technique today due to efficiency, simplicity, and versatility. By simple cloning of a 20-nt sgRNA into a readily available Cas9 vector, it can be adapted to target your gene of interest in a short time frame. However, there are situations where this approach is not suitable, for instance, when a higher degree of precision is required due to a high degree of homology between genes, or for therapeutic applications where there is no room for unintended edits or off-target effects.

This is where prime editing shines, as it enables precise edits without relying on DSB, resulting in scarless genome editing with minimal risk of unintentional alterations to the genome. This precision does come at a cost, as the technique has a significantly lower efficiency compared to CRISPR/Cas9 and requires a more complex and involved vector design, making the genome editing approach more technically demanding.

Another method that offers some advantages over a standard CRISPR-based approach is Base Editing. Similar to prime editing, it builds upon a modified Cas9 enzyme. The technique allows for precise single-nucleotide changes without DSB. While useful for correcting specific point mutations, it's scope is constrained to a particular subset of base conversions, typically A to G or C to G, and has a limited editing window relative to the PAM site. There is also a risk of introducing so-called bystander mutations, which are unintentional mutations adjacent to the on-target cut site (Rees & Liu, 2018).

Thus, CRISPR-Cas9 remains the preferred method for large insertions and gene KO and will likely remain the standard approach for general genome editing until another technique becomes as versatile, efficient, and simple to use. Prime editing is an increasingly prominent alternative for precise and scarless corrections, where DSB can be detrimental. It therefore holds considerable therapeutic potential. Other techniques, such as Base editing and protein-based systems, including TALENs and Zinc finger nucleases, still hold value for targeted and niche applications but were not suitable for the scope of this thesis. Careful considerations regarding specificity, editing efficiency, and downstream applications should be taken when deciding on the optimal genome editing strategy for a specific project.

Table 2: Human pluripotent stem cell lines used in this thesis.
Summary of hPSC lines generated, edited and utilized across Paper I-IV, including cell type, donor sex, biobank ID, genotype and disease status. *Not generated as part of this thesis.

Line Name	Donor sex	ID Coriell/WiCell	Genotype		Disease	Comment
			Allele 1	Allele 2		
UNC AxD Cl 1 hiPSC	Male	GM16825 (Coriell	*GFAP* R239C	WT	AxD	Paper I *
UNC Iso Cl 11 hiPSC	Male	Derived from GM16825	WT	WT	Healthy, Isogenic control	Paper I *
H1 hESC	Male	WA01 (WiCell)	WT	WT	Healthy	Paper II, chromosomal variations *
MLC1 S93L A8 hESC	Male	Derived from WA01	*MLC1* S93L	*MLC1* S93L	MLC	Paper II, chromosomal variations
MLC1 S93L A8 hESC	Male	Derived from WA01	*MLC1* S93L	*MLC1* S93L	MLC	Paper II, chromosomal variations
HC hiPSC	Male	GM08398 (Coriell)	WT	WT	Healthy	Paper III and IV
SNCA N-Terminus HA hiPSC	Male	Derived from GM08398	*SNCA* Exon 1-HA	*SNCA* HA-Exon 1	Healthy	Paper III
SNCA C-Terminus HA hiPSC	Male	Derived from GM08398	*SNCA* Exon 6-HA	*SNCA* Exon 6-HA	Healthy	Paper III
SNCA C-Terminus mCh hiPSC	Male	Derived from GM08398	*SNCA* Exon 6-mCh	*SNCA* Exon 6-mCh	Healthy	Paper III
HC-KO hiPSC	Male	Derived from GM08398	*GBA1* Exon 9-KO	*GBA1* Exon 9-KO	GD	Paper IV
GD hiPSC	Female	GM01260 (Coriell)	*GBA1* P415R	*GBA1* L444P	GD	Paper IV
GD-Iso hiPSC	Female	Derived from GM01260	WT	WT	Healthy, isogenic control	Paper IV

Quality control of hPSC lines

When working with any new hPSC line, QC is crucial. In the scope of this thesis, QC of new hPSC lines has been performed repeatedly in accordance with the ISSCR Standards for Human Stem Cell Use in Research (Ludwig et al., 2023), outlining core principles relating to basic characterization of the cells, assessment of undifferentiated state and pluripotency, genomic characterization as well as further recommendations for stem cell-based model systems and how all these parameters should be reported.

Basic characterization

The basic characterization encompasses the acquisition of materials, cell line biobanking, cell line authentication, verification of transgene elimination, and assessment of cell hygiene.

Following ethical guidelines and to ensure traceability of source material, all fibroblasts and hPSCs used in this thesis were acquired through Material Transfer Agreements between the donating institutions and responsible principal investigators.

A well-structured cell banking system is essential for ensuring the long-term utility, traceability, and reproducibility of hPSCs in a research environment. A two-tiered system, comprising of a master cell bank and a working cell bank, provides a well-characterized, tightly controlled reserve of early-passage cells, as well as a working stock derived from these that is used for routine experiments. This hierarchical approach minimizes the need for repeated thawing of the original vials, reduces the experimental variability, and supports the long-term preservation of cell line integrity.

All cryopreservation of hPSCs was performed following enzymatic dissociation of the cells to ensure consistent single-cell suspensions. Cells were resuspended in cryoprotective media containing 10% DMSO, such as Cryostor CS10 from STEMCELL Technologies. Each vial was prelabeled with relevant identifiers and passage number to ensure traceability. Following this, cell vials were gradually cooled at a rate of -1°C/min until they reached -80°C, after which they were stored in liquid nitrogen or -150 °C freezers for long-term storage.

Cell line authentication is essential for traceability, reproducibility, and experimental integrity. To verify the identity of the generated hPSC lines and detect potential cross-contamination, STR profiling was performed using the Cell Line Authentication service by Eurofins Genomics, which employed PCR-based single-locus technology on sixteen independent loci with genomic DNA.

Verification of transgene elimination was not deemed necessary for the generated hiPSCs in this thesis, as they were all generated through mRNA-based reprogramming, which relies on the transient expression of mRNA encoding the reprogramming factors. The risk of stable expression or genomic integration of these factors is therefore minimal.

Routine screening of mycoplasma contamination is critical for laboratories handling human cells, as these contaminations are a common occurrence and can easily go undetected due to their subtle effects. These small bacteria lack cell walls, allowing them to infiltrate host cells and evade detection by standard means. Their parasitic lifestyle enables interference with key cellular processes such as growth, metabolism, and gene expression, thereby severely compromising the integrity of experimental data. Routine in-house mycoplasma testing was performed using the MycoAlert Plus detection kit from Lonza, which detects mycoplasma-specific enzymatic activity, allowing for rapid identification. In addition to in-house testing, cell lines were externally screened via the Eurofins Genomics MycoplasmaCheck service for further verification.

Assessment of undifferentiated state and functional pluripotency

The evaluation of the undifferentiated state is key to the quality control workflow of hPSCs. To ensure an in-depth appraisal of marker expression, both ICC and quantitative PCR (qPCR) were employed in this thesis. ICC provides single-cell resolution, allowing for the detection of heterogeneous expression and spontaneously differentiated cells. qPCR provides a quantitative assessment of cell populations, thereby offering a broader overview of cell state.

For the generation of lines in Papers II-IV, we performed immunocytochemical analysis using well-established markers of undifferentiated state, including staining for SOX2, NANOG, OCT3/4, as well as Tra-1-81 in Paper III and IV.

For all stainings of 2D cultures in this thesis, cells were washed before being fixed in 4% paraformaldehyde for 15 min, followed by washing. The cells were then blocked and permeabilized with blocking solution for an hour before overnight incubation with primary antibodies at 4°C. This was followed by washing and a 2-hour incubation of secondary antibodies, and a nuclear stain incubation, such as DAPI, to allow for visualization of the nuclei. After staining, cells were mounted using a mounting medium such as PVA:DABCO.

When it comes to cell fixation, while several different chemicals can be used, most techniques rely on either crosslinking or precipitation/dehydration, or a combination of the two. PFA is a crosslinking fixative, forming covalent bonds between proteins to preserve cellular and nuclear structures. This approach is generally better for preserving cellular morphology, ideal for detecting cytoplasmic and membrane proteins, and is compatible with a wide range of antibodies. However, it can mask

some epitopes, and additional permeabilization steps are required for intracellular targets. Methanol is an example of a precipitating/dehydrating fixative, which precipitates proteins while simultaneously permeabilizing cells through dehydration of membranes. This approach is straightforward, making it well-suited for high-throughput applications. It tends to preserve specific epitopes more effectively than crosslinking alternatives but can cause morphological distortion and is not suitable for detecting surface or membrane-bound proteins. Additionally, it requires access to freezer space for both methanol storage and the fixation process itself.

In this thesis, the choice of fixation method was guided by downstream applications and practical lab considerations. PFA-based fixation was the preferred approach, as it worked reliably with the model system used and was well-suited for many of the targets of interest. Methanol fixation was explored for specific applications later in the PhD studies, but these results were not included in the thesis.

For additional characterization in Papers III and IV, we performed RT-qPCR to analyze the gene expression of markers of undifferentiated state using TaqMan assays. RNA from independent experiments was used to synthesize cDNA through reverse transcriptase reactions prior to setting up the qPCR reactions.

All reactions were conducted with technical replicates and included appropriate negative controls. Reference gene assays (GAPDH or YWHAZ) were used for normalization, while TaqMan assays for SOX2, NANOG and POU5F1 served as markers of undifferentiated state.

qPCR amplifies target DNA through thermal cycling and enables real-time detection by measuring fluorescence that increases proportionally with product accumulation in each cycle. TaqMan probes support this by using fluorophore-quencher-labeled oligonucleotides that hybridize with the target sequence with high specificity and only emit their fluorescence when the probe is cleaved during amplification. This specificity is achieved through dual hybridization; both primers and the probe must bind to the correct target sequence for signal generation, minimizing the risk of detecting non-specific products. Additionally, the probe is designed to anneal within the amplicon, and fluorescence is only released upon probe degradation during the extension phase, further enhancing target discrimination.

The most widely used alternative is SYBR Green, which binds to any double-stranded DNA and produces a fluorescent signal upon binding. Unlike TaqMan, SYBR Green cannot distinguish between specific and non-specific products, such as primer dimers and off-target amplicons. This method is straightforward to implement and more cost-effective than TaqMan probe-based approaches, but it offers lower specificity. To verify the accuracy of the amplified product, post-PCR melting curve analysis is required. A TaqMan-based approach was chosen for these projects to ensure high specificity, avoid non-specific amplification interference, and eliminate the need for additional post-PCR analysis.

Functional pluripotency is a defining characteristic of hPSCs and should therefore be verified through trilineage differentiation to confirm the capacity to generate endoderm, ectoderm, and mesoderm fate. Following this, cell fate should be confirmed by examining two or more established markers of the three germ layers, either by immunocytochemistry or by qPCR analysis. For Papers II-IV, we differentiated our hPSC using the STEMdiff Trilineage Differentiation Kit from STEMCELL Technologies, which differentiates towards the three germ layers for 5-7 days prior to fixing the cultures. We then performed immunocytochemical analysis of endodermal, ectodermal, and mesodermal markers.

This type of kit offers rapid and directed 2D differentiation into the three germ layers under defined conditions, making them scalable and easily reproducible. However, they are expensive and only assess early lineage commitment by generating progenitor cells of each germ layer. As an alternative, we have previously employed embryoid body formation, a 3D self-organizing method that enables more unbiased differentiation and allows for insights into lineage bias. It is cost-effective due to simple media composition, but requires longer differentiation, is more variable, and analysis is more complex.

To assess functional pluripotency across multiple lines in parallel, we used the STEMdiff Trilineage Differentiation Kit. This assay provided reproducible results within a short timeframe and offered a practical approach for evaluating differentiation into the three germ layers.

Genomic characterization

Another critical aspect of hPSC quality control is the assessment of genetic integrity, as genetic anomalies may arise following reprogramming, passaging, or prolonged culture. Such variations can impact cellular behavior, affect differentiation capabilities, and compromise experimental outcomes. Thus, karyotypic analysis is crucial for ensuring the suitability of hPSCs for downstream applications and long-term stability. In this thesis, we employed G-band karyotyping for Papers III and IV, while we used molecular karyotyping for Paper II.

To prepare cells for G-band karyotyping, cells at approximately 70% confluency were treated with Colcemid for 45 min before collection. Cells were then lysed using a hypotonic solution prior to being pelleted. The pellet was resuspended in Carnoy fixative and kept at -20. The G-banding analysis was outsourced to a karyotyping service provider, which analyzed 20 metaphases per chromosome to assess the genetic integrity of the sample.

Colcemid is a methylated derivative of colchicine that blocks mitosis by binding to the tubulin heterodimer and preventing microtubule polymerization. This arrests cells in metaphase, when chromosomes are highly condensed and most suitable for cytogenetic analysis. Its use is essential in G-band karyotyping to increase the yield of clearly defined metaphase spreads, facilitating accurate assessment of chromosomal structure in stem cell cultures.

The molecular karyotype was assessed by copy number variation analysis of genomic DNA using SNP array, through outsourced sequencing and genotyping analysis by the Cell and Gene Therapy Core at Lund University.

G-band karyotyping is a conventional cytogenetic method that relies on visual examination of metaphase chromosomes using a microscope. The technique enables the detection of large-scale chromosomal defects or abnormalities at a resolution greater than 5-10 Mb. It's a relatively inexpensive technique that's well-established for detecting sensitive chromosomal rearrangements, but it's limited by its low resolution, which allows small copy number variations to go undetected.

In comparison, molecular karyotyping relies on DNA microarrays, thereby allowing for a much higher resolution, down to the range of 50-100 kb. This enables the detection of smaller genetic defects and copy number variations associated with hiPSC reprogramming and prolonged cell culture. These techniques can be performed using genomic DNA, so in contrast to G-band karyotyping, they do not require dividing cells. However, molecular karyotyping cannot detect balanced chromosomal rearrangements, as it relies on detecting quantitative changes, as opposed to structural ones, which G-band karyotyping can detect.

Each cytogenetic method offers distinct advantages, depending on the type of chromosomal abnormality being investigated. G-banding remains well suited for identifying large-scale structural rearrangements, such as translocations, deletions, and duplications. In contrast, molecular karyotyping provides significantly higher resolution, enabling the detection of submicroscopic copy number variations that are not visible by conventional banding techniques. Rather than serving as alternatives, these approaches are best viewed as complementary to one another. When used together under appropriate conditions, they offer a more comprehensive assessment of genomic integrity and stability.

While these are steps are taken as a necessity following in-house generation of new lines, they are also important aspects to consider before starting experiments with previously established lines acquired through cell banks, or older lines that have not been characterized recently, as became evident in Paper II, where we discovered chromosomal abnormalities in the parental line following the generation of MLC disease lines, that had not been detected by previous g-band karyotyping.

Further quality control following genome editing

Following genome editing, many of the quality control steps mirror those used when deriving new hiPSC lines. However, additional QC measures are required in addition to genome editing verification, such as assessment of both on- and off-target effects.

On-target analysis

Genome editing can produce unintentional on-target effects, such as large rearrangements, insertions, deletions or LOH, that can go undetected by standard sequencing approaches. If there are heterozygous SNPs present near the edited locus, these can be used to detect large-scale rearrangements, or LOH. When suitable SNPs are absent, quantitative genotyping PCR may be necessary to accurately assess allele counts and on-target genomic integrity. This was the case for Paper II, where we used qgPCR to confirm LOH in several clones that had previously gone undetected.

This technique, based on Weisheit et al. (2020), utilizes custom qPCR assays that were multiplexed with a TaqMan Copy Number reference assay. Primers for the custom assay were designed using Benchling Software to amplify a 300-450 bp region spanning the genome-edited site. Probes targeting this amplicon were designed using IDT's PrimerQuest tool and included a 5' fluorescent reporter and a 3' quencher, thereby functioning in the same manner as standard TaqMan probes. Assay efficiency was calculated from the slope of a standard curve generated from serial dilutions of genomic DNA. The assays were performed in triplicate using genomic DNA from both parental and genome-edited hPSC lines. Subsequent analysis, based on the assay efficiency calculations, provided estimates of allele copy number at the targeted site.

Off-target analysis

To assess potential off-target effects following CRISPR/Cas9-based genome editing, the five genomic regions with the highest predicted risk were identified using the aggregation scoring approach developed by Hsu et al. (2013) through Benchling software. Off-target effects refer to unintended DNA cleavage events at loci that share partial sequence homology with the intended target site. These events can lead to undesired insertions, deletions, or chromosomal rearrangements, potentially confounding experimental outcomes or introducing artifacts in downstream analyses. Assessing off-target activity is essential following CRISPR/Cas9 genome editing, as DSBs can lead to unintended mutations, and is particularly important in stem cell models that require high genomic stability for developmental accuracy and reproducibility.

Forward programming

Lentiviral vectors

Lentiviral delivery is a widely used method for transcription factor-based differentiation of both fibroblasts and hiPSCs. When applied to hiPSCs to generate differentiated cells, this process is referred to as forward programming. The ability of lentiviruses to integrate into the host genome permits sustained transgene expression, while inducible systems, such as doxycycline-responsive promoters, enable precise temporal regulation of transcription factor activity. Third-generation lentiviral vectors were generated by transient transfection of packaging plasmids and lentivectors into HEK 293T cells. After incubation, viral particles were harvested from the culture supernatant and concentrated via ultracentrifugation. Unlike earlier systems, third-generation lentiviral vectors support the use of inducible promoters, such as the doxycycline-sensitive TetOn promoter. This compatibility, along with improved biosafety, was essential for the forward programming strategy employed in this thesis. The self-inactivating design of these vectors further reduces the risk of insertional mutagenesis by preventing unintended activation of adjacent host genes. Furthermore, the separation of essential viral components across four plasmids minimizes the likelihood of recombination events that could generate replication-competent virus. These safety enhancements have positioned third-generation lentiviral system as a well-established tool for stable gene delivery in stem cell research and select clinical applications, such as *ex vivo* gene therapies for rare diseases like MLD. In summary, the versatility, safety features, and tight control over gene expression make third-generation lentiviral vectors particularly well-suited for experimental applications that require precise and sustained transgene delivery in stem cell-based research.

Alternative delivery of forward programming factors

A compelling alternative to lentiviral vectors for forward programming approaches is the PiggyBac transposon system, which enables stable genomic integration of cassettes containing transcription factors, allowing for scarless excision following the establishment of desired cell identity. Lentiviral vectors preferentially integrate into transcriptionally active regions of the genome, which can lead to variable transgene expression and increase the risk of insertional mutagenesis. In contrast, the PiggyBac transposon system integrates at defined TTAA sites and is delivered via co-transfection of two plasmids. This approach provides greater control over genomic insertion and enables more flexible construct design, including the use of larger polycistronic cassettes. Although transfection efficiency may decline with increasing vector size, this limitation is generally less restrictive than the packaging constraints associated with viral systems.

When this thesis work was initiated, the PiggyBac system was not yet widely adopted for use in hiPSC, especially in the context of forward programming applications. Therefore, a lentiviral approach was chosen due to its robust, reliable, and well-characterized expression of transcription factors. While PiggyBac now represents a promising alternative, the system still requires further optimization and validation before it can replace lentiviral systems.

Cocultures of induced neurons and astrocytes

Induced neurons and astrocytes were generated from human iPSCs as described in Zhang et al. (2013) and Canals et al. (2018) with minor modifications. In brief, hiPSCs were dissociated and replated on Matrigel-coated plates prior to transduction with lentiviral vectors carrying transcription factors under the control of a doxycycline-inducible promoter. For neuronal induction, cells were transduced with NGN2. For astrocyte differentiation, cells were transduced with SOX9 and NFIB. Doxycycline was added the day following transduction to induce lineage-specific gene expression. For iNs, neuronal media was used from the first day of differentiation. For the iAs protocol, early-phase differentiation involved serum-containing conditions for initial cell expansion, followed by a gradual transition to defined, growth-factor-enriched differentiation media over several days. Selection for each of the transcription factors was applied during the initial stages to enrich for transduced cells. To establish cocultures, both cell types were harvested following seven days of differentiation. Cells were pelleted, and the iNs were strained to remove cell aggregates before being replated at a defined ratio of 3:1 neurons to astrocytes. Following this, a mixed medium of equal parts neuronal and astrocytic differentiation media was used. From day 9 and onward, the astrocytic media was replaced with a maturation media, and half-media changes were performed every 2-3 days. To limit unwanted cell proliferation, FUDR was added between days 9 and 21.

hiPSCs can be directed to form neurons and astrocytes through various differentiation strategies, each with distinct advantages and limitations. In this thesis, a forward programming approach was employed, utilizing inducible overexpression of lineage-specific transcription factors. This method enabled rapid and efficient generation of defined neural and glial identities, offering a practical alternative to more prolonged differentiation protocols. The astrocytic protocol enables the generation of a mature morphology and functional properties after only 21 days in culture, whereas the induced neurons reach functional maturity following five weeks of coculture with the astrocytes.

In comparison, small molecule-based differentiation schemes aim to mimic developmental signaling cues to guide hiPSCs towards neural fates. While these methods may produce cell populations that resemble intermediate developmental stages and mirror natural developmental progression more closely, they typically require longer protocols, are more variable across different cell lines, and can result in heterogeneous cultures unless additional enrichment steps are taken.

The forward programming-based modeling system was chosen for its relative speed, scalability, and reproducibility. This approach enables synchronized generation of both cell types, making it well-suited for establishing defined cocultures of neurons and astrocytes, which was essential for studying the underlying astrocytic mechanisms contributing to rare diseases.

3D brain organoids

While not the primary methodological approach for my experimental work, organoid models have played a crucial role in this thesis by validating the neurodevelopmental phenotype observed in our 2D AxD model (Paper I) and by enabling the discovery of an interneuron deficit in GD (Paper IV).

Organoid models offer valuable insights into human neurodevelopment by more closely recapitulating cellular diversity and tissue architecture than conventional monolayer cultures. Their 3D organization enables the study of developmental processes such as temporal patterning, regional specification, and cell migration within a spatially relevant context. This is particularly useful for investigating rare CNS disorders where progenitor behavior or localized phenotypes may be missed in 2D systems. Organoids also provide access to early stages of human brain development that are otherwise inaccessible *in vivo*, enabling observation of prenatal lineage specification and neurogenesis. In early-onset conditions, pathological changes often precede the appearance of clinical symptoms, underscoring the need for models that capture disease processes at their earliest stages. 3D *in vitro* models offer a robust experimental framework for investigating early pathogenic events, facilitating the identification of candidate biomarkers, identifying sensitive developmental windows and assessing therapeutic approaches intended to limit developmental disruption and support normal maturation. For the studies in this thesis, three different protocols for generating human brain organoids were employed.

Unguided neural organoids were derived through spontaneous self-organization of hPSCs in the absence of exogenous patterning signals. This approach yields heterogeneous neural structures with variable regional identities, resembling multiple brain areas to varying degrees (Lancaster & Knoblich, 2014; Ormel et al., 2018). Although the composition can be inconsistent, this method established the foundation for organoid-based neurodevelopmental studies. Given the neural differentiation defect observed in the forward programming model of AxD, unguided neural organoids were the first type generated in Paper I.

Directed differentiation toward a dorsal forebrain identity was used to generate cortical organoids. Patterning during early differentiation promoted the formation of more uniform populations of radial glia and excitatory neurons, closely recapitulating telencephalic development (Yoon et al., 2019). This regional specificity makes the model particularly suitable for studying disease phenotypes with cortical involvement. This strategy was employed in Paper I to assess whether forebrain patterning could rescue the fate commitment defect seen in unguided AxD organoids, and in Paper IV, given the cortical degeneration observed in GD patients.

Subpallial organoids, generated through ventral patterning, facilitated the production of GABAergic interneuron progenitors (Sloan et al., 2018). This approach enabled investigation of interneuron development and migratory behavior *in vitro*. In Paper IV, it was used as a complementary model alongside cortical organoids to examine the dynamic interactions between interneurons and cortical architecture during neurodevelopment.

Organoid models have significantly advanced the ability to study human brain development and CNS disorders by providing a multicellular, 3D environment that closely reflects *in vivo* physiology. However, these systems also present notable limitations. Unguided organoids can display substantial variability in cellular composition, size, and maturation, which can compromise reproducibility and phenotypic consistency. This variability is reduced in patterned organoids, where defined signaling cues promote more reliable regional specification and developmental trajectories. However, the lack of vascularization restricts oxygen and nutrient diffusion, which can lead to necrotic cores and may affect long-term viability and maturation. Moreover, depending on the protocol used, the absence of cell types, such as microglia may limit the physiological relevance for disease modeling.

Alternative methods for 3D modeling, including scaffold-based cultures and organ-on-a-chip platforms, offer greater control over cell composition, microenvironment, and perfusion; however, they lack the self-organizing complexity of organoids. These systems often require specialized materials and technical expertise, which can limit their accessibility. Organoid approaches, despite their biological complexity, are supported by commercially available reagents and standardized protocols, which facilitate their integration into a wide range of stem cell research laboratories.

Taking these considerations into account, an organoid-based approach was selected for 3D modeling in this thesis due to the unparalleled ability to model early human neurodevelopment with a high degree of cellular and spatial complexity, allowing for unique insights into rare CNS disease pathology.

Single-cell RNA sequencing

Single-cell RNA sequencing has become an indispensable tool for studying rare diseases, enabling the unbiased identification of complex cell populations and transcriptional states at a single-cell resolution. In the context of this thesis, scRNAseq libraries of hiPSC-derived cocultures and 3D brain organoids were prepared using the 10x Genomics Chromium platform. Although I was not directly responsible for the sequencing and bioinformatic analysis, the resulting data and downstream interpretations were crucial to the findings presented in Papers I and IV.

By identifying distinct populations and cellular heterogeneity based on transcriptomic profiles, scRNAseq has enabled a deeper understanding of cell fates and disease-associated phenotypes in both 2D and 3D model systems. For the cocultures, it enabled the detection of disease-relevant subpopulations of cells, while in organoids it facilitated the exploration of altered fate commitment and the detection of shifts in cell populations between conditions.

Downstream analysis includes stringent quality control, clustering informed by canonical marker genes, and differential gene expression analysis across conditions. These steps enabled the identification of population-specific markers as well as shared transcriptional features across coculture and organoid systems. Thus, scRNAseq served as a valuable approach to elucidate underlying disease mechanisms and cellular phenotypes in hiPSC models of rare CNS disorders.

Despite the widespread applications and strengths of scRNAseq, there are some inherent limitations to the technique that need to be considered. The generation of the single-cell suspension required for the library preparation may introduce a cell population bias through the selective loss of more fragile cells, and this process can also induce stress-related changes to the transcriptome. Furthermore, the data may be affected by batch effects and dropout events, where many transcripts go undetected in individual cells, producing technical noise that requires meticulous bioinformatic handling. Additionally, scRNAseq lacks spatial information, resulting in the loss of the anatomical context within tissue architecture. However, this limitation can be addressed with complementary spatial transcriptomics techniques.

Alternative methods require different trade-offs. Single-nucleus RNA sequencing circumvents the challenges associated with enzymatic dissociation and cell viability, allowing for the use of frozen tissues. Single-nucleus RNA sequencing captures only nuclear transcripts, which typically results in reduced transcript diversity and lower sensitivity compared to whole-cell methods. Spatial transcriptomics preserves anatomical context by mapping gene expression directly within intact tissue sections, offering a distinct advantage for studying spatial organization. However, this comes at the expense of cellular resolution and throughput, which remain lower than those achieved with single-cell RNA sequencing. In contrast, bulk RNA sequencing provides a cost-effective and technically robust overview of average gene expression across samples, yet lacks cellular resolution, thereby obscuring transcriptional heterogeneity and rare cell populations within the tissue.

Thus, while there are limitations related to the use of this technique, given the heterogeneity and complexity associated with these hiPSC-derived model systems used in these studies of rare CNS disorders, scRNAseq was chosen over other methods due to its capacity to unravel cellular complexity, capture subtle phenotypes, and dissect disease-relevant mechanisms at a single-cell level.

Use of generative AI models

Artificial intelligence tools, including ChatGPT and Microsoft Copilot, were used throughout the development of this thesis to support literature searches, content organization, and language processing. These models functioned as language assistants and enhanced search tools, aiding in the identification of relevant publications, the synthesis of complex information, and improving the clarity and readability of the text. All content generated with the assistance of AI tools was carefully reviewed and verified by the author. The tools were employed strictly in supportive, non-decision-making roles and did not replace the author’s critical analysis, original interpretation, or direct engagement with primary sources.

Acknowledgements

I would like to thank both my supervisors, Henrik and Isaac, for their guidance, insight, and support throughout my PhD. Henrik, thank you for giving me the opportunity to pursue this work and for your thoughtful mentorship and advice over the years. Isaac, thank you for your guidance, for helping me navigate my PhD studies, and for making my research visit to Zurich possible. Your careful attention to detail has shaped my approach to scientific questions. Together, your combined expertise and complementary styles of supervision and leadership have been instrumental in shaping both my research direction and the values that guide my work.

To all my colleagues, past and present: Frenki, James, Ella, Jonas, Enrico, Aline, Laura, Alicja, Madalina, and the ones I may have unintentionally omitted, thank you for your valuable discussions, collaboration, and support throughout these years. Sharing this time with you, both in the lab and beyond, made the experience more enjoyable and rewarding. From scientific conversations to coffee breaks, lunches, and time spent outside of work, your company and support have meant a lot. I would also like to thank my students, Samira, Kavya, Roger, Marieke, and Ot, for their contributions to projects included in this thesis.

To everyone at BMC who made the lab a collaborative and welcoming environment, thank you. Thank you to Nika and the Medical Doctoral Student Council for their hard work on behalf of PhD students. I would also like to acknowledge the Lund Stem Cell Center Imaging Core and Anna Hammarlund for their assistance with microscopy and image analysis, the Cell and Gene Therapy Core for their support in generating iPSC lines, and the Electrophysiology Core for help with electrophysiological experiments. Additionally, I am grateful to the Center for Translational Genomics at Lund University for their contributions to single-cell RNA sequencing.

Thank you to Christine and Jenny for welcoming me into the 2021–2022 Professional Development Program. The experience contributed meaningfully to my personal and professional growth during my PhD studies and will continue to impact me moving forward. To my fellow participants in the program, thank you for the shared discussions on science, career choices, and the struggles of a PhD student. The retreats, courses, and write-ups offered a valuable sense of perspective and community during this part of the PhD.

Thank you to the researchers and staff at the University Children's Hospital Zurich for the warm welcome and supportive environment during my visit. I appreciated the chance to learn from your work and to contribute in a collaborative setting. My time in Zurich was an important and memorable part of this PhD, and I'm grateful to everyone who made it meaningful. Thank you to Anna-Giorgia and Sevi, it was great seeing you again during my stay in Zurich.

Thank you to my family for your support and understanding throughout this PhD. Your encouragement helped me stay grounded along the way. A special thank you to Livi for your patience, care, and support. Being by my side throughout this process made a real difference and helped me see it through.

References

Abbott, N. J., Rönnbäck, L., & Hansson, E. (2006). Astrocyte–endothelial interactions at the blood–brain barrier. *Nature Reviews Neuroscience*, *7*(1), 41–53. https://doi.org/10.1038/nrn1824

Aflaki, E., Stubblefield, B. K., McGlinchey, R. P., McMahon, B., Ory, D. S., & Sidransky, E. (2020). A characterization of Gaucher iPS-derived astrocytes: Potential implications for Parkinson's disease. *Neurobiology of Disease*, *134*, 104647. https://doi.org/10.1016/j.nbd.2019.104647

Andersen, J. V., Markussen, K. H., Jakobsen, E., Schousboe, A., Waagepetersen, H. S., Rosenberg, P. A., & Aldana, B. I. (2021). Glutamate metabolism and recycling at the excitatory synapse in health and neurodegeneration. *Neuropharmacology*, *196*, 108719. https://doi.org/10.1016/j.neuropharm.2021.108719

Anderson, R. H., & Francis, K. R. (2018). Modeling rare diseases with induced pluripotent stem cell technology. *Molecular and Cellular Probes*, *40*, 52–59. https://doi.org/10.1016/j.mcp.2018.01.001

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., & Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, *576*(7785), 149–157. https://doi.org/10.1038/s41586-019-1711-4

Awad, O., Panicker, L. M., Deranieh, R. M., Srikanth, M. P., Brown, R. A., Voit, A., Peesay, T., Park, T. S., Zambidis, E. T., & Feldman, R. A. (2017). Altered Differentiation Potential of Gaucher's Disease iPSC Neuronal Progenitors due to Wnt/β-Catenin Downregulation. *Stem Cell Reports*, *9*(6), 1853–1867. https://doi.org/10.1016/j.stemcr.2017.10.029

Awad, O., Sarkar, C., Panicker, L. M., Miller, D., Zeng, X., Sgambato, J. A., Lipinski, M. M., & Feldman, R. A. (2015). Altered TFEB-mediated lysosomal biogenesis in Gaucher disease iPSC-derived neuronal cells.

Human Molecular Genetics, *24*(20), 5775–5788. https://doi.org/10.1093/hmg/ddv297

Babcock, M. C., Mikulka, C. R., Wang, B., Chandriani, S., Chandra, S., Xu, Y., Webster, K., Feng, Y., Nelvagal, H. R., Giaramita, A., Yip, B. K., Lo, M., Jiang, X., Chao, Q., Woloszynek, J. C., Shen, Y., Bhagwat, S., Sands, M. S., & Crawford, B. E. (2021). Substrate reduction therapy for Krabbe disease and metachromatic leukodystrophy using a novel ceramide galactosyltransferase inhibitor. *Scientific Reports*, *11*(1), 14486. https://doi.org/10.1038/s41598-021-93601-1

Ballabio, A., & Gieselmann, V. (2009). Lysosomal disorders: From storage to cellular damage. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1793*(4), 684–696. https://doi.org/10.1016/j.bbamcr.2008.12.001

Basu, S., Adams, L., Guhathakurta, S., & Kim, Y.-S. (2017). A novel tool for monitoring endogenous alpha-synuclein transcription by NanoLuciferase tag insertion at the 3′end using CRISPR-Cas9 genome editing technique. *Scientific Reports*, *7*, 45883. https://doi.org/10.1038/srep45883

Battaglia, R. A., Beltran, A. S., Delic, S., Dumitru, R., Robinson, J. A., Kabiraj, P., Herring, L. E., Madden, V. J., Ravinder, N., Willems, E., Newman, R. A., Quinlan, R. A., Goldman, J. E., Perng, M.-D., Inagaki, M., & Snider, N. T. (2019). Site-specific phosphorylation and caspase cleavage of GFAP are new markers of Alexander disease severity. *eLife*, *8*, e47789. https://doi.org/10.7554/eLife.47789

Benetó, N., Cozar, M., Castilla-Vallmanya, L., Zetterdahl, O. G., Sacultanu, M., Segur-Bailach, E., García-Morant, M., Ribes, A., Ahlenius, H., Grinberg, D., Vilageliu, L., & Canals, I. (2020). Neuronal and Astrocytic Differentiation from Sanfilippo C Syndrome iPSCs for Disease Modeling and Drug Development. *Journal of Clinical Medicine*, *9*(3). https://doi.org/10.3390/jcm9030644

Boddupalli, C. S., Nair, S., Belinsky, G., Gans, J., Teeple, E., Nguyen, T.-H., Mehta, S., Guo, L., Kramer, M. L., Ruan, J., Wang, H., Davison, M., Kumar, D., Vidyadhara, D., Zhang, B., Klinger, K., & Mistry, P. K. (2022). Neuroinflammation in neuronopathic Gaucher disease: Role of microglia and NK cells, biomarkers, and response to substrate reduction therapy. *eLife*, *11*, e79830. https://doi.org/10.7554/eLife.79830

Brignone, M. S., Lanciotti, A., Camerini, S., De Nuccio, C., Petrucci, T. C., Visentin, S., & Ambrosini, E. (2015). MLC1 protein: A likely link between

leukodystrophies and brain channelopathies. *Frontiers in Cellular Neuroscience*, *9*, 66. https://doi.org/10.3389/fncel.2015.00106

Brown, R. A., Voit, A., Srikanth, M. P., Thayer, J. A., Kingsbury, T. J., Jacobson, M. A., Lipinski, M. M., Feldman, R. A., & Awad, O. (2019). mTOR hyperactivity mediates lysosomal dysfunction in Gaucher's disease iPSC-neuronal cells. *Disease Models & Mechanisms*, *12*(10), dmm038596. https://doi.org/10.1242/dmm.038596

Bugiani, M., & Breur, M. (2018). Leukodystrophies due to astroyctic dysfunction. *Brain Pathology*, *28*(3), 369–371. https://doi.org/10.1111/bpa.12607

Canals, I., Ginisty, A., Quist, E., Timmerman, R., Fritze, J., Miskinyte, G., Monni, E., Hansen, M. G., Hidalgo, I., Bryder, D., Bengzon, J., & Ahlenius, H. (2018). Rapid and efficient induction of functional astrocytes from human pluripotent stem cells. *Nature Methods*, *15*(9), 693–696. https://doi.org/10.1038/s41592-018-0103-2

Ceravolo, G., Zhelcheska, K., Squadrito, V., Pellerin, D., Gitto, E., Hartley, L., & Houlden, H. (2024). Update on leukodystrophies and developing trials. *Journal of Neurology*, *271*(1), 593–605. https://doi.org/10.1007/s00415-023-11996-5

Chandrasegaran, S., & Carroll, D. (2016). Origins of Programmable Nucleases for Genome Engineering. *Journal of Molecular Biology*, *428*(5, Part B), 963–989. https://doi.org/10.1016/j.jmb.2015.10.014

Chanoumidou, K., Hernández-Rodríguez, B., Windener, F., Thomas, C., Stehling, M., Mozafari, S., Albrecht, S., Ottoboni, L., Antel, J., Kim, K.-P., Velychko, S., Cui, Q. L., Xu, Y. K. T., Martino, G., Winkler, J., Schöler, H. R., Evercooren, A. B.-V., Boespflug-Tanguy, O., Vaquerizas, J. M., … Kuhlmann, T. (2021). One-step Reprogramming of Human Fibroblasts into Oligodendrocyte-like Cells by SOX10, OLIG2, and NKX6.2. *Stem Cell Reports*, *16*(4), 771–783. https://doi.org/10.1016/j.stemcr.2021.03.001

Chen, A. E., Egli, D., Niakan, K., Deng, J., Akutsu, H., Yamaki, M., Cowan, C., Fitz-Gerald, C., Zhang, K., Melton, D. A., & Eggan, K. (2009). Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell*, *4*(2), 103–106. https://doi.org/10.1016/j.stem.2008.12.001

Chen, S.-W., & Wong, Y.-H. (2023). Directed Differentiation of Human iPSCs into Microglia-Like Cells Using Defined Transcription Factors. In Y.-W. A.

Huang & C. Pak (Eds.), *Stem Cell-Based Neural Model Systems for Brain Disorders* (pp. 53–68). Springer US. https://doi.org/10.1007/978-1-0716-3287-1_5

Colacurcio, D. J., & Nixon, R. A. (2016). Disorders of lysosomal acidification—The emerging role of v-ATPase in aging and neurodegenerative disease. *Ageing Research Reviews*, *32*, 75–88. https://doi.org/10.1016/j.arr.2016.05.004

Darios, F., & Stevanin, G. (2020). Impairment of Lysosome Function and Autophagy in Rare Neurodegenerative Diseases. *Journal of Molecular Biology*, *432*(8), 2714–2734. https://doi.org/10.1016/j.jmb.2020.02.033

de Waard, D. M., & Bugiani, M. (2020). Astrocyte–Oligodendrocyte–Microglia Crosstalk in Astrocytopathies. *Frontiers in Cellular Neuroscience*, *14*. https://doi.org/10.3389/fncel.2020.608073

Diao, J., Yip, C. K., & Zhong, Q. (2024). Molecular structures and function of the autophagosome-lysosome fusion machinery. *Autophagy Reports*, *3*(1), 2305594. https://doi.org/10.1080/27694127.2024.2305594

Eaton, S. L., & Wishart, T. M. (2017). Bridging the gap: Large animal models in neurodegenerative research. *Mammalian Genome*, *28*(7), 324–337. https://doi.org/10.1007/s00335-017-9687-6

Ferreira, C. R. (2019). The burden of rare diseases. *American Journal of Medical Genetics Part A*, *179*(6), 885–892. https://doi.org/10.1002/ajmg.a.61124

Firdaus, Z., & Li, X. (2024). Unraveling the Genetic Landscape of Neurological Disorders: Insights into Pathogenesis, Techniques for Variant Identification, and Therapeutic Approaches. *International Journal of Molecular Sciences*, *25*(4), Article 4. https://doi.org/10.3390/ijms25042320

Freel, B. A., Sheets, J. N., & Francis, K. R. (2020). iPSC modeling of rare pediatric disorders. *Journal of Neuroscience Methods*, *332*, 108533. https://doi.org/10.1016/j.jneumeth.2019.108533

Fu, Y., Yang, M., Yu, H., Wang, Y., Wu, X., Yong, J., Mao, Y., Cui, Y., Fan, X., Wen, L., Qiao, J., & Tang, F. (2021). Heterogeneity of glial progenitor cells during the neurogenesis-to-gliogenesis switch in the developing human cerebral cortex. *Cell Reports*, *34*(9), 108788. https://doi.org/10.1016/j.celrep.2021.108788

Fujikake, N., Shin, M., & Shimizu, S. (2018). Association Between Autophagy and Neurodegenerative Diseases. *Frontiers in Neuroscience*, *12*. https://doi.org/10.3389/fnins.2018.00255

Fumagalli, F., Calbi, V., Sora, M. G. N., Sessa, M., Baldoli, C., Rancoita, P. M. V., Ciotti, F., Sarzana, M., Fraschini, M., Zambon, A. A., Acquati, S., Redaelli, D., Attanasio, V., Miglietta, S., Mattia, F. D., Barzaghi, F., Ferrua, F., Migliavacca, M., Tucci, F., … Aiuti, A. (2022). Lentiviral haematopoietic stem-cell gene therapy for early-onset metachromatic leukodystrophy: Long-term results from a non-randomised, open-label, phase 1/2 trial and expanded access. *The Lancet*, *399*(10322), 372–383. https://doi.org/10.1016/S0140-6736(21)02017-1

Furderer, M. L., Hertz, E., Lopez, G. J., & Sidransky, E. (2022). Neuropathological Features of Gaucher Disease and Gaucher Disease with Parkinsonism. *International Journal of Molecular Sciences*, *23*(10), 5842. https://doi.org/10.3390/ijms23105842

García-León, J. A., Kumar, M., Boon, R., Chau, D., One, J., Wolfs, E., Eggermont, K., Berckmans, P., Gunhanlar, N., de Vrij, F., Lendemeijer, B., Pavie, B., Corthout, N., Kushner, S. A., Dávila, J. C., Lambrichts, I., Hu, W.-S., & Verfaillie, C. M. (2018). SOX10 Single Transcription Factor-Based Fast and Efficient Generation of Oligodendrocytes from Human Pluripotent Stem Cells. *Stem Cell Reports*, *10*(2), 655–672. https://doi.org/10.1016/j.stemcr.2017.12.014

Geerts, H. (2009). Of Mice and Men. *CNS Drugs*, *23*(11), 915–926. https://doi.org/10.2165/11310890-000000000-00000

Hagemann, T. L. (2022). Alexander disease: Models, mechanisms, and medicine. *Current Opinion in Neurobiology*, *72*, 140–147. https://doi.org/10.1016/j.conb.2021.10.002

Hagemann, T. L., Powers, B., Lin, N.-H., Mohamed, A. F., Dague, K. L., Hannah, S. C., Bachmann, G., Mazur, C., Rigo, F., Olsen, A. L., Feany, M. B., Perng, M.-D., Berman, R. F., & Messing, A. (2021). Antisense therapy in a rat model of Alexander disease reverses GFAP pathology, white matter deficits, and motor impairment. *Science Translational Medicine*, *13*(620), eabg4711. https://doi.org/10.1126/scitranslmed.abg4711

Haggarty, S. J., Silva, M. C., Cross, A., Brandon, N. J., & Perlis, R. H. (2016). Advancing Drug Discovery for Neuropsychiatric Disorders Using Patient-

Specific Stem Cell Models. *Molecular and Cellular Neurosciences*, *73*, 104–115. https://doi.org/10.1016/j.mcn.2016.01.011

Hansen, C., Björklund, T., Petit, G. H., Lundblad, M., Murmu, R. P., Brundin, P., & Li, J.-Y. (2013). A novel α-synuclein-GFP mouse model displays progressive motor impairment, olfactory dysfunction and accumulation of α-synuclein-GFP. *Neurobiology of Disease*, *56*, 145–155. https://doi.org/10.1016/j.nbd.2013.04.017

Hertz, E., Chen, Y., & Sidransky, E. (2024). Gaucher disease provides a unique window into Parkinson disease pathogenesis. *Nature Reviews Neurology*, *20*(9), 526–540. https://doi.org/10.1038/s41582-024-00999-z

Hoegg-Beiler, M. B., Sirisi, S., Orozco, I. J., Ferrer, I., Hohensee, S., Auberson, M., Gödde, K., Vilches, C., de Heredia, M. L., Nunes, V., Estévez, R., & Jentsch, T. J. (2014). Disrupting MLC1 and GlialCAM and ClC-2 interactions in leukodystrophy entails glial chloride channel dysfunction. *Nature Communications*, *5*(1), 3475. https://doi.org/10.1038/ncomms4475

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., & Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, *31*(9), 827–832. https://doi.org/10.1038/nbt.2647

Ilja Boor, P. K., Groot, K. de, Mejaski-Bosnjak, V., Brenner, C., van der Knaap, M. S., Scheper, G. C., & Pronk, J. C. (2006). Megalencephalic leukoencephalopathy with subcortical cysts: An update and extended mutation analysis of MLC1. *Human Mutation*, *27*(6), 505–512. https://doi.org/10.1002/humu.20332

Jones, J. R., Kong, L., Hanna, M. G., Hoffman, B., Krencik, R., Bradley, R., Hagemann, T., Choi, J., Doers, M., Dubovis, M., Sherafat, M. A., Bhattacharyya, A., Kendziorski, C., Audhya, A., Messing, A., & Zhang, S.-C. (2018). Mutations in GFAP Disrupt the Distribution and Function of Organelles in Human Astrocytes. *Cell Reports*, *25*(4), 947-958.e4. https://doi.org/10.1016/j.celrep.2018.09.083

Jorge, M. S., & Bugiani, M. (2019). Astroglia in Leukodystrophies. In A. Verkhratsky, M. S. Ho, R. Zorec, & V. Parpura (Eds.), *Neuroglia in Neurodegenerative Diseases* (pp. 199–225). Springer. https://doi.org/10.1007/978-981-13-9913-8_9

Jovanovic, V. M., Weber, C., Slamecka, J., Ryu, S., Chu, P.-H., Sen, C., Inman, J., De Sousa, J. F., Barnaeva, E., Hirst, M., Galbraith, D., Ormanoglu, P., Jethmalani, Y., Mercado, J. C., Michael, S., Ward, M. E., Simeonov, A., Voss, T. C., Tristan, C. A., & Singeç, I. (2023). A defined roadmap of radial glia and astrocyte differentiation from human pluripotent stem cells. *Stem Cell Reports*, *18*(8), 1701–1720. https://doi.org/10.1016/j.stemcr.2023.06.007

Jucker, M. (2010). The benefits and limitations of animal models for translational research in neurodegenerative diseases. *Nature Medicine*, *16*(11), 1210–1214. https://doi.org/10.1038/nm.2224

Kaufmann, P., Pariser, A. R., & Austin, C. (2018). From scientific discovery to treatments for rare diseases – the view from the National Center for Advancing Translational Sciences – Office of Rare Diseases Research. *Orphanet Journal of Rare Diseases*, *13*(1), 196. https://doi.org/10.1186/s13023-018-0936-x

Kernohan, K. D., & Boycott, K. M. (2024). The expanding diagnostic toolbox for rare genetic diseases. *Nature Reviews Genetics*, *25*(6), 401–415. https://doi.org/10.1038/s41576-023-00683-w

Kim, E. Y., Hong, Y. B., Go, S. H., Lee, B., & Jung, S.-C. (2006). Downregulation of neurotrophic factors in the brain of a mouse model of Gaucher disease; implications for neuronal loss in Gaucher disease. *Experimental & Molecular Medicine*, *38*(4), 348–356. https://doi.org/10.1038/emm.2006.41

Kondo, T., Funayama, M., Miyake, M., Tsukita, K., Era, T., Osaka, H., Ayaki, T., Takahashi, R., & Inoue, H. (2016). Modeling Alexander disease with patient iPSCs reveals cellular and molecular pathology of astrocytes. *Acta Neuropathologica Communications*, *4*(1), 69. https://doi.org/10.1186/s40478-016-0337-0

Kruyer, A., Kalivas, P. W., & Scofield, M. D. (2023). Astrocyte regulation of synaptic signaling in psychiatric disorders. *Neuropsychopharmacology*, *48*(1), 21–36. https://doi.org/10.1038/s41386-022-01338-w

Kumar, M., Srikanth, M. P., Deleidi, M., Hallett, P. J., Isacson, O., & Feldman, R. A. (2023). Acid ceramidase involved in pathogenic cascade leading to accumulation of α-synuclein in iPSC model of GBA1-associated Parkinson's disease. *Human Molecular Genetics*, *32*(11), 1888–1900. https://doi.org/10.1093/hmg/ddad025

Kumar, S. T., Jagannath, S., Francois, C., Vanderstichele, H., Stoops, E., & Lashuel, H. A. (2020). How specific are the conformation-specific α-synuclein antibodies? Characterization and validation of 16 α-synuclein conformation-specific antibodies using well-characterized preparations of α-synuclein monomers, fibrils and oligomers with distinct structures and morphology. *Neurobiology of Disease*, *146*, 105086. https://doi.org/10.1016/j.nbd.2020.105086

Lalo, U., Koh, W., Lee, C. J., & Pankratov, Y. (2021). The tripartite glutamatergic synapse. *Neuropharmacology*, *199*, 108758. https://doi.org/10.1016/j.neuropharm.2021.108758

Lancaster, M. A., & Knoblich, J. A. (2014). Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science*, *345*(6194), 1247125. https://doi.org/10.1126/science.1247125

Lancaster, M. A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L. S., Hurles, M. E., Homfray, T., Penninger, J. M., Jackson, A. P., & Knoblich, J. A. (2013). Cerebral organoids model human brain development and microcephaly. *Nature*, *501*(7467), 373–379. https://doi.org/10.1038/nature12517

Lanciotti, A., Brignone, M. S., Macioce, P., Visentin, S., & Ambrosini, E. (2022). Human iPSC-Derived Astrocytes: A Powerful Tool to Study Primary Astrocyte Dysfunction in the Pathogenesis of Rare Leukodystrophies. *International Journal of Molecular Sciences*, *23*(1), Article 1. https://doi.org/10.3390/ijms23010274

Lee, J. S. (2024). Molecular diagnostic approach to rare neurological diseases from a clinician viewpoint. *Genomics & Informatics*, *22*, 18. https://doi.org/10.1186/s44342-024-00025-0

Li, K., Wei, Q., Liu, F.-F., Hu, F., Xie, A., Zhu, L.-Q., & Liu, D. (2018). Synaptic Dysfunction in Alzheimer's Disease: Aβ, Tau, and Epigenetic Alterations. *Molecular Neurobiology*, *55*(4), 3021–3032. https://doi.org/10.1007/s12035-017-0533-3

Li, L., Tian, E., Chen, X., Chao, J., Klein, J., Qu, Q., Sun, G., Sun, G., Huang, Y., Warden, C. D., Ye, P., Feng, L., Li, X., Cui, Q., Sultan, A., Douvaras, P., Fossati, V., Sanjana, N. E., Riggs, A. D., & Shi, Y. (2018). GFAP Mutations in Astrocytes Impair Oligodendrocyte Progenitor Proliferation and Myelination in an hiPSC Model of Alexander Disease. *Cell Stem Cell*, *23*(2), 239-251.e6. https://doi.org/10.1016/j.stem.2018.07.009

Lin, H.-C., He, Z., Ebert, S., Schörnig, M., Santel, M., Nikolova, M. T., Weigert, A., Hevers, W., Kasri, N. N., Taverna, E., Camp, J. G., & Treutlein, B. (2021). *NGN2* induces diverse neuron types from human pluripotency. *Stem Cell Reports*, *16*(9), 2118–2127. https://doi.org/10.1016/j.stemcr.2021.07.006

Lin, Y., Zhao, X., Liou, B., Fannin, V., Zhang, W., Setchell, K. D. R., Wang, X., Pan, D., Grabowski, G. A., Liu, C., & Sun, Y. (2024). Intrinsic link between PGRN and Gba1 D409V mutation dosage in potentiating Gaucher disease. *Human Molecular Genetics*, *33*(20), 1771–1788. https://doi.org/10.1093/hmg/ddae113

Liu, B., Teschemacher, A. G., & Kasparov, S. (2017). Astroglia as a cellular target for neuroprotection and treatment of neuro-psychiatric disorders. *Glia*, *65*(8), 1205–1226. https://doi.org/10.1002/glia.23136

Lopes-Júnior, L. C., Ferraz, V. E. F., Lima, R. A. G., Schuab, S. I. P. C., Pessanha, R. M., Luz, G. S., Laignier, M. R., Nunes, K. Z., Lopes, A. B., Grassi, J., Moreira, J. A., Jardim, F. A., Leite, F. M. C., Freitas, P. de S. S., & Bertolini, S. R. (2022). Health Policies for Rare Disease Patients: A Scoping Review. *International Journal of Environmental Research and Public Health*, *19*(22), Article 22. https://doi.org/10.3390/ijerph192215174

López-Hernández, T., Sirisi, S., Capdevila-Nortes, X., Montolio, M., Fernández-Dueñas, V., Scheper, G. C., van der Knaap, M. S., Casquero, P., Ciruela, F., Ferrer, I., Nunes, V., & Estévez, R. (2011). Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts. *Human Molecular Genetics*, *20*(16), 3266–3277. https://doi.org/10.1093/hmg/ddr238

Luce, E., & Duclos-Vallee, J.-C. (2025). Stem Cells and Organoids: A Paradigm Shift in Preclinical Models Toward Personalized Medicine. *Pharmaceuticals*, *18*(7), Article 7. https://doi.org/10.3390/ph18070992

Ludwig, T. E., Andrews, P. W., Barbaric, I., Benvenisty, N., Bhattacharyya, A., Crook, J. M., Daheron, L. M., Draper, J. S., Healy, L. E., Huch, M., Inamdar, M. S., Jensen, K. B., Kurtz, A., Lancaster, M. A., Liberali, P., Lutolf, M. P., Mummery, C. L., Pera, M. F., Sato, Y., … Mosher, J. T. (2023). ISSCR standards for the use of human stem cells in basic research. *Stem Cell Reports*, *18*(9), 1744–1752. https://doi.org/10.1016/j.stemcr.2023.08.003

Luo, C., Keown, C. L., Kurihara, L., Zhou, J., He, Y., Li, J., Castanon, R., Lucero, J., Nery, J. R., Sandoval, J. P., Bui, B., Sejnowski, T. J., Harkins, T. T., Mukamel, E. A., Behrens, M. M., & Ecker, J. R. (2017). Single-cell methylomes identify neuronal subtypes and regulatory elements in mammalian cortex. *Science*, *357*(6351), 600–604. https://doi.org/10.1126/science.aan3351

Malik, B. R., Maddison, D. C., Smith, G. A., & Peters, O. M. (2019). Autophagic and endo-lysosomal dysfunction in neurodegenerative disease. *Molecular Brain*, *12*(1), 100. https://doi.org/10.1186/s13041-019-0504-x

Marwaha, S., Knowles, J. W., & Ashley, E. A. (2022). A guide for the diagnosis of rare and undiagnosed disease: Beyond the exome. *Genome Medicine*, *14*(1), 23. https://doi.org/10.1186/s13073-022-01026-w

Massaro, G., Geard, A. F., Liu, W., Coombe-Tennant, O., Waddington, S. N., Baruteau, J., Gissen, P., & Rahim, A. A. (2021). Gene Therapy for Lysosomal Storage Disorders: Ongoing Studies and Clinical Development. *Biomolecules*, *11*(4), 611. https://doi.org/10.3390/biom11040611

Mayhew, C. N., & Singhania, R. (2022). A review of protocols for brain organoids and applications for disease modeling. *STAR Protocols*, *4*(1), 101860. https://doi.org/10.1016/j.xpro.2022.101860

Mazzulli, J. R., Xu, Y.-H., Sun, Y., Knight, A. L., McLean, P. J., Caldwell, G. A., Sidransky, E., Grabowski, G. A., & Krainc, D. (2011). Gaucher disease glucocerebrosidase and α-synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell*, *146*(1), 37–52. https://doi.org/10.1016/j.cell.2011.06.001

Mertens, J., Paquola, A. C. M., Ku, M., Hatch, E., Böhnke, L., Ladjevardi, S., McGrath, S., Campbell, B., Lee, H., Herdy, J. R., Gonçalves, J. T., Toda, T., Kim, Y., Winkler, J., Yao, J., Hetzer, M. W., & Gage, F. H. (2015). Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects. *Cell Stem Cell*, *17*(6), 705–718. https://doi.org/10.1016/j.stem.2015.09.001

Momenzadeh, S., & Jami, M.-S. (2021). Remyelination in PNS and CNS: Current and upcoming cellular and molecular strategies to treat disabling neuropathies. *Molecular Biology Reports*, *48*(12), 8097–8110. https://doi.org/10.1007/s11033-021-06755-6

Morales, J. E., De, A., Miller, A. A., Chen, Z., & McCarty, J. H. (2022). Mlc1-Expressing Perivascular Astrocytes Promote Blood–Brain Barrier Integrity. *Journal of Neuroscience*, *42*(8), 1406–1416. https://doi.org/10.1523/JNEUROSCI.1918-21.2021

Morris, H. R., Spillantini, M. G., Sue, C. M., & Williams-Gray, C. H. (2024). The pathogenesis of Parkinson's disease. *The Lancet*, *403*(10423), 293–304. https://doi.org/10.1016/S0140-6736(23)01478-2

Muthusamy, K., Sivadasan, A., Dixon, L., Sudhakar, S., Thomas, M., Danda, S., Wszolek, Z. K., Wierenga, K., Dhamija, R., & Gavrilova, R. (2023). Adult-onset leukodystrophies: A practical guide, recent treatment updates, and future directions. *Frontiers in Neurology*, *14*. https://doi.org/10.3389/fneur.2023.1219324

Nalysnyk, L., Rotella ,Philip, Simeone ,Jason C., Hamed ,Alaa, & and Weinreb, N. (2017). Gaucher disease epidemiology and natural history: A comprehensive review of the literature. *Hematology*, *22*(2), 65–73. https://doi.org/10.1080/10245332.2016.1240391

Nguengang Wakap, S., Lambert, D. M., Olry, A., Rodwell, C., Gueydan, C., Lanneau, V., Murphy, D., Le Cam, Y., & Rath, A. (2020). Estimating cumulative point prevalence of rare diseases: Analysis of the Orphanet database. *European Journal of Human Genetics*, *28*(2), 165–173. https://doi.org/10.1038/s41431-019-0508-0

Oberheim, N. A., Takano, T., Han, X., He, W., Lin, J. H. C., Wang, F., Xu, Q., Wyatt, J. D., Pilcher, W., Ojemann, J. G., Ransom, B. R., Goldman, S. A., & Nedergaard, M. (2009). Uniquely Hominid Features of Adult Human Astrocytes. *Journal of Neuroscience*, *29*(10), 3276–3287. https://doi.org/10.1523/JNEUROSCI.4707-08.2009

Olabarria, M., Putilina, M., Riemer, E. C., & Goldman, J. E. (2015). Astrocyte pathology in Alexander disease causes a marked inflammatory environment. *Acta Neuropathologica*, *130*(4), 469–486. https://doi.org/10.1007/s00401-015-1469-1

Ormel, P. R., Vieira de Sá, R., van Bodegraven, E. J., Karst, H., Harschnitz, O., Sneeboer, M. A. M., Johansen, L. E., van Dijk, R. E., Scheefhals, N., Berdenis van Berlekom, A., Ribes Martínez, E., Kling, S., MacGillavry, H. D., van den Berg, L. H., Kahn, R. S., Hol, E. M., de Witte, L. D., & Pasterkamp, R. J. (2018). Microglia innately develop within cerebral

organoids. *Nature Communications*, *9*(1), 4167. https://doi.org/10.1038/s41467-018-06684-2

Orvisky, E., Sidransky, E., McKinney, C. E., Lamarca, M. E., Samimi, R., Krasnewich, D., Martin, B. M., & Ginns, E. I. (2000). Glucosylsphingosine Accumulation in Mice and Patients with Type 2 Gaucher Disease Begins Early in Gestation. *Pediatric Research*, *48*(2), 233–237. https://doi.org/10.1203/00006450-200008000-00018

Passchier, E. M. J., Bisseling, Q., Helman, G., van Spaendonk, R. M. L., Simons, C., Olsthoorn, R. C. L., van der Veen, H., Abbink, T. E. M., van der Knaap, M. S., & Min, R. (2024). Megalencephalic leukoencephalopathy with subcortical cysts: A variant update and review of the literature. *Frontiers in Genetics*, *15*. https://doi.org/10.3389/fgene.2024.1352947

Passchier, E. M. J., Kerst, S., Brouwers, E., Hamilton, E. M. C., Bisseling, Q., Bugiani, M., Waisfisz, Q., Kitchen, P., Unger, L., Breur, M., Hoogterp, L., de Vries, S. I., Abbink, T. E. M., Kole, M. H. P., Leurs, R., Vischer, H. F., Brignone, M. S., Ambrosini, E., Feillet, F., … van der Knaap, M. S. (2023). Aquaporin-4 and GPRC5B: Old and new players in controlling brain oedema. *Brain*, *146*(8), 3444–3454. https://doi.org/10.1093/brain/awad146

Pauwels, K., Gijsbers, R., Toelen, J., Schambach, A., Willard-Gallo, K., Verheust, C., Debyser, Z., & Herman, P. (2009). State-of-the-Art Lentiviral Vectors for Research Use: Risk Assessment and Biosafety Recommendations. *Current Gene Therapy*, *9*(6), 459–474. https://doi.org/10.2174/156652309790031120

Pekny, M., & Pekna, M. (2014). Astrocyte Reactivity and Reactive Astrogliosis: Costs and Benefits. *Physiological Reviews*, *94*(4), 1077–1098. https://doi.org/10.1152/physrev.00041.2013

Peng, Y., Liou, B., Lin, Y., Mayhew, C. N., Fleming, S. M., & Sun, Y. (2023). iPSC-derived neural precursor cells engineering GBA1 recovers acid β-glucosidase deficiency and diminishes α-synuclein and neuropathology. *Molecular Therapy Methods & Clinical Development*, *29*, 185–201. https://doi.org/10.1016/j.omtm.2023.03.007

Pereda, A. E. (2014). Electrical synapses and their functional interactions with chemical synapses. *Nature Reviews Neuroscience*, *15*(4), 250–263. https://doi.org/10.1038/nrn3708

Platt, F. M., Boland, B., & van der Spoel, A. C. (2012). Lysosomal storage disorders: The cellular impact of lysosomal dysfunction. *The Journal of Cell Biology*, *199*(5), 723–734. https://doi.org/10.1083/jcb.201208152

Platt, F. M., d'Azzo, A., Davidson, B. L., Neufeld, E. F., & Tifft, C. J. (2018). Lysosomal storage diseases. *Nature Reviews Disease Primers*, *4*(1), 1–25. https://doi.org/10.1038/s41572-018-0025-4

Potokar, M., Morita, M., Wiche, G., & Jorgačevski, J. (2020). The Diversity of Intermediate Filaments in Astrocytes. *Cells*, *9*(7), Article 7. https://doi.org/10.3390/cells9071604

Prust, M., Wang, J., Morizono, H., Messing, A., Brenner, M., Gordon, E., Hartka, T., Sokohl, A., Schiffmann, R., Gordish-Dressman, H., Albin, R., Amartino, H., Brockman, K., Dinopoulos, A., Dotti, M. T., Fain, D., Fernandez, R., Ferreira, J., Fleming, J., … Vanderver, A. (2011). GFAP mutations, age at onset, and clinical subtypes in Alexander disease. *Neurology*, *77*(13), 1287–1294. https://doi.org/10.1212/WNL.0b013e3182309f72

Quist, E., Trovato, F., Avaliani, N., Zetterdahl, O. G., Gonzalez-Ramos, A., Hansen, M. G., Kokaia, M., Canals, I., & Ahlenius, H. (2022). Transcription factor-based direct conversion of human fibroblasts to functional astrocytes. *Stem Cell Reports*, *17*(7), 1620–1635. https://doi.org/10.1016/j.stemcr.2022.05.015

Ran, F. A., Hsu, P. D., Lin, C.-Y., Gootenberg, J. S., Konermann, S., Trevino, A. E., Scott, D. A., Inoue, A., Matoba, S., Zhang, Y., & Zhang, F. (2013). Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. *Cell*, *154*(6), 1380–1389. https://doi.org/10.1016/j.cell.2013.08.021

Rees, H. A., & Liu, D. R. (2018). Base editing: Precision chemistry on the genome and transcriptome of living cells. *Nature Reviews. Genetics*, *19*(12), 770–788. https://doi.org/10.1038/s41576-018-0059-1

Reinhard, C., Bachoud-Lévi, A.-C., Bäumer, T., Bertini, E., Brunelle, A., Buizer, A. I., Federico, A., Gasser, T., Groeschel, S., Hermanns, S., Klockgether, T., Krägeloh-Mann, I., Landwehrmeyer, G. B., Leber, I., Macaya, A., Mariotti, C., Meissner, W. G., Molnar, M. J., Nonnekes, J., … Graessner, H. (2021). The European Reference Network for Rare Neurological Diseases. *Frontiers in Neurology*, *11*. https://doi.org/10.3389/fneur.2020.616569

Revel-Vilk, S., Fuller, M., & Zimran, A. (2020). Value of Glucosylsphingosine (Lyso-Gb1) as a Biomarker in Gaucher Disease: A Systematic Literature Review. *International Journal of Molecular Sciences*, *21*(19), Article 19. https://doi.org/10.3390/ijms21197159

Rosety, I., Zagare, A., Saraiva, C., Nickels, S., Antony, P., Almeida, C., Glaab, E., Halder, R., Velychko, S., Rauen, T., Schöler, H. R., Bolognin, S., Sauter, T., Jarazo, J., Krüger, R., & Schwamborn, J. C. (2023). Impaired neuron differentiation in GBA-associated Parkinson's disease is linked to cell cycle defects in organoids. *Npj Parkinson's Disease*, *9*(1), 166. https://doi.org/10.1038/s41531-023-00616-8

Roshan Lal, T., & Sidransky, E. (2017). The Spectrum of Neurological Manifestations Associated with Gaucher Disease. *Diseases*, *5*(1), Article 1. https://doi.org/10.3390/diseases5010010

Saftig, P., & Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function. *Nature Reviews Molecular Cell Biology*, *10*(9), 623–635. https://doi.org/10.1038/nrm2745

Schiera, G., Di Liegro, C. M., Schirò, G., Sorbello, G., & Di Liegro, I. (2024). Involvement of Astrocytes in the Formation, Maintenance, and Function of the Blood–Brain Barrier. *Cells*, *13*(2), Article 2. https://doi.org/10.3390/cells13020150

Schöndorf, D. C., Aureli, M., McAllister, F. E., Hindley, C. J., Mayer, F., Schmid, B., Sardi, S. P., Valsecchi, M., Hoffmann, S., Schwarz, L. K., Hedrich, U., Berg, D., Shihabuddin, L. S., Hu, J., Pruszak, J., Gygi, S. P., Sonnino, S., Gasser, T., & Deleidi, M. (2014). iPSC-derived neurons from GBA1-associated Parkinson's disease patients show autophagic defects and impaired calcium homeostasis. *Nature Communications*, *5*(1), 4028. https://doi.org/10.1038/ncomms5028

Sevin, C., & Deiva, K. (2021). Clinical Trials for Gene Therapy in Lysosomal Diseases With CNS Involvement. *Frontiers in Molecular Biosciences*, *8*. https://doi.org/10.3389/fmolb.2021.624988

Sicca, F., Ambrosini, E., Marchese, M., Sforna, L., Servettini, I., Valvo, G., Brignone, M. S., Lanciotti, A., Moro, F., Grottesi, A., Catacuzzeno, L., Baldini, S., Hasan, S., D'Adamo, M. C., Franciolini, F., Molinari, P., Santorelli, F. M., & Pessia, M. (2016). Gain-of-function defects of astrocytic Kir4.1 channels in children with autism spectrum disorders and epilepsy. *Scientific Reports*, *6*(1), 34325. https://doi.org/10.1038/srep34325

Sidransky, E., Nalls, M. A., Aasly, J. O., Aharon-Peretz, J., Annesi, G., Barbosa, E. R., Bar-Shira, A., Berg, D., Bras, J., Brice, A., Marco, E. V. D., Dürr, A., Eblan, M. J., Fahn, S., Farrer, M. J., Gan-Or, Z., Gasser, T., Gershoni-Baruch, R., Giladi, N., … Wolfsberg, T. G. (2009). Multicenter Analysis of Glucocerebrosidase Mutations in Parkinson's Disease. *The New England Journal of Medicine*.

Siracusa, R., Fusco, R., & Cuzzocrea, S. (2019). Astrocytes: Role and Functions in Brain Pathologies. *Frontiers in Pharmacology*, *10*. https://doi.org/10.3389/fphar.2019.01114

Sloan, S. A., Andersen, J., Paşca, A. M., Birey, F., & Paşca, S. P. (2018). Generation and assembly of human brain region-specific three-dimensional cultures. *Nature Protocols*, *13*(9), 2062–2085. https://doi.org/10.1038/s41596-018-0032-7

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: Biology and pathology. *Acta Neuropathologica*, *119*(1), 7–35. https://doi.org/10.1007/s00401-009-0619-8

Sosunov, A. A., Guilfoyle, E., Wu, X., McKhann, G. M., & Goldman, J. E. (2013). Phenotypic Conversions of "Protoplasmic" to "Reactive" Astrocytes in Alexander Disease. *Journal of Neuroscience*, *33*(17), 7439–7450. https://doi.org/10.1523/JNEUROSCI.4506-12.2013

Sosunov, A. A., McKhann, G. M., & Goldman, J. E. (2017). The origin of Rosenthal fibers and their contributions to astrocyte pathology in Alexander disease. *Acta Neuropathologica Communications*, *5*(1), 27. https://doi.org/10.1186/s40478-017-0425-9

Sosunov, A., Olabarria, M., & Goldman, J. E. (2018). Alexander disease: An astrocytopathy that produces a leukodystrophy. *Brain Pathology*, *28*(3), 388–398. https://doi.org/10.1111/bpa.12601

Spruston, N. (2008). Pyramidal neurons: Dendritic structure and synaptic integration. *Nature Reviews Neuroscience*, *9*(3), 206–221. https://doi.org/10.1038/nrn2286

Stahl, F., Denner, P., Piston, D., Evert, B. O., de Boni, L., Schmitt, I., Breuer, P., & Wüllner, U. (2021). Activators of alpha synuclein expression identified by reporter cell line-based high throughput drug screen. *Scientific Reports*, *11*(1), 19857. https://doi.org/10.1038/s41598-021-98841-9

Staudt, C., Puissant, E., & Boonen, M. (2017). Subcellular Trafficking of Mammalian Lysosomal Proteins: An Extended View. *International Journal of Molecular Sciences*, *18*(1), Article 1. https://doi.org/10.3390/ijms18010047

Stirnemann, J., Belmatoug, N., Camou, F., Serratrice, C., Froissart, R., Caillaud, C., Levade, T., Astudillo, L., Serratrice, J., Brassier, A., Rose, C., Billette de Villemeur, T., & Berger, M. G. (2017). A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments. *International Journal of Molecular Sciences*, *18*(2), Article 2. https://doi.org/10.3390/ijms18020441

Südhof, T. C. (2017). Synaptic Neurexin Complexes: A Molecular Code for the Logic of Neural Circuits. *Cell*, *171*(4), 745–769. https://doi.org/10.1016/j.cell.2017.10.024

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*, *131*(5), 861–872. https://doi.org/10.1016/j.cell.2007.11.019

Taruscio, D., & Gahl, W. A. (2024). Rare diseases: Challenges and opportunities for research and public health. *Nature Reviews Disease Primers*, *10*(1), 13. https://doi.org/10.1038/s41572-024-00505-1

Telias, M. (2023). Neural differentiation protocols: How to choose the correct approach. *Neural Regeneration Research*, *18*(6), 1273. https://doi.org/10.4103/1673-5374.360171

Valori, C. F., Possenti, A., Brambilla, L., & Rossi, D. (2021). Challenges and Opportunities of Targeting Astrocytes to Halt Neurodegenerative Disorders. *Cells*, *10*(8), Article 8. https://doi.org/10.3390/cells10082019

van der Knaap, M. S., Barth, P. G., Stroink, H., van Nieuwenhuizen, O., Arts, W. F. M., Hoogenraad, F., & Valk, J. (1995). Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. *Annals of Neurology*, *37*(3), 324–334. https://doi.org/10.1002/ana.410370308

van der Knaap, M. S., & Bugiani, M. (2017). Leukodystrophies: A proposed classification system based on pathological changes and pathogenetic mechanisms. *Acta Neuropathologica*, *134*(3), 351–382. https://doi.org/10.1007/s00401-017-1739-1

van der Knaap, M. S., & Bugiani, M. (2018). Leukodystrophies—Much more than just diseases of myelin. *Nature Reviews Neurology*, *14*(12), 747–748. https://doi.org/10.1038/s41582-018-0093-9

Vaquer, G., Dannerstedt, F. R., Mavris, M., Bignami, F., Llinares-Garcia, J., Westermark, K., & Sepodes, B. (2013). Animal models for metabolic, neuromuscular and ophthalmological rare diseases. *Nature Reviews Drug Discovery*, *12*(4), 287–305. https://doi.org/10.1038/nrd3831

Viedma-Poyatos, Á., González-Jiménez, P., Pajares, M. A., & Pérez-Sala, D. (2022). Alexander disease GFAP R239C mutant shows increased susceptibility to lipoxidation and elicits mitochondrial dysfunction and oxidative stress. *Redox Biology*, *55*, 102415. https://doi.org/10.1016/j.redox.2022.102415

Walker, A. K., Daniels, C. M. L., Goldman, J. E., Trojanowski, J. Q., Lee, V. M.-Y., & Messing, A. (2014). Astrocytic TDP-43 Pathology in Alexander Disease. *Journal of Neuroscience*, *34*(19), 6448–6458. https://doi.org/10.1523/JNEUROSCI.0248-14.2014

Walkley, S. U. (2021). Rethinking Lysosomes and Lysosomal Disease. *Neuroscience Letters*, 136155. https://doi.org/10.1016/j.neulet.2021.136155

Weisheit, I., Kroeger, J. A., Malik, R., Wefers, B., Lichtner, P., Wurst, W., Dichgans, M., & Paquet, D. (2021). Simple and reliable detection of CRISPR-induced on-target effects by qgPCR and SNP genotyping. *Nature Protocols*, *16*(3), Article 3. https://doi.org/10.1038/s41596-020-00481-2

Wippold, F. J., Perry, A., & Lennerz, J. (2006). Neuropathology for the Neuroradiologist: Rosenthal Fibers. *American Journal of Neuroradiology*, *27*(5), 958–961.

Wolf, N. I., Knaap, M. S. van der, & Engelen, M. (2025). Treatment of leukodystrophies: Advances and challenges. *European Journal of Paediatric Neurology*, *56*, 46–50. https://doi.org/10.1016/j.ejpn.2025.03.016

Wong, K., Sidransky, E., Verma, A., Mixon, T., Sandberg, G. D., Wakefield, L. K., Morrison, A., Lwin, A., Colegial, C., Allman, J. M., & Schiffmann, R. (2004). Neuropathology provides clues to the pathophysiology of Gaucher disease. *Molecular Genetics and Metabolism*, *82*(3), 192–207. https://doi.org/10.1016/j.ymgme.2004.04.011

Woo, E. G., Tayebi, N., & Sidransky, E. (2021). Next-Generation Sequencing Analysis of GBA1: The Challenge of Detecting Complex Recombinant Alleles. *Frontiers in Genetics*, *12*. https://doi.org/10.3389/fgene.2021.684067

Woodman, P. G., & Futter, C. E. (2008). Multivesicular bodies: Co-ordinated progression to maturity. *Current Opinion in Cell Biology*, *20*(4), 408–414. https://doi.org/10.1016/j.ceb.2008.04.001

Wright, C. F., FitzPatrick, D. R., & Firth, H. V. (2018). Paediatric genomics: Diagnosing rare disease in children. *Nature Reviews Genetics*, *19*(5), 253–268. https://doi.org/10.1038/nrg.2017.116

Yang, N., Chanda, S., Marro, S., Ng, Y.-H., Janas, J. A., Haag, D., Ang, C. E., Tang, Y., Flores, Q., Mall, M., Wapinski, O., Li, M., Ahlenius, H., Rubenstein, J. L., Chang, H. Y., Buylla, A. A., Südhof, T. C., & Wernig, M. (2017). Generation of pure GABAergic neurons by transcription factor programming. *Nature Methods*, *14*(6), 621–628. https://doi.org/10.1038/nmeth.4291

Yarkova, E. S., Grigor'eva, E. V., Medvedev, S. P., Pavlova, S. V., Zakian, S. M., & Malakhova, A. A. (2024). IPSC-Derived Astrocytes Contribute to In Vitro Modeling of Parkinson's Disease Caused by the GBA1 N370S Mutation. *International Journal of Molecular Sciences*, *25*(1), Article 1. https://doi.org/10.3390/ijms25010327

Yi, H., Zhang, J., Gao, K., Yan, W., Chu, H., Zhang, J., Zhang, F., Jiang, Y., Wang, J., & Wu, Y. (2025). Morphological Characteristics and Extracellular Matrix Abnormalities in Astrocytes Derived From iPSCs of Children With Alexander Disease. *CNS Neuroscience & Therapeutics*, *31*(1), e70240. https://doi.org/10.1111/cns.70240

Yoon, S.-J., Elahi, L. S., Pașca, A. M., Marton, R. M., Gordon, A., Revah, O., Miura, Y., Walczak, E. M., Holdgate, G. M., Fan, H. C., Huguenard, J. R., Geschwind, D. H., & Pașca, S. P. (2019). Reliability of human cortical organoid generation. *Nature Methods*, *16*(1), 75–78. https://doi.org/10.1038/s41592-018-0255-0

Zeng, J., Indajang, J., Pitt, D., & Lo, C. H. (2025). Lysosomal acidification impairment in astrocyte-mediated neuroinflammation. *Journal of Neuroinflammation*, *22*(1), 72. https://doi.org/10.1186/s12974-025-03410-w

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., Xu, W., Yang, N., Danko, T., Chen, L., Wernig, M., & Südhof, T. C. (2013). Rapid Single-Step Induction of Functional Neurons from Human Pluripotent Stem Cells. *Neuron*, *78*(5), 785–798. https://doi.org/10.1016/j.neuron.2013.05.029

Zhou, Z., Zhou, J., Liao, J., Chen, Z., & Zheng, Y. (2022). The Emerging Role of Astrocytic Autophagy in Central Nervous System Disorders. *Neurochemical Research*, *47*(12), 3697–3708. https://doi.org/10.1007/s11064-022-03714-w

Zimran, A., Ruchlemer, R., & Revel-Vilk, S. (2020). A patient with Gaucher disease and plasma cell dyscrasia: Bidirectional impact. *Hematology*, *2020*(1), 389–394. https://doi.org/10.1182/hematology.2020000123

Zini, S., Muscò, A., Longhena, F., Faustini, G., Tobia, C., Borsani, G., Spillantini, M. G., Zizioli, D., & Bellucci, A. (2025). A novel stable transgenic zebrafish line expressing mCherry-tagged human alpha-synuclein in the nervous system and exhibiting all the key features of Lewy body disorders at larval stage. *Neurobiology of Disease*, *213*, 107018. https://doi.org/10.1016/j.nbd.2025.107018