Generation of human astrocytes for disease modeling. A study based on stem cells, direct conversion and genome engineering to dissect the role of astrocytes in leukodystrophies

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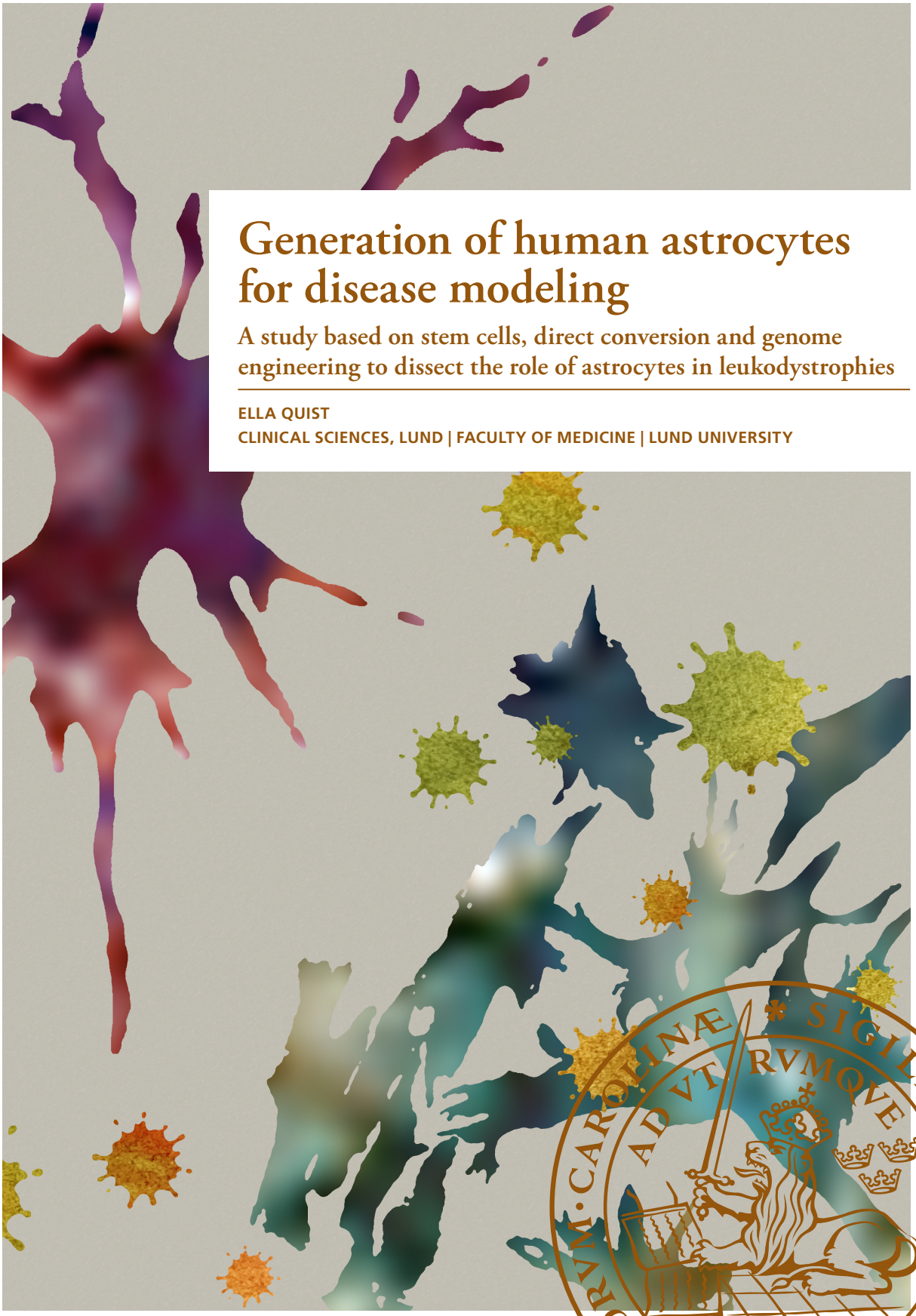
ELLA QUIST
CLINICAL SCIENCES, LUND | FACULTY OF MEDICINE | LUND UNIVERSITY



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LUND
UNIVERSITY

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Abstract:

Astrocytes are one of the major cell types in the central nervous system and are indispensable for brain development and function. The human brain and human astrocytes have unique structures and functions that are not present in other animal species. Studies of fundamental astrocyte biology in humans and their role in neurological disease have been hindered by shortage of native human astrocytes for research purposes and inadequate animal and cell models. With advancements in stem cell technology, the possibility to generate astrocytes *in vitro* from human pluripotent stem cells (hPSCs), ultimately derived from patient cells, emerged. However, traditional differentiation protocols of hPSCs to functional astrocytes, based on external cues to mimic development, are complex and time-consuming. In contrast, ectopic overexpression of cell lineage-specific transcription factors can fast forward this process.

Here we have developed a rapid and efficient method to generate functional and mature astrocytes from hPSCs through overexpression of the gliogenic transcription factors *Sox9* and *Nfib*. We have performed extensive phenotypic and functional characterization to confirm an astrocytic identity of the obtained cells. This method reduces the time to generate mature astrocytes from months to weeks. By combining our method with CRISPR/Cas9 genome editing we demonstrate that our method is feasible for disease modeling of the leukodystrophies Alexander disease (AxD) and Megalencephalic leukoencephalopathy with subcortical cysts (MLC).

Furthermore, we have developed an efficient method to directly convert human fibroblasts to astrocytes. We show that our method can be used with fibroblasts obtained from the entire human lifespan. We also, for the first time, show a co-culture system of astrocytes and neurons obtained through direct conversion of the same starting fibroblast populations. Finally, we provide proof-of-principle that our direct conversion method can be used for disease modeling by directly converting AxD patient fibroblasts to astrocytes.

The methods developed in this thesis allow for rapid generation of patient specific astrocytes which have the potential to uncover the role of astrocytes in neurological disorders and reveal novel targets for therapeutic interventions.

Key words: Astrocytes, stem cells, transcription factor programming, direct conversion, genome engineering, CRISPR/Ca9, leukodystrophies

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Artistic interpretation of fibroblasts encountering lentiviral particles carrying gliogenic transcription factors and through gradual morphological and phenotypic changes convert to functional astrocytes with a stellate morphology.

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Till Ludvig,

du är min ögonsten, solstråle
och största kärlek

Table of Contents

Original papers, manuscripts, and additional scientific output

Papers included in the thesis

Paper I

Rapid and efficient induction of functional astrocytes from human pluripotent stem cells.

Isaac Canals, Aurélie Ginisty, **Ella Quist**, Raissa Timmerman, Jonas Fritze, Giedre Miskinyte, Emanuela Monni, Marita G. Hansen, Isabel Hidalgo, David Bryder, Johan Bengzon and Henrik Ahlenius.

Nature Methods volume 15, pages 693–696, 2018.

Paper II

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

Ella Quist, Francis Paul, Isaac Canals and Henrik Ahlenius.

Manuscript

Paper III

Transcription factor-based direct conversion of human fibroblasts to functional astrocytes.

Ella Quist, Francesco Trovato, Natalia Avaliani, Oskar G. Zetterdahl, Ana Gonzalez-Ramos, Marita G. Hansen, Merab Kokaia, Isaac Canals and Henrik Ahlenius.

Stem Cell Reports, volume 17, issue 7, pages 1620-1635, 2022.

Paper IV

Modeling Alexander disease using direct conversion of patient derived fibroblasts to astrocytes.

Ella Quist, Niklas Darin, Pontus Wasling, Milos Pekny, Isaac Canals and Henrik Ahlenius.

Manuscript

Papers not included in the thesis

Transcription Factor Programming of Human Pluripotent Stem Cells to Functionally Mature Astrocytes for Monocultures and Cocultures with Neurons.

Ella Quist, Henrik Ahlenius and Isaac Canals

In: Ahlenius, H. (eds) Neural Reprogramming. Methods in Molecular Biology, volume 2352. Humana, New York, NY, pages 133-148, 2021.

Transcription Factor-Based Strategies to Generate Neural Cell Types from Human Pluripotent Stem Cells.

Isaac Canals, **Ella Quist** and Henrik Ahlenius.

Cellular Reprogramming volume 23, issue 44, pages 206-220, 2021.

Loss of Cxcr5 alters neuroblast proliferation and migration in the aged brain.

Jonas Fritze, Aurélie Ginisty, Rebecca McDonald, **Ella Quist**, Eleanor Stamp, Emanuela Monni, Parashar Dhapola, Stefan Lang and Henrik Ahlenius.

Stem Cells, volume 38, issue 9, pages 1175-1187, 2020.

Patent application

Direct conversion of human somatic cells to functional astrocytes.

Ella Quist and Henrik Ahlenius.

Swedish patent application number 2230105-5.

Popular scientific article

Så kan hudcellen lära oss om hjärnan.

Ella Quist.

Vetenskap & hälsa, June 13th, 2018.

Preface

The scene took place during Christmas celebrations at my parents' house six years ago. The atmosphere was filled with holiday spirit along with my infectious enthusiasm. I had just been accepted to do my master's thesis at Lund Stem Cell Center. The living room was filled with printed copies of various project proposals. One caught my attention in a way that stuck in my mind. "Astrocytes. Hmm, I think I have heard that word before". A quick Google-search later and indeed, I had heard about this star-shaped cell before. Once.

I could recall that this unique event had occurred during one of the courses I had taken during my exchange studies in Canada. In fact, I would never have enrolled to this course unless I was convinced by a newly met friend. My doubts were partly based on my inability to pronounce, or even worse, spell the name of the course, but also because it felt far off from my biotechnology engineering programme. But there it was, in one of the lectures notes from “Physiological Psychology”. Astrocytes - a supportive cell. That's it.

I know more about astrocytes now. A lot more.

This story illustrates how overlooked astrocytes have been historically. Since then, there has been a tremendous increase in our understanding of what an astrocyte is, appreciation of their heterogeneity and their involvement in neurological disease. These past years, I came to embrace astrocytes with my entire soul. I did not only see them in the microscope, I saw them in the form of leaves and water puddles while walking back and forth to the lab. Now my PhD studies are coming to an end, and instead I am watching from the outside how astrocytes are conducting their essential tasks in brain development and function in my 5-month-old son as he grows.

Abstract

Astrocytes are one of the major cell types in the central nervous system and are indispensable for brain development and function. The human brain and human astrocytes have unique structures and functions that are not present in other animal species. Studies of fundamental astrocyte biology in humans and their role in neurological disease have been hindered by shortage of native human astrocytes for research purposes and inadequate animal and cell models. With advancements in stem cell technology, the possibility to generate astrocytes *in vitro* from human pluripotent stem cells (hPSCs), ultimately derived from patient cells, emerged. However, traditional differentiation protocols of hPSCs to functional astrocytes, based on external cues to mimic development, are complex and time-consuming. In contrast, ectopic overexpression of cell lineage-specific transcription factors can fast forward this process.

Here we have developed a rapid and efficient method to generate functional and mature astrocytes from hPSCs through overexpression of the gliogenic transcription factors *Sox9* and *Nfib.* We have performed extensive phenotypic and functional characterization to confirm an astrocytic identity of the obtained cells. This method reduces the time to generate mature astrocytes from months to weeks. By combining our method with CRISPR/Cas9 genome editing we demonstrate that our method is feasible for disease modeling of the leukodystrophies Alexander disease (AxD) and Megalencephalic leukoencephalopathy with subcortical cysts (MLC).

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The methods developed in this thesis allow for rapid generation of patient specific astrocytes which have the potential to uncover the role of astrocytes in neurological disorders and reveal novel targets for therapeutic interventions.

Lay summary

The brain is built up by several different cell types, each with important functions. The teamwork between the different cell types is the foundation for us to have thoughts, feelings and be able to move our bodies. If any of the cell types in the brain stop working properly, the cooperation is disturbed, leading to a compromised function of the brain. This may result in difficulties to control movements, communicate with others, and lead to that patient becomes dependent on assistance to cope with everyday life. In the worst cases, diseases affecting the brain are fatal due to loss of life supporting functions.

Historically, research has been focused on understanding how neurons, the cell type that sends electrical signals through the brain, function and how their functions can be restored to normal with drugs to treat diseases affecting the brain. However, during the last 20 years, research has identified an additional cell type as a key player, essential for proper function of neurons and thus the brain, namely the astrocyte. Although astrocytes have proved important for proper brain function, there is still an incomplete understanding on how the teamwork between astrocytes, neurons and other cells in the brain is carried out and what consequences occur when astrocytes stop working properly.

Research on the various cell types in the brain, such as astrocytes, have been hindered by difficulties in obtaining the actual cells to study. There is extremely limited access to brain tissue for research purposes, both from healthy individuals as well as from patients with brain disease. As an alternative, researchers have studied astrocytes in animals. These studies have proved useful in obtaining general knowledge about the brain and astrocytes. However, the human brain and the astrocytes themselves, are substantially more complex in humans than in other species. Hence, knowledge gained from studying animal models might not hold true for the human brain. The limitation of animals to replicate the complex human brain, and its associated diseases, constitute a challenge in developing treatments for diseases affecting the brain.

A breakthrough came with the establishment of stem cell technology and the discovery that it was possible to, in a controlled manner, change one cell type into another cell type in the lab. A stem cell has the potential to develop into any other cell type present in the body, and methods were developed to guide this process to produce any desired cell type. Following this discovery, methods to transform cell

types that are easily accessible from the human body, such as skin cells and blood cells, to stem cells were developed. These could subsequently be guided to form any other cell type in the human body. These new technologies enabled production of cell types otherwise difficult to obtain for research, such as astrocytes, in a laboratory setting. However, one challenge remained, the methods to produce astrocytes in the laboratory were time-consuming, taking several months, and it was not certain that the produced cells really were the desired astrocytes.

By using a new approach, based on activation of parts of the genome that control development of astrocytes, we have in this study developed methods for rapid and efficient production of astrocytes from human stem cells as well as directly from human skin cells. We have examined the resulting cells stringently to be confident that they have functions that are expected from astrocytes. We have also introduced disease-causing mutations, identified in a group of disabling and fatal diseases called leukodystrophies that are thought to be caused by malfunctional astrocytes, in human stem cells, using the genome editing technology CRISPR/Cas9. Following the generation of leukodystrophy-mutated human stem cells, we have used our methods to produce astrocytes in order to generate cell-based astrocyte models of these diseases. These models allow for studies of biological, and possibly disease-causing processes, that occur in astrocytes.

The new methods to produce astrocytes in the laboratory, that we have developed in this thesis, allow for rapid production of human astrocytes of high quality. Our methods have the potential to reveal new insight into what happens when astrocytes stop working properly and how this contributes to brain disease. Knowledge gained from studying the role of astrocytes in disease might lead to the discovery and development of new efficient treatments for leukodystrophies, that today are lacking, but also for many other diseases affecting the brain due to the central role of astrocytes in brain function.

Populärvetenskaplig sammanfattning

Hjärnan består av flera olika celltyper som var och en har viktiga funktioner. Samspelet mellan de olika celltyperna gör att vi kan tänka, känna och röra oss. När någon av hjärnans celler slutar fungera påverkas samspelet och hela hjärnans funktion. Detta kan leda till svårigheter att kontrollera kroppsrörelser, kommunicera med andra och att man blir beroende av assistans för att klara vardagen. I värsta fall är sjukdomar som drabbar hjärnan dödliga då kontroll över livsuppehållande funktioner förloras.

Historiskt sett har forskningen fokuserat på att förstå hur nervceller, den celltyp som skickar elektriska signaler i hjärnan, fungerar och hur man på medicinsk väg kan påverka deras funktion för att behandla sjukdomar som drabbar hjärnan. De senaste 20 åren har forskningsstudier identifierat ytterligare en celltyp som nyckelspelare, de stjärnformade astrocyterna, som visat sig vara oumbärlig för nervcellernas och hjärnans funktion. Men exakt hur samspelet mellan astrocyter, nervceller och resten av celltyperna i hjärnan ser ut och vad som händer då astrocyter slutar fungera har länge varit okänt.

Forskning på hjärnans celler, så som astrocyter, har varit hindrad på grund av stora svårigheter att få tag i just själva cellerna. Då tillgången till hjärnvävnad från både friska människor och personer drabbade av hjärnsjukdom är extremt begränsad har forskare istället studerat astrocyter i djur. Trots att man har fått mycket kunskap om hjärnan och astrocyter genom dessa studier så är människans hjärna, och även själva astrocyterna, betydligt mer komplexa i människa än i andra djurarter. Det är därför inte säkert att kunskapen från djurmodeller även gäller i människans hjärna. Detta utgör ett hinder för studier av den komplexa mänskliga hjärnan och utveckling av behandlingar mot hjärnans sjukdomar.

Ett genombrott kom i och med att stamcellsteknologin etablerades och att forskare upptäckte att man under kontrollerade former i laboratoriet kan ändra en celltyp till en annan vald celltyp. En stamcell har potential att bilda alla celltyper i kroppen och metoder utvecklades för att kunna styra vilken celltyp stamcellen skulle bilda. Efter detta utvecklades även tillvägagångssätt för att isolera celler som är lättillgänglig från människans kropp, t ex hudceller och blodceller, och i laboratoriet omvandla dessa till stamceller och därefter vidare till vilken celltyp som helst som finns i människans kropp. Dessa metodutvecklingar gjorde det möjligt att tillverka celltyper som normalt sett är svåra att isolera, så som astrocyter. Ett problem

kvarstod dock, de metoder som användes för att tillverka astrocyter i laboratoriet var tidsödande och osäkra. Det kunde ta upp till ett halvår och det var då inte ens helt säkert att de producerade cellerna verkligen var de önskade astrocyterna.

Genom ett nytt tillvägagångsätt baserat på att aktivera de delar av arvsmassan (DNA) som styr bildandet av astrocyter har vi i det här arbetet utvecklat metoder för att snabbt och effektivt tillverka astrocyter från både mänskliga stamceller och direkt från mänskliga hudceller. Vi har undersökt de resulterande cellerna för att säkerhetsställa att de har de funktioner som förväntas av astrocyter. Med hjälp av den så kallade gensaxen CRISPR/Cas9 har vi fört in sjukdomsframkallande mutationer i arvsmassan i mänskliga stamceller. Dessa mutationer har hittats i patienter drabbade av leukodystrofi, en grupp funktionsnedsättande och dödliga sjukdomar som tros bero på felaktiga astrocyter. Vi har sedan använt de metoder vi har utvecklat för att tillverka astrocyter från muterade stamceller och på så sätt skapat astrocytiska cellmodeller av dessa sjukdomar för att kunna undersöka de biologiska och eventuellt sjukdomsframkallande mekanismer som sker i astrocyter.

De nya metoderna för att tillverka astrocyter som vi har utvecklat i detta arbete tillåter en snabb och kvalitetssäker tillverkning av mänskliga astrocyter. Dessa metoder har potential att öka förståelsen för vad som händer när astrocyter slutar fungera som de ska och hur detta bidrar till sjukdom i hjärnan. Denna kunskap kan i förlängningen bidra till att utveckla nya effektiva behandlingar av leukodystrofier, som idag saknas, men även många andra sjukdomar som drabbar hjärnan.

Abbreviations

ALS	Amyotrophic lateral sclerosis
AP	Action potential
AxD	Alexander disease
Cas9	CRISPR associated protein 9
Cas9n	CRISPR associated protein 9 nickase
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
DSB	Double-stranded break
FBS	Fetal bovine serum
HEF	Human embryonic fibroblast
HDR	Homology directed repair
hESC	Human embryonic stem cell
hES-iAs	Human embryonic stem cell induced astrocytes
hOLS	Human oligodendrocyte spheroids
hiPSC	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell
iAs	Induced astrocyte
iN	Induced neuron
iNPC	Induced neural progenitor cell
iOL	Induced myelinating oligodendrocyte
iOPC	Induced oligodendrocyte progenitor cell
iPSC	Induced pluripotent stem cell
MLC	Megalencephalic leukoencephalopathy with subcortical cysts
MRI	Magnetic resonance imaging

NEAA	Non-Essential Amino Acids
NSC	Neural stem cell
NHEJ	Non-homologous end joining
OPC	Oligodendrocyte progenitor cell
PAM	Protospacer adjacent motif
R&D	Research and development
SAB	*Sox9*, *Nfia* and *Nfib*
SB	*Sox9* and *Nfib*
sgRNA	Single guide RNA
sPSCs	Spontaneous postsynaptic currents
ssODN	Single-stranded oligodeoxynucleotide
TF	Transcription factor
VRAC	Volume-regulated anion current
WT	Wild type

Introduction

Rationale for this thesis

Neurological disorders are a leading cause of death and disability worldwide and constitute an increasing economic burden for society as the global population continues to expand and the lifespan of the population increases (Feigin et al., 2017). At the same time, drug discovery in the pharmaceutical industry has been facing a productivity crisis, including development of treatments for central nervous system (CNS) disorders (Kola and Landis, 2004; Pammolli et al., 2020). The costs per drug spent in Research and development (R&D) doubled every 9th year between 1950 and 2010 while failure rates of candidate drugs remained similar, or even increased (Hay et al., 2014; Scannell et al., 2012). During the years 1991-2000 the success rate for CNS disorder drugs were only 8% (Kola and Landis, 2004). The main reasons for failure were due to lack of efficacy in humans and safety concerns (Kola and Landis, 2004). Interestingly, failure rates seemed to correlate with animal models having poor predictability of human physiology in certain therapeutic areas, in particular for oncology and neurological diseases (Kola and Landis, 2004).

The pharmaceutical companies had to take action to solve this productivity crisis. It was hypothesized that the commonly used simplified models had insufficient predicational potential and by instead using models of higher predictive value of human pathology, better treatments would result (Scannell et al., 2012). One of several strategies was to change the mindset from quantity to quality by focusing on understanding the target (Cook et al., 2014). Investments in stem cell technology and genome editing platforms were done to improve biological translatability (Morgan et al., 2018). In addition, rare diseases with no existing treatments were given more attention (Ringel et al., 2020). Indeed, a greater understanding of disease biology has been hypothesized to, at least partly, be contributing to breaking the negative trend in R&D productivity for most therapeutic areas (Hurle et al., 2016; Pammolli et al., 2020; Ringel et al., 2020). However, attrition rates for CNS drug candidates have not decreased and still constitute one of the highest among all therapeutic areas (Pammolli et al., 2020). This highlights the challenges in studying the human CNS due to its exceptional complexity, which is difficult to fully capture using simplified models (Pammolli et al., 2020). Thus, models with high biological relevance and predicational value for human CNS pathologies are highly warranted (Trudler et al., 2021).

Astrocytes

Astrocytes in CNS development and function

Astrocytes are one of the major cell types in the mammalian central nervous system (CNS) and are crucial for normal brain development and functionality (Sofroniew and Vinters, 2010; Verkhratsky and Nedergaard, 2018). Historically, astrocytes were thought to be passive support cells for neurons but research over the last decades have changed that view. Today it is widely accepted that astrocytes are a heterogenous group of cells with active roles that cannot be neglected when attempting to understand the human brain, its associated diseases and how to treat them (Valori et al., 2019). Importantly, this new knowledge has highlighted that without the supportive functions from astrocytes, the human CNS, as we know it, can simply not exist (Verkhratsky and Nedergaard, 2018).

Astrocytes participate in formation of neuronal networks by promoting synaptogenesis and synapse stabilization as well as refinement through elimination of redundant synapses during development (Allen and Eroglu, 2017; Barker and Ullian, 2010). They also promote proliferation and migration of oligodendrocyte precursor cells (OPCs) and take part in myelin formation, maintenance, remodeling and remyelination following lesions to the CNS (Camargo et al., 2017; Hughes, 2021; Traiffort et al., 2020).

Astrocytes are involved in synaptic transmission by taking up and recycling released neurotransmitters, such as glutamate, from the synaptic cleft (Allen and Eroglu, 2017; Barker and Ullian, 2010; Magistretti et al., 1999). It has also been proposed that astrocytes directly modulate synaptic activity through release of synaptically active molecules such as ATP and D-serine (Barker and Ullian, 2010). Furthermore, astrocytes display regulated intracellular calcium transients which have functional effects on both astrocyte-astrocyte communication as well as neuronal function (Sofroniew and Vinters, 2010).

One of the most crucial functions of astrocytes is to maintain pH, ion and water homeostasis through various cell membrane transporters and channels on their surfaces (Benfenati and Ferroni, 2010). They also form large syncytia through astrocyte-astrocyte gap junction coupling allowing them to transport ions and various molecules (Verkhratsky and Nedergaard, 2018). These features result in a prominent buffering capacity of potassium ions and regulation of osmotic changes that arise upon neuronal signaling (Benfenati and Ferroni, 2010; Simard and Nedergaard, 2004).

Astrocyte endfeet enwrap blood vessels to form a gliovascular unit through which exchange of nutrients and metabolites with the bloodstream and CNS cells occurs (Magistretti et al., 1999). It has also been shown that astrocytes secrete factors that

regulate changes in local blood flow in response to neuronal activity (Nedergaard et al., 2003). Furthermore, astrocytes participate in forming the selective blood-brain barrier, hindering pathogens and large molecules to enter the CNS (Sofroniew and Vinters, 2010).

Astrocytes in disease

Astrocytes respond to all forms of injury, infection or disease that affect the CNS, through a process called reactive astrogliosis (Sofroniew and Vinters, 2010). This includes a wide range of transcriptional, morphological, and functional changes such as release of pro- and anti-inflammatory cytokines, hypertrophy of cell processes and formation of physical barriers (Escartin et al., 2021). These responses can lead to both beneficial and detrimental effects on surrounding cells and CNS function, and is a hallmark for many neurological diseases (Escartin et al., 2021).

In addition, due to the essential functions performed by astrocytes and their close interplay with neurons, oligodendrocytes, and other CNS cells it is easy to understand that astrocytes modulate and, in some cases, can even be a causative factor for neurological diseases. Indeed, astrocytes have emerged as key players in a wide range of neurodevelopmental, neuropsychiatric, and neurodegenerative diseases such as autism, schizophrenia, epilepsy, depression, multiple sclerosis (MS), and Alzheimer's disease (Lee et al., 2022; Pekny et al., 2016; Seifert et al., 2010; Sloan and Barres, 2014). Currently, there is insufficient understanding of the defined roles of astrocytes and the molecular mechanism causing pathology. However, the contribution of astrocytes to some diseases have started to be uncovered (Almad and Maragakis, 2018; Lee et al., 2022). For instance, there is emerging evidence indicating that astrocytes have a causative role in the motor neuron degeneration seen in amyotrophic lateral sclerosis (ALS) patients (Bruijn et al., 1997; Di Giorgio et al., 2007; Nagai et al., 2007; Qian et al., 2017; Serio et al., 2013). However, today there are only a few physically and mentally debilitating white matter disorders, called leukodystrophies, in which astrocytes have been suggested to be the primary origin of disease and due to mutations in genes with enriched expression in astrocytes (Lanciotti et al., 2013).

Traditional models for studies of astrocytes

Studies of astrocyte biology have been hampered by the limited availability of neural cells isolated from living healthy individuals and patients with neurological disease. Traditionally, astrocytes have been studied using brain tissue samples, animal models, primary rodent astrocytes, and human astrocytoma cell lines (Ren and Dunaevsky, 2021; Trudler et al., 2021). However, each of these has major limitations for modeling of human CNS disorders.

Autopsy and biopsy materials are of extremely limited availability and are restricted in the external manipulation that can be done in attempting to understand disease mechanisms. In addition, postmortem tissue only represents the end stage of disease and does not give information on how the disease was initiated or progressed.

Animal models such as rodents, zebrafish and drosophila have been widely used to study astrocytes and provided many important insights (Guttenplan and Liddelow, 2019). Although some biological aspects are preserved through evolution, the human brain has a unique structure and higher complexity compared to other species. Furthermore, human astrocytes have a diameter at least twice as large and with 10-fold more processes as compared to mouse counterparts (Oberheim et al., 2009). The increased size and complexity enable human astrocytes to contact and regulate 270,000-2,000,000 synapses compared to 20,000-120,000 synapses in rodents (Oberheim et al., 2009). Differences in gene expression and importantly, functional differences, in for instance calcium signaling response and inflammatory response, have been described between rodent and human astrocytes (Li et al., 2021; Oberheim et al., 2009; Zhang et al., 2016). Moreover, in addition to grey matter protoplasmic and white matter fibrous astrocytes, there are subtypes of astrocytes present in the human CNS that are not found in rodents, namely interlaminar astrocytes and varicose projection astrocytes (Colombo and Reisin, 2004; Oberheim et al., 2009; Oberheim et al., 2006). Thus, findings obtained using animal models might not be a proper biological representation of what is truly occurring in the human CNS.

Studies based on cell cultures have traditionally been done through ectopic overexpression of the gene of interest in primary rat astrocytes, astrocytes derived from transgenic animals and human astrocytoma cell lines (Guttenplan and Liddelow, 2019; Lanciotti et al., 2022). However, these models exhibit abnormally high levels of the gene/protein in either a non-human or tumoral background. Thus, one cannot exclude that findings made in these systems do not fully reflect biological processes occurring in the brain of a living human being, leading to confounding results.

The neuroscience field has recognized that these traditional models are not sufficient to fully elucidate definite roles of astrocytes and their contributions to neurological disease (Valori et al., 2019). As new technologies such as stem cell reprogramming and genome engineering have been developed, researchers have been provided with tools that can generate accessible models with high predictive value for human biology and pathology, and thus facilitate the development of novel therapies targeting astrocytes (Soldner and Jaenisch, 2018; Trudler et al., 2021; Whiteley et al., 2022).

Generation of human astrocytes using stem cell technologies

Historically, cell differentiation, the process through which a cell acquires its specialized functions in a certain tissue, was thought to be an irreversible process. However, this idea was challenged and proven wrong with somatic nuclear transfer experiments, performed in the middle of the 20th century, showing that cellular fates are not permanent and can be changed with environmental factors present in oocytes (Briggs and King, 1952; Gurdon, 1962; Gurdon et al., 1958). Later it was shown that fully differentiated fibroblast could be induced to pluripotent cells by overexpressing embryonic stem cell enriched-transcription factors (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). With these findings, new possibilities to generate cells otherwise difficult to obtain from living humans arose (Fig. 1).

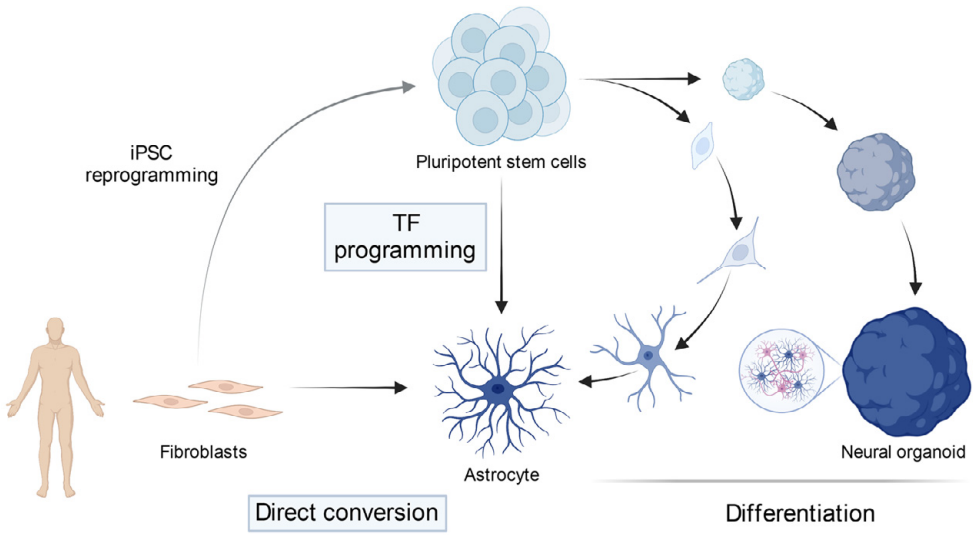


Fig. 1. Overview of different methods to generate human astrocytes *in vitro*.
Human pluripotent stem cells (hPSCs), either embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs), derived from fibroblasts, can be differentiated to astrocytes in monolayer cultures or as neural organoids (right side). In this work, we have developed methods to induce astrocytes through transcription factor (TF) programming of hPSCs and through direct conversion of human fibroblasts (in boxes). Created with BioRender.com.

Over the last decade, the field of stem cell technology has accelerated rapidly as more robust methods to differentiate human embryonic stem cells (hESCs) and patient-derived induced pluripotent stem cells (hiPSCs) have been developed, and today are common tools to generate cells for studies of human biology. Importantly, these new experimental approaches have shown their potential in CNS disease

modeling (Rowe and Daley, 2019; Trudler et al., 2021) and drug discovery (Silva and Haggarty, 2020), and constitute a promising avenue for regenerative medicine (Hastings et al., 2022; Tomishima and Kirkeby, 2021) and personalized medicine (Soldner and Jaenisch, 2018).

However, astrocyte differentiation protocols, either in monolayer (Krencik et al., 2011; Leventoux et al., 2020; Shaltouki et al., 2013; Tcw et al., 2017) or 3D culture (Sloan et al., 2017), which use instructive factors and molecules to mimic neural development are time-consuming, labor intensive and at the time this thesis was initiated, the resulting astrocytes were rarely well-characterized (Chandrasekaran et al., 2016). An alternative to traditional differentiation is to ectopically overexpress cell lineage-specific key transcription factors (TFs) in human pluripotent stem cells (hPSCs) (Canals et al., 2021). This process is referred to as transcription factor (TF) programming, or forward programming, and has been demonstrated to efficiently and rapidly generate induced neurons (iNs) (Yang et al., 2017; Zhang et al., 2013), oligodendrocyte precursor cells (iOPCs) (Garcia-Leon et al., 2018) and microglia (Chen et al., 2021) from hPSCs (reviewed in Canals et al., 2021). However, the possibility to generate induced astrocytes (iAs) from hPSCs through TF programming had, at the time this thesis work was initiated, not been explored.

An alternative approach to differentiation or TF programming of hPSCs is to directly convert already fully differentiated somatic cells, such as fibroblasts, to other cell fates without passing through a progenitor or pluripotent state (Ahlenius, 2022). This approach is called direct conversion, or direct cellular reprogramming, and can be accomplished through overexpression of TFs, short hairpin RNAs, application of instructive molecules, or a combination of these (Drouin-Ouellet et al., 2017; Traxler et al., 2019). Several methods have been developed to directly convert mouse and human fibroblasts to induced neurons (iNs) (Pang et al., 2011), oligodendrocyte precursor cells (iOPCs) (Yang et al., 2013) and myelinating oligodendrocytes (iOLS) (Chanoumidou et al., 2021). Interestingly, studies on iNs and iOLs obtained through direct conversion have shown that they retain age-related and possibly disease-associated features such as epigenetic marks, gene expression, and mitochondrial defects, whereas hPSC-derived cells represents a rejuvenated state (Chanoumidou et al., 2021; Kim et al., 2018; Mertens et al., 2015; Miller et al., 2013; Tang et al., 2017). In addition, hiPSC generation from patient fibroblasts is based on clonal expansion. Thus, only one fibroblast is represented in the final hiPSC clone and the subsequent progeny. This might be a concern since several cell types, including fibroblasts, neurons and astrocytes, have somatic copy number variations, suggesting an extensive genetic mosaicism which must be considered when using iPSC-derived cells (Abyzov et al., 2012; Cuevas-Diaz Duran et al., 2019; McConnell et al., 2013). In contrast, by using direct conversion, all or the majority of fibroblasts are represented in the final population, thus better preserving the genetic mosaicism (Mertens et al., 2016). For these reasons, using direct conversion to obtain cellular disease models, in particular for age-related and/or

sporadic diseases, might yield more meaningful data as it better reflects the age and variation within the donor fibroblast population (Mertens et al., 2016). Directly converted iNs have shown their potential in disease modeling of, for example, Huntington's disease and ALS (Pircs et al., 2022; Tang et al., 2017). Furthermore, it was recently shown that pathological hallmarks of Parkinson's disease were present in iNs obtained through direct conversion of patient fibroblasts but absent in hiPSC-derived neurons generated from the same fibroblasts, highlighting the importance of direct conversion as a methodology in disease modeling (Drouin-Ouellet et al., 2022).

When this thesis work was initiated, it had recently been shown that iAs could be generated through direct conversion of mouse fibroblasts by overexpressing the gliogenic TFs *Sox9*, *Nfia* and *Nfib* (Caiazzo et al., 2015), and subsequently, by using a cocktail of small molecules (Tian et al., 2016). However, generation of iAs directly from human fibroblasts was inefficient and proof that TF-mediated directly converted human iAs were functional as well as their use for disease modeling were still missing.

CRISPR/Cas9 genome engineering technology

Advancements in stem cell technology have been accompanied by a revolution in genome engineering technology using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein 9 (Cas9) systems. These have opened new possibilities to generate biologically relevant human cell-based disease models (Li et al., 2020).

The CRISPR system was originally discovered in bacteria and archaea, where it constitutes an adaptive defense system to protect from invading viruses (Bhaya et al., 2011). Later, it was harnessed to be used as a genome engineering tool and adapted to be used in mammalian cells (Cong et al., 2013). CRISPR/Cas9 genome engineering is based on a Cas9 nuclease that is guided by a short RNA molecule (sgRNA), complementary to a target DNA sequence (Jinek et al., 2012). If there is a protospacer adjacent motif (PAM) located downstream of the sgRNA targeting sequence, Cas9 introduces a double-strand break (DSB) in the DNA (Jinek et al., 2012). Cas9 nucleases from different bacterial strains that recognize different PAMs have been isolated and the most commonly used Cas9, isolated from *Streptococcus pyogenes* (SpCas9), recognize the PAM sequence 5'-NGG-3' and cut the DNA three nucleotides upstream of this PAM (Adli, 2018; Jinek et al., 2012). Once a DSB is introduced in the DNA, the cell repairs the break by using endogenous DNA repair pathways. The repair is accomplished either through non-homologous end joining (NHEJ), which typically results in random insertions or deletions, or with homology directed repair (HDR) using a DNA repair template, normally consisting

of the other allele in mammalian cells during replication (Sander and Joung, 2014). By providing a synthetically produced DNA repair template with a certain degree of homology to the target region, changes in the DNA sequence, such as point mutations or molecular tags, can be introduced (Fig. 2) (Cong et al., 2013).

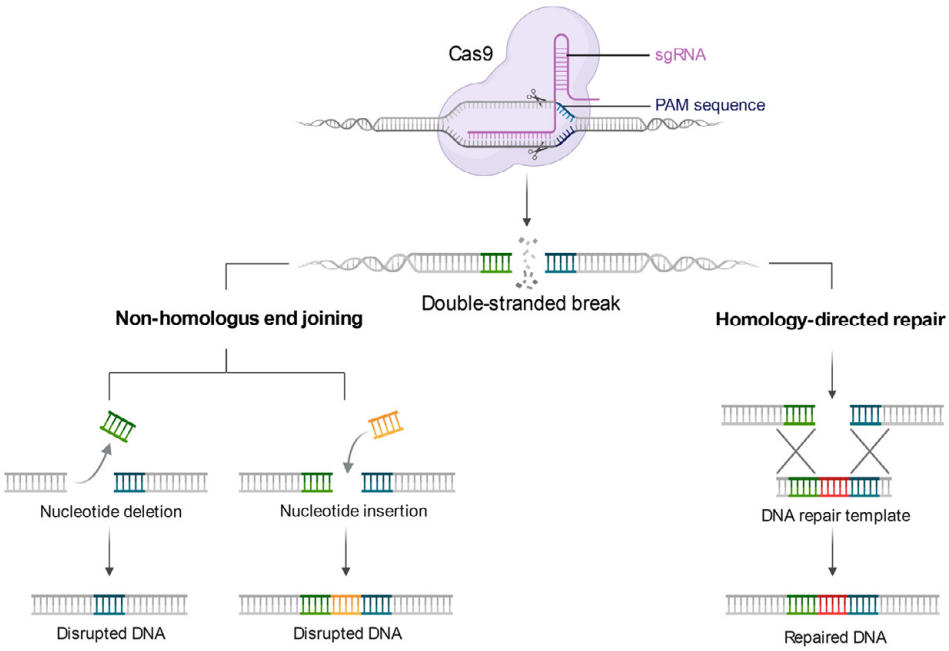


Fig. 2. Schematic illustration of genome editing with CRISPR/Cas9.
Cas9 is guided by a single guide RNA molecule (sgRNA), designed to be complementary to a target region in the genome. If there is a protospacer adjacent motif (PAM) located downstream of the sgRNA targeting sequence, Cas9 introduce a double-stranded break in the DNA. The double-stranded break is repaired by either non-homologous end joining, resulting in random insertions or deletions of nucleotides, or homology-directed repair, resulting in high fidelity repair of the DNA. By providing a synthetically designed DNA repair template, specific mutations can be inserted in the genome with high precision through HDR mechanisms. Created with BioRender.com.

The specificity for Cas9 binding is highly dependent on the uniqueness of the sgRNA sequence followed by a PAM in the genome. However, Cas9 binding tolerates some mismatches, which can result in off-target binding and cleavage (Fu et al., 2013). To increase the specificity of genome editing, mutated versions of Cas9, in which one of the catalytic domains has been inactivated, have been developed to transform the Cas9 nuclease to a DNA nickase (Cas9n) that only cuts one strand (Cong et al., 2013; Jinek et al., 2012). Therefore, genome engineering using this approach requires two Cas9n, guided by a pair of sgRNA complementary to regions in close proximity on opposite DNA strands, to generate a DSB (Ran et al., 2013). By being dependent on two sgRNAs to generate a DSB, the total number

of bases needed to be matched are increased, thereby yielding a higher precision and reducing off-target cutting (Ran et al., 2013).

The CRISPR/Cas9 genome engineering toolbox is continuously expanding with new versions of Cas9, which allows for base editing, prime editing, activation or repression of gene expression and epigenetic modifications (Anzalone et al., 2020; Hilton et al., 2015; La Russa and Qi, 2015). Importantly, genome editing using CRISPR/Cas9 has become a highly valuable tool to study human biology and has been used to generate new knowledge for many diseases such as Alzheimer's disease (Lin et al., 2018), Parkinson's disease (Soldner et al., 2016), and a wide range of heritable diseases (Li et al., 2020).

Leukodystrophies

Overview

Leukodystrophies are a group of rare genetically determined neurological disorders primarily affecting CNS white matter (Kevelam et al., 2016). These disorders mainly affect children but can present at any age (Kohlschütter and Eichler, 2011; Lanciotti et al., 2022; van der Knaap et al., 2019). Leukodystrophies are rare diseases but as a group they have an incidence rate of 10-200 out of 1 million children depending on region and classification criteria (Bonkowsky et al., 2010; Heim et al., 1997; Vanderver et al., 2012). Leukodystrophies are heterogeneous in their pathology but commonly lead to cognitive and motor disability (Lanciotti et al., 2013). Some leukodystrophies are progressive and fatal diseases, whereas others, typically those with onset in adulthood, have more benign clinical manifestations (van der Knaap et al., 2019). Current treatments consist of relief of symptoms with, for example, antiepileptic drugs or supportive care such as wheelchairs, speech therapy and nutritional support (van der Knaap et al., 2019).

Brain white matter constitutes half of the human brain and is comprised of mainly myelinated axons, and is a crucial component of functional neuronal networks governing cognitive and motor functions (Filley and Fields, 2016). The term leukodystrophy include conditions that show deficits in formation of myelin, degeneration of myelin, presence of structural or biochemical abnormal myelin, or vacuolization of myelin (van der Knaap and Bugiani, 2017). Myelin is an extension of oligodendrocyte plasma membranes that wraps around axons to form an insulated layer allowing for rapid transmission of neuronal electrical signals (Nave and Werner, 2014). Historically, leukodystrophies have been diagnosed using magnetic resonance imaging (MRI). Due to the white matter changes and myelination defects seen in patients, leukodystrophies were thought to be disorders originating from oligodendrocytes. However, as DNA sequencing technologies have become faster,

cheaper, and more available, mutations in genes normally not expressed in oligodendrocytes have been identified as disease-causative in some leukodystrophy patients (Brenner et al., 2001; Leegwater et al., 2001; Rademakers et al., 2011). These genes are instead expressed in astrocytes, microglia, neuronal axons, or blood vessel cells, and changed the view that all leukodystrophies are due to malfunctional oligodendrocytes or myelination. Based on the findings made using DNA sequencing, a new classification system was proposed based on in which cell type the mutated gene is expressed in, or playing a central part in pathology, namely myelin disorders, astrocytopathies, microgliopathies, leuko-axonopathies and leuko-vasculopathies (van der Knaap and Bugiani, 2017). To date seven leukodystrophies have been suggested to be due to malfunctional astrocytes, namely Alexander disease (AxD), Megalencephalic leukoencephalopathy with subcortical cysts (MLC), Vanishing white matter, ClC-2-related disease, Aicardi-Goutières syndrome, Oculodentodigital dysplasia and Giant axonal neuropathy (van der Knaap and Bugiani, 2017). Today, dysfunction of astrocytes in AxD and MLC have the clearest genetic link (van der Knaap and Bugiani, 2017). However, how astrocyte dysfunction results in disturbance of myelin and white matter alterations remain largely unknown (Lanciotti et al., 2022).

Alexander disease

AxD is a neurodegenerative, progressive and fatal disease (Messing, 2018). It is extremely rare, with an estimated prevalence of 1 out of 2.7 million people during a 5-year period (Yoshida et al., 2011). AxD can arise throughout life and has been classified into two forms based on clinical manifestations (Prust et al., 2011). Type I AxD patients have infantile disease onset and normally present macrocephaly, seizures, delayed development of motor and cognitive functions, and have a median survival of 14 years from onset (Prust et al., 2011; Yoshida et al., 2011). Type II AxD patients have disease onset later in childhood or as adults and have milder symptoms related to cerebellar and brainstem dysfunction such as autonomic dysfunction and ataxia, and a median survival of 25 years (Prust et al., 2011; Yoshida et al., 2011). Today there are no available treatments for AxD, only relief of symptoms and supportive care exist (Hagemann, 2022).

MRI imaging and histological analyses of AxD patient brains have shown white matter abnormalities, swelling, demyelination, reactive astrogliosis and, in some cases, axonal and neuronal loss (Borrett and Becker, 1985; Klein and Anzil, 1994; Tian et al., 2010). A pathological hallmark of AxD is formation of cytoplasmic inclusions called Rosenthal fibers, which consist of aggregates of GFAP together with heat shock proteins such as αB-crystallin and HSP27 (Borrett and Becker, 1985; Sosunov et al., 2018; Tanaka et al., 2007; Tomokane et al., 1991). Extensive degenerative features and large number of Rosenthal fibers are particularly prominent in AxD patients with early disease onset (Sosunov et al., 2018). These

abnormalities are observed in the frontal brain regions in type I patients, whereas in type II it has cerebellar, brainstem and spinal cord prevalence (Prust et al., 2011).

AxD is caused by dominant toxic gain-of-function mutations in the *GFAP* gene, encoding an intermediate filament protein, that has a highly specific expression in astrocytes (Brenner et al., 2001). How mutations in an intermediate filament protein result in formation of Rosenthal fibers, myelin deficits and abnormal white matter remains puzzling (Hagemann, 2022). Several transgenic and knock-in mouse models of AxD have been generated to increase the understanding of AxD pathology. These have provided mechanistic insights in the biology of Rosenthal fiber formation and composition (Hagemann et al., 2006; Heaven et al., 2016), as well as astrocyte dysfunction in AxD, such as aberrant calcium signaling (Saito et al., 2018), increased oxidative stress (Hagemann et al., 2005) and downregulation of potassium channels and glutamate transporters (Minkel et al., 2015). Interestingly, elevation of either wild type GFAP or mutant GFAP expression levels in AxD transgenic mouse models result in formation of Rosenthal fibers (Messing et al., 1998; Tanaka et al., 2007). Although informative, mouse models fail to robustly recapitulate the demyelination phenotype observed in AxD patients with early disease onset (Hagemann et al., 2006; Rutherford and Hamilton, 2019; Sosunov et al., 2018; Tanaka et al., 2007). Recently, a rat model of AxD was generated that better mimic disease phenotypes of AxD patients (Hagemann et al., 2021). Interestingly, treatment using antisense oligonucleotides (ASOs) targeting *Gfap* could reverse pathology. However, myelination deficits were only investigated in spinal cord, and it is currently unknown if the rat model display white matter changes in the brain (Hagemann et al., 2021)

Studies on AxD have also been performed in astrocytoma cell lines overexpressing mutant *GFAP*, which have revealed mitochondrial dysfunction and susceptibility to oxidative stress (Viedma-Poyatos et al., 2022).

However, given that the AxD mouse models fail to fully recapitulate patient phenotypes and AxD cellular models originate from tumors and are engineered to overexpress abnormally high *GFAP* levels, one can question the biological relevance of these models. Instead, we and others have since generated AxD *in vitro* astrocyte models based on hPSCs. These have shown to be able to form GFAP inclusions and aggregates (Canals et al., 2018; Jones et al., 2018), have altered localization of endoplasmic reticulum and lysosomes and deficits in ATP release resulting in abnormal calcium signaling (Jones et al., 2018). Furthermore, hPSC-derived AxD astrocyte models have been found to have an increased inflammatory state (Kondo et al., 2016) and interestingly, inhibit proliferation of hPSC-derived OPCs and reduce myelination (Li et al., 2018a).

Megalencephalic leukoencephalopathy with subcortical cysts

MLC is an extremely rare disease with onset at the first year of life (van der Knaap et al., 1995a). Children display cerebral white matter edema, macrocephaly, cysts, developmental delay of cognitive functions, motor deteriorations, epileptic seizures, and progressive cerebellar ataxia (Hamilton et al., 2018; van der Knaap et al., 2012). In some cases, mild head traumas trigger seizures, motor deterioration or leaves the patient unconscious for months (Bugiani et al., 2003; Riel-Romero et al., 2005). Current treatments only give symptomatic relief such as administration of drugs controlling epileptic seizures (van der Knaap et al., 2012).

MRI imaging of patient brains shows a swollen white matter and subcortical cysts, mainly in the anterior temporal regions (van der Knaap et al., 1995a; van der Knaap et al., 2012; van der Knaap et al., 1995b). Through examination of brain tissue samples, presence of swollen astrocytes and gliosis, as well as vacuolization of myelin sheets and astrocyte endfeet contacting blood vessels have been found (Duarri et al., 2011; Miles et al., 2009; van der Knaap et al., 1996). In some biopsies, the myelin content has been normal, whereas in others, thinner layers of myelin have been observed (Miles et al., 2009; van der Knaap et al., 1996).

MLC is caused by recessive mutations in *MLC1* or *HEPACAM*, genes that are mainly expressed in astrocytes (Leegwater et al., 2001; Lopez-Hernandez et al., 2011a). MLC1 is a membrane bound protein with putative ion channel function, while HEPACAM is a cell adhesion protein (Leegwater et al., 2001; Ridder et al., 2011). Analyses of brain samples have shown that MLC1 and HEPACAM interact and co-localize at astrocyte end-feet as well as astrocyte-astrocyte junctions, and that expression of MLC1 in MLC patients is largely abolished (Duarri et al., 2008; Lopez-Hernandez et al., 2011a; Lopez-Hernandez et al., 2011b). An additional form of MLC, caused by dominant mutation in *HEPACAM*, has been identified and, interestingly, these patients show normalization of motor and cognitive abilities over time as well as improvement of initial MRI abnormalities together with reduction of cysts (Hamilton et al., 2018; Lopez-Hernandez et al., 2011a; van der Knaap et al., 2010).

MLC mouse models have been generated and show intramyelinic edema, vacuolization of white matter, and astrocyte swelling (Dubey et al., 2014; Hoegg-Beiler et al., 2014; Sugio et al., 2017). These models have for instance provided insight into how MLC1 affects the function of the gliovascular unit (Gilbert et al., 2021). An MLC zebrafish model has also been generated to investigate the species-specific relationship between MLC1 and HEPACAM (Sirisi et al., 2014). However, MLC animal models do not show motor abnormalities as seen in patients, vacuolization only in cerebellum, and temporal expression of MLC1 and HEPACAM, correlating with appearance of vacuoles, differ between humans and other species (Dubey et al., 2014; Hoegg-Beiler et al., 2014; Sirisi et al., 2014).

Majority of *in vitro* studies have been based on overexpression or knockdown systems in primary rat astrocytes, HEK293 cells, HeLa cells, Sf9 insect cells or astrocytoma cell lines (Lanciotti et al., 2022; Lopez-Hernandez et al., 2011a; Ridder et al., 2011). These have provided mechanistic insight in MLC1 function. Reduced expression of MLC1 has been found to cause deficits in generation of chloride currents, reduce the ability to regulate cell volume upon osmotic stress, and cause intracellular vacuolization (Duarri et al., 2011; Ridder et al., 2011). These findings suggests that MLC1 has an association to volume-regulated anion current (VRAC) activity, a process that is involved in the temporal swelling occurring in astrocytes to regulate osmotic changes upon neuronal activity (Benfenati and Ferroni, 2010). Supporting this hypothesis, reduced VRAC activity as well as a decreased rate, by which the temporal swelling induced upon hypotonic treatment, takes to normalize has also been observed in MLC patient lymphoblasts, cells that endogenously express MLC1 (Ridder et al., 2011). Furthermore, studies using cellular models have identified that HEPACAM act as a chaperone for MLC1, and that mutations in *HEPACAM* cause trafficking defects of both MLC1 and HEPACAM, leading to deficits in activation of VRACs and providing an explanation to why mutations in *MLC1* and *HEPACAM* result in the same disease (Capdevila-Nortes et al., 2013; Lopez-Hernandez et al., 2011b). Cellular models have also been used to identify that MLC1 is part of a protein complex with channels and transporters such as KIR4.1, AQP4, TRPV4 and Na^+/K^+ ATPase pumps that are expressed at the gliovascular unit and involved in controlling ion and water homeostasis (Brignone et al., 2011; Lanciotti et al., 2010; Lanciotti et al., 2012).

Several lines of evidence suggest that astrocytes have a role in MLC pathology and that MLC is caused by defects in regulation of ion and water homeostasis. However, available animal models do not display all disease phenotypes seen in patients and further investigations are needed to fully understand MLC pathogenesis. Stem cell technology, such as those methods developed in this thesis, constitute a promising avenue to generate relevant and easily accessible MLC astrocyte models. However, to our knowledge, the results presented in this thesis and preliminary results from another group, are the only hPSC-based astrocyte models available today (Lanciotti et al., 2022). Nevertheless, these constitute promising platforms to dissect pathology and facilitate identifications of new therapeutic approaches that today are missing for MLC patients.

Aims of the thesis

This thesis aimed to develop *in vitro* methods to generate human astrocytes to study their role in neurological disorders.

Specific aims:

- To establish a method to rapidly generate functional and mature astrocytes from human pluripotent stem cells through transcription factor programming (Paper I).
- To generate astrocyte disease models of AxD and MLC by combining CRISPR/Cas9 genome editing of human embryonic stem cells with the method developed in Paper I (Paper I and Paper II).
- To establish an efficient method to generate astrocytes from human fibroblasts through direct conversion (Paper III).
- To generate an astrocyte disease model of AxD based on direct conversion of patient fibroblasts (Paper IV).

Summary of key results

Transcription factor programming of human pluripotent stem cells to induced astrocytes (Paper I)

When this work was initiated, current protocols for differentiation of human pluripotent stem cells (hPSCs) to astrocytes were very time-consuming and the resulting cells were poorly characterized (Chandrasekaran et al., 2016). This hindered research on human astrocyte biology and their involvement in neurological disease. Thus, we set out to develop a rapid and efficient transcription factor (TF) driven method to induce functional and mature human astrocytes (iAs) from hPSCs.

Previous studies on direct conversion of mouse fibroblasts to astrocytes had identified that overexpression of *Nfia*, *Nfib* and *Sox9*, from a selection of transcription factors enriched in astrocytes, was the optimal combination to induce an astrocytic cell fate (Caiazzo et al., 2015). Based on these findings, we performed a screen by overexpressing these TFs alone, in pairs and all three in combination in H1 hESCs using doxycycline-inducible lentiviral vectors. In contrast to mouse fibroblasts, our TF screen revealed that combined overexpression of *Sox9* and *Nfib* in hESCs resulted in the most robust astrocytic identity (hES-iAs). Furthermore, to have more physiological relevant culturing conditions, we gradually introduced serum-free cell culture media adapted for astrocytes.

Using immunocytochemistry, we observed that seven days after induction of transgenes, almost all cells (>90%) expressed the well-established astrocyte markers S100B, GFAP and VIMENTIN (Fig. 3A and 3B). We could also confirm that there was no induction of neural stem cells, neurons, or oligodendrocytes.

Mature astrocytes are post-mitotic and we observed that proliferation, assessed by KI67 expression, was clearly reduced with time to undetectable levels 21 days after induction of transgenes (Fig. 3C) (Zhang et al., 2016). At the same time, cells became more process bearing with complex morphology resembling mature astrocytes (Fig. 3D) (Zhang et al., 2016). Single cell RT-qPCR of hES-iAs and human primary adult astrocytes of a selected set of genes expressed in astrocytes, oligodendrocytes, neurons, neural stem cells or pluripotent cells, showed that hES-iAs are a homogenous population with a gene expression similar to human adult astrocytes.

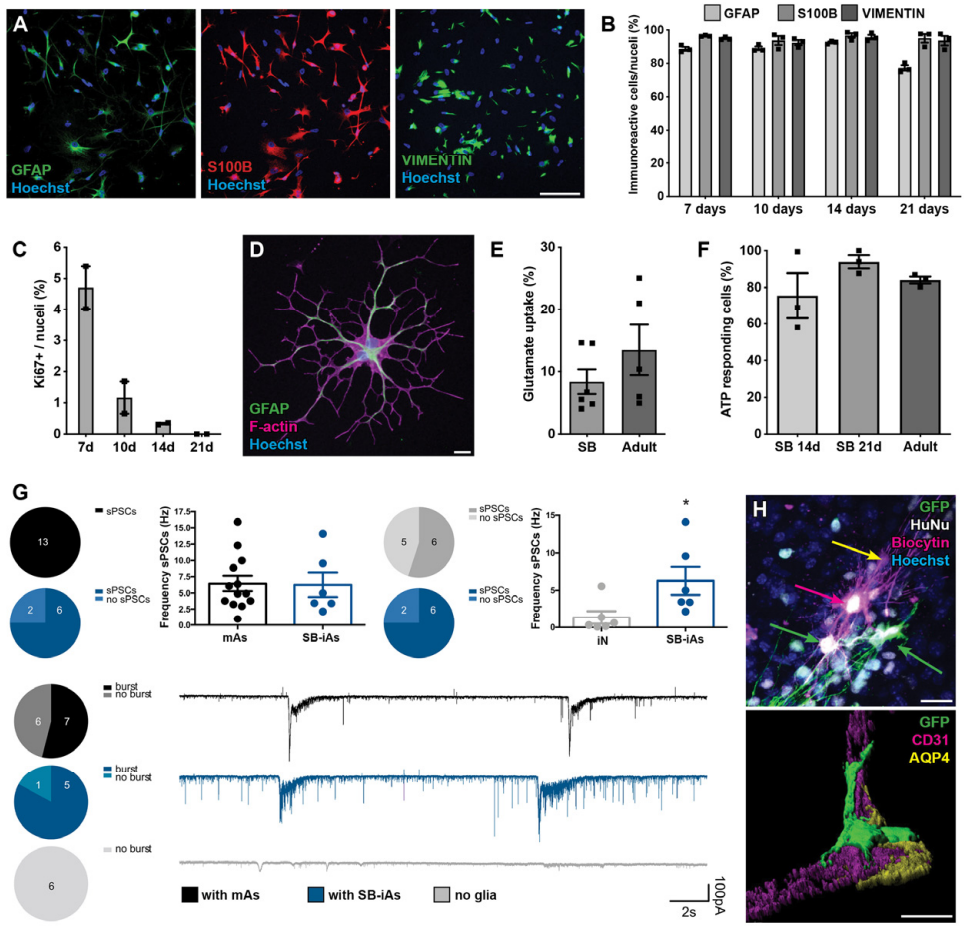


Fig. 3. Generation of induced astrocytes through transcription factor programming of human embryonic stem cells.

(**A**) Representative immunofluorescence images of GFAP, S100B, and VIMENTIN expression 7 days after induction of *Sox9* and *Nfib* (SB) in hESCs. (**B**) Quantification of GFAP+, S100B+ and VIMENTIN+ cells at 7, 10, 14, or 21 days. (**C**) Quantification of KI67+ cells. (**D**) Representative immunofluorescence of hES-iAs at day 21. (**E**) Glutamate uptake of hES-iAs (SB) at 14 days, and primary human adult astrocytes (Adult) (**F**) Percentage of Fluo-4 loaded cells with ATP-induced calcium response in hES-iAs (SB), at 14 and 21 days, and primary human adult astrocytes (Adult). (**G**) Electrophysiological recordings of induced neurons (iNs) cultured with mouse astrocytes (mAs, black, n=13 cells), with hES-iAs (SB-iAs), blue, n=6 iNs) or alone (iNs, grey, n=6 iNs) alone showing frequency of spontaneous postsynaptic current (sPSCs, top) and bursting activity (bottom). (**H**) Immunofluorescence images of GFP-labelled hES-iAs transplanted into mouse brain. GFP+ transplanted cells filled with biocytin 13 weeks after transplantation, showing diffusion of biocytin to GFP+/HuNu+ transplanted as well as GFP-/HuNu- host cells (top). Magenta arrow marks injected cell, green and yellow arrows mark transplanted and host cells, respectively, with biocytin. 3D reconstruction of z-stack acquired using confocal imaging showing GFP-labelled transplanted hES-iAs co-labelled with the astrocytic water channel protein AQP4 and the endothelial marker CD31 (bottom). Data are presented as mean ± SEM for n=2-3 independent induction experiments unless otherwise started. One-way ANOVA comparing all groups, with Tukey correction for multiple comparisons (E, F) and unpaired two-tailed Mann–Whitney test (G) were performed for statistical analysis. * $p<0.05$. Scale bars, 100 µm (A), 10 µm (D, H;bottom), 20 µm (H; top).

To investigate if hES-iAs could perform crucial astrocytic functions we performed an extensive functional characterization of obtained cells 14 and 21 days after induction of transgenes. We found that hES-iAs could take up glutamate at similar levels as human primary adult astrocytes and that they formed functional gap junctions (Fig. 3E). We performed live cell Ca^{2+} imaging experiments and observed that hES-iAs both had spontaneous and ATP-induced Ca^{2+} elevations similar to human adult astrocytes (Fig. 3F). Furthermore, we showed that hES-iAs upregulated expression of cytokine and chemokine transcripts following stimulation with interleukin-1β (IL-1β), similar to what has been described in human primary astrocytes, indicating that hES-iAs are immunocompetent (Harikumar et al., 2014). Furthermore, one of the main functions of astrocytes is to support synapse formation and neuronal function. To test this, we set up a co-culture system with hES-iAs and iNs derived from hESCs using *Ngn2* overexpression according to a previous study (Zhang et al., 2013). We observed that the frequency of spontaneous postsynaptic currents (sPSCs) was similar in iNs as when cultured with mouse astrocytes, which at the time was the gold standard for co-cultures with neurons. In addition, this frequency was significantly improved compared to when culturing iNs alone, in which only half of the recorded iNs had sPSCs. Analysis of bursting activity of iNs revealed that more iNs cultured with hES-iAs had increased activity compared to when cultured alone, and interestingly, when cultured with mouse astrocytes (Fig. 3G). The beneficial effect of hES-iAs on iNs was further supported by immunocytochemical analysis and quantification of overlap of the pre- and postsynaptic markers Bassoon and PSD95 on MAP2+ neurites. This showed a significant increase in marker overlap, indicative of an increased number of synapses, in iNs when co-cultured with hES-iAs as compared to when no astrocytes were present.

To test if hES-iAs could engraft, survive, and integrate in the brain we transplanted GFP labelled hES-iAs into the brains of neonatal and adult mice. Analysis using immunohistochemistry revealed that approximately 25% of transplanted cells had engrafted 4 weeks after transplantation and the cells retained their astrocytic identity. We observed that the transplanted hES-iAs expressed the gap junction protein CX43 and that it was sometimes located at iAs-iAs contacts. To test if functional gap junctions were formed *in vivo,* we performed biocytin diffusion experiments of GFP+ hES-iAs in acute brain slices. We observed that biocytin spread both to neighboring hES-iAs but also to host cells, confirming that gap junctions formed were functional and suggesting that hES-iAs participated in a panglial syncytia with both transplanted cells and host astrocytes. Co-staining of the main astrocytic water transporter AQP4 and endothelial cell marker CD31 revealed that transplanted GFP+ hES-iAs were in close contact with blood vessels and, in some cases, wrapping around them (Fig. 3H). These results indicate that transplanted hES-iAs can functionally integrate in the mouse brain and that they participate in formation of the gliovascular unit and blood-brain barrier.

We and others have now applied our method on more than 10 hPSC lines and demonstrated that our method robustly induces an astrocytic cell fate, although the efficacy varies slightly depending on the hPSC line used (Dobrindt et al., 2021).

In summary, we have established a rapid and efficient method to generate functional and mature human astrocytes from hPSCs through overexpression of the gliogenic TFs *Sox9* and *Nfib*. This method reduces the time to generate functional human astrocytes *in vitro* from months to weeks. We have performed extensive characterization of hES-iAs and have found high resemblance to human adult astrocytes on morphological, phenotypic, molecular, and functional levels. Furthermore, we have shown through transplantation studies, that hES-iAs survive, integrate, and maintain their astrocytic identity in the mouse brain.

Generation of AxD and MLC disease models using CRISPR/Cas9 genome engineering (Paper I and II)

Having established our TF programming method, we aimed to investigate if hES-iAs could be used for disease modeling of neurological disorders. We decided to generate models of the leukodystrophies AxD and MLC, disorders in which dysfunctional astrocytes have been suggested to be the primary cause. The models were generated by introducing disease-causing mutations in hESCs, and subsequently inducing an astrocytic cell fate using our TF programming method.

AxD astrocyte disease model (Paper I)

Here we performed CRISPR/Cas9 genome editing with the D10A mutant Cas9 nickase (Cas9n) and a pair of sgRNAs to reduce the risk of off-target mutagenesis compared to wild-type (WT) Cas9 and a single sgRNA (Ran et al., 2013). We introduced the R239C patient mutation in *GFAP* in H1 hESC by providing a single-stranded oligodeoxynucleotide (ssODN) HDR repair template, containing the R239C mutation and silent mutation in one of the PAM sequences to avoid repetitive editing (Fig. 4A). This allowed us to isolate one homozygous line, however we did not find any hESC clones that had a heterozygous mutation, as it is found in patients (Fig. 4B). We validated the specificity of genome editing by confirming that none of the top three most likely off-target sites for each sgRNA had been altered. Next, we subjected the homozygous AxD hESC line and isogenic non-edited parental H1 hESCs to our TF programming protocol. Gene expression using RT-qPCR showed that there was no difference in *S100B* expression between the lines, suggesting that ability to generate iAs was unaltered by the R239C *GFAP* mutation. We proceeded by analyzing resulting cells for previously described disease phenotypes. Immunocytochemical analysis revealed presence of GFAP

inclusions in AxD hES-iAs, similar to those previously described using overexpression of mutated *GFAP* in astrocytoma cell lines (Fig. 4C) (Chen et al., 2013; Tang et al., 2010), mouse primary astrocytes (Mignot et al., 2007) and since then, in other hPSC-derived astrocyte AxD models (Gao et al., 2019; Jones et al., 2018; Li et al., 2018a). Gene expression analysis showed downregulation of *KCNJ10* encoding KIR4.1, the main potassium channel in astrocytes, in AxD hES-iAs similar to what has been described in a transgenic AxD mouse model (Minkel et al., 2015) and *ATP1B2,* encoding the beta 2 subunit of Na^+/K^+ ATPase, that is involved in transporting Na^+ and K^+ over the cell membrane (Fig. 4D). Furthermore, aberrant calcium signaling has been reported in a mouse model of AxD (Saito et al., 2018). Thus, we performed Ca^{2+} imaging experiments of AxD hES-iAs. These experiments showed no difference in number of ATP responding cells but revealed that AxD hES-iAs had a higher amplitude of the ATP-induced response and a slower recovery to basal levels compared to isogenic controls (Fig. 4E).

These results show that genetically edited hES-iAs can recapitulate AxD disease phenotypes, providing a proof-of-principle that hES-iAs can be used for modeling of neurological disorders.

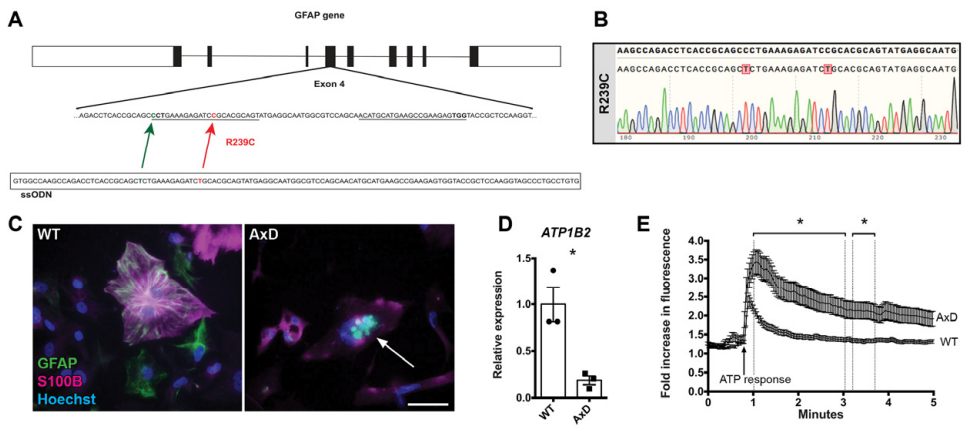


Fig. 4. Generation and characterization of Alexander disease (AxD) mutated hES-iAs.
(**A**) CRISPR/Cas9 genome editing strategy for introducing the R239C mutation in *GFAP*. sgRNA sequences are underlined and PAM sequences indicated in bold. ssODN repair template is shown in a box with disease mutation indicated with red font and silent mutation in PAM in green font. (**B**) DNA alignment of one sequenced homozygous AxD hESC clone, to unedited *GFAP* gene (top row in bold), showing both the disease mutation and silent PAM mutation in *GFAP*, both indicated in red. (**C**) Example immunofluorescence images of GFAP and S100B of unedited parental hES-iAs (WT) and AxD hES-iAs 28 days after induction of *Sox9* and *Nfib.* Arrow indicate GFAP inclusions. (**D**) Gene expression of *ATP1B2* relative to *GAPDH* in WT and AxD hES-iAs normalized to WT hES-iAs. (**E**) Fluo-4-based Ca^{2+} imaging results showing the fold increase in fluorescence over time, before and after ATP addition. Data are presented as mean ± SEM for n=3 independent induction experiments. Unpaired two-tailed t-test with Welch's correction for unequal variances (D) and multiple t-test comparing WT hES-iAs to AxD hES-iAs at each time point (E) were performed for statistical analysis. *p<0.05. Scale bar 50 µm (B). ssODN= single-stranded oligodeoxynucleotide.

MLC astrocyte disease model (Paper II)

To generate models of MLC, we introduced two known recessive patient homozygous point mutations in the *MLC1* gene, the mild but more common S93L mutation, and the severe but less frequent S280L (Ilja Boor et al., 2006). We designed and performed CRISPR/Cas9 genome editing for each locus in a similar manner as for AxD (Fig. 5A). However, we could not isolate any correctly edited hESC clones using the Cas9n and pairs of sgRNAs, likely due to that the distance between the two sgRNAs within each pair was too long (Ran et al., 2013). Instead, by using WT Cas9 and a single sgRNA we isolated one homozygous hESC line for each mutation (Fig. 5B). We confirmed that the MLC hESC lines had a normal karyotype, were of pure H1 hESC origin, were free from mycoplasma, had retained pluripotent properties during genome editing and that none of the top five most likely off-target sites had been altered.

Having validated the genome editing and integrity of the MLC hESC lines, we subjected them to our TF programming method to generate *MLC1* mutated hES-iAs. Immunocytochemical analysis and quantification of VIMENTIN, S100B and GFAP revealed that iAs could be generated from the MLC hESC lines similarly as parental unedited H1 hESCs in experiments performed in parallel (Fig. 5C). Next, we analyzed obtained cells for presence of vacuoles, that has been observed in MLC brain tissue as well as in primary rat astrocytes (Capdevila-Nortes et al., 2013; Duarri et al., 2011; Miles et al., 2009; van der Knaap et al., 1996), and mislocalization of MLC1, a phenotype previously described in several *in vitro* models (Duarri et al., 2008; Lopez-Hernandez et al., 2011b). However, we did not observe any obvious vacuoles or mislocalization of MLC1 under these experimental conditions (Fig. 5D and 5E).

Although astrocytes are likely key players in pathogenesis, MLC is an infantile onset disease affecting myelin, which is produced by oligodendrocytes (van der Knaap and Bugiani, 2017). Therefore, there is a need for models that recapitulate brain development and the crosstalk between different neural cell types. Thus, we decided to differentiate MLC hESC lines and unedited hESCs to neural organoids, which resemble brain development, and contain neurons and oligodendrocytes as well as astrocytes. We used a previously established method, specifically designed for guiding oligodendrocyte development and myelin formation, referred to as human oligodendrocyte spheroids (hOLS) (Marton et al., 2019).

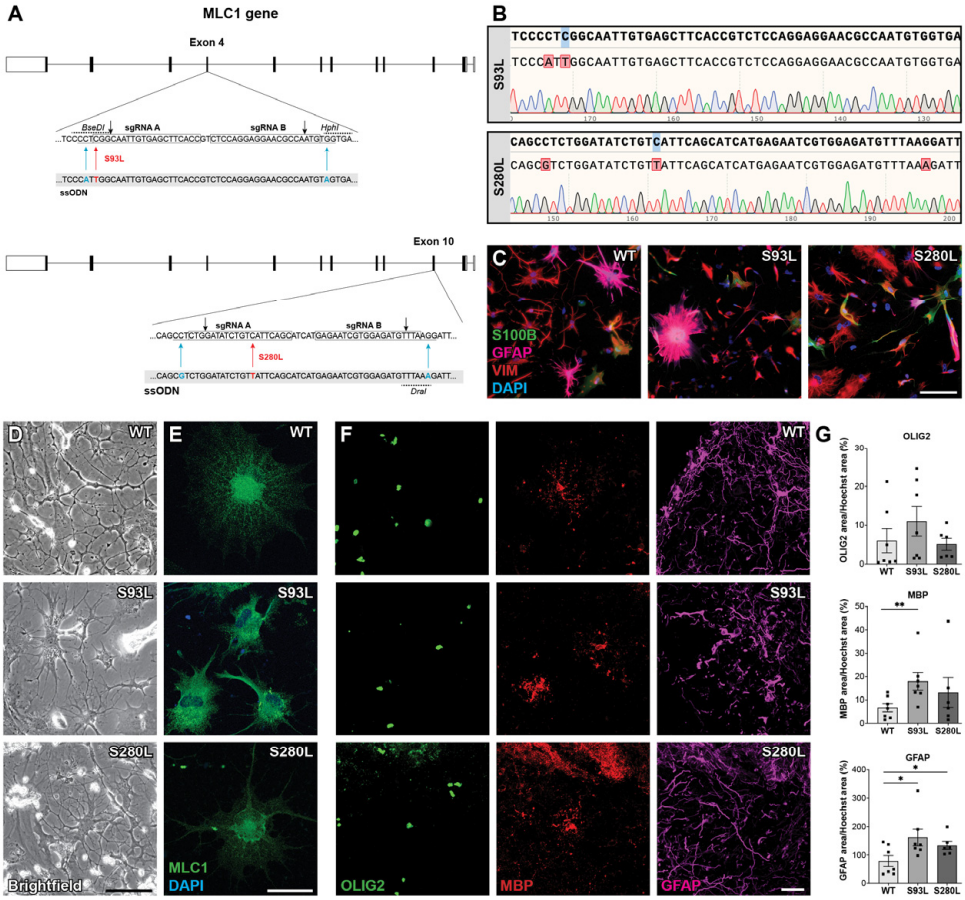


Fig. 5. Generation and characterization of megalencephalic leukoencephalopathy with subcortical cysts (MLC) mutated hES-iAs and hOLS.

(**A**) Design and strategy for CRISPR/Cas9 genome editing. sgRNAs are shown in boxes, PAM sequences are underlined and Cas9 cut site indicated by black arrows. ssODN repair templates are shown in grey boxes. Disease mutations are indicated with red font and silent mutations in PAM in blue font. Relevant restriction enzyme recognition sites are indicated with dotted lines. (**B**) DNA alignment of sequenced MLC hESC clones to unedited *MLC1* gene, showing both mutations in red and disease mutations indicated in blue. (**C**) Representative immunofluorescence images of S100B, GFAP and VIMENTIN (VIM) of unedited parental hES cells (WT) and hES cell lines with S93L or S280L *MLC1* mutations 28 days after induction of *Sox9* and *Nfib*. (**D**) Representative brightfield images of WT hES-iAs and MLC hES-iAs. (**E**) Representative immunofluorescence images of MLC1 of WT hES-iAs and MLC hES-iAs. (**F**) Representative immunofluorescence images of hOLS sections, immunostained for OLIG2, MBP and GFAP at 118 days of differentiation, from unedited parental hESCs (WT) and hESC lines with S93L or S280L *MLC1* mutations. (**G**) Quantification of OLIG2, MBP and GFAP immunoreactive area normalized to area covered by Hoechst. Data are presented as mean ± SEM of n=6-7 hOLS from 2 independent differentiation experiments (G). t-tests were performed on lognormal transformed data for statistical analysis between MLC hESC lines to WT (G). Scale bars, 100 µm (C, D), 50 µm (E, F).

Analysis of immunostained cryosections at day 59 of differentiation revealed no obvious difference in marker expression of intermediate progenitors (TBR2), neural stem cells (SOX2), neuronal cells (MAP2) or oligodendrocyte lineage cells, including progenitors, (OLIG2). At this time point there was very low marker expression of myelin (MBP) and no GFAP expression was observed, indicating that hOLS were too immature to contain myelinating oligodendrocytes and astrocytes. However, analysis at day 118 revealed a substantial increase of OLIG2, MBP and GFAP expression (Fig. 5F). Surprisingly, quantification of these glia markers revealed a higher level of MBP expression in hOLS carrying the *MLC1* S93L mutation as well as more GFAP expression in hOLS from both MLC hESCs as compared to unedited controls (Fig. 5G).

Altogether, we have generated and validated two hESC lines, harboring either the *MLC1* S93L or S280L patient mutations, using CRISPR/Cas9 genome editing. We have shown that these can be used to generate astrocytes in monolayer cultures as well as neural organoids containing astrocytes, oligodendrocytes, and myelin.

Direct conversion of human fibroblasts to induced astrocytes (Paper III)

Direct conversion constitutes an attractive alternative approach to generate human astrocyte models with the potential to capture age and genetic diversity of donors (Mertens et al., 2016). However, direct conversion of human fibroblasts to astrocytes was, at the time this study was initiated, poorly explored. Only one study existed in which S100B and GFAP expression was induced in only 1-2% of the starting fibroblasts population, resulting cells had no convincing astrocyte morphology, and functional characterization was lacking (Caiazzo et al., 2015). Thus, we set out to establish a method for efficient direct conversion of human fibroblasts to functional induced astrocytes (iAs).

We initiated our experiments by replicating the proof-of-principle study by overexpressing *Sox9*, *Nfia* and *Nfib* in human fibroblasts. However, we did two major adaptations that we speculated could improve conversion. First, we cloned each of the TFs into doxycycline-inducible lentiviral vectors containing blasticidin, hygromycin and puromycin resistance genes, respectively, to enable selection of transduced cells. Second, we gradually replaced the standard serum-containing media with serum-free media adapted for astrocyte differentiation and maturation. These optimizations allowed us to obtain cultures consisting of approximately 30 % iAs, assessed by immunostaining and quantification of S100B and GFAP, 3 weeks after induction of transgenes in human embryonic fibroblasts (HEFs) (Fig. 6A). In relation to the initial number of cells used for conversion experiments, this corresponded to a yield of around 80% S100B+, 50% GFAP+, and 40% S100B+

GFAP+ co-labelled cells. By extending the time in culture by 2 weeks, some iAs had developed stellate process bearing morphologies similar to bona fide astrocytes.

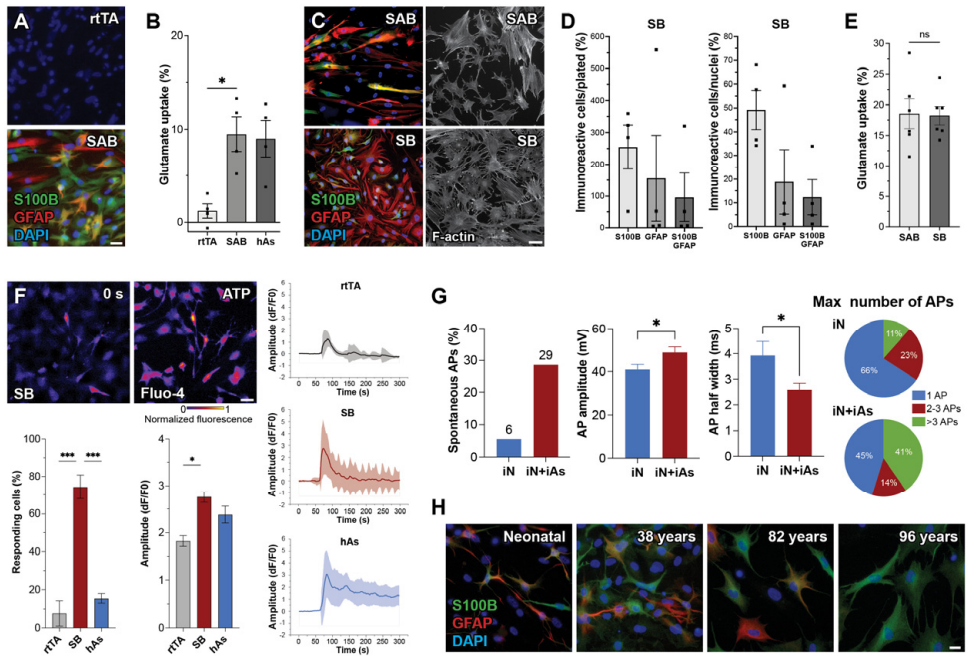


Fig. 6. Direct conversion of human fibroblasts to induced astrocytes.
(**A**) Representative immunofluorescence images of S100B and GFAP 3 weeks after induction of *Sox9*, *Nfia* and *Nfib* (SAB), in human embryonic fibroblasts (HEF) or rtTA-transduced control HEFs (rtTA). (**B**) Glutamate uptake of rtTA-transduced control HEFs and SAB-HEF-iAs, at 5 weeks, and primary human fetal astrocytes (hAs). (**C**) Representative immunofluorescence images of S100B and GFAP 5 weeks after induction of *Sox9*, *Nfia* and *Nfib* (SAB) or *Sox9* and *Nfib* (SB) in HEFs. (**D**) Quantification of yield and purity of S100B, GFAP and S100B/GFAP immunoreactive cells in SB-transduced HEFs at 3 weeks. (**E**) Glutamate uptake in SAB- and SB-HEF-iAs at 5 weeks. (**F**) Characterization of ATP-induced calcium response in Fluo-4 loaded rtTA-transduced control HEFs and SB-HEF-iAs, at 5 weeks, and primary human fetal astrocytes (hAs). (**G**) Electrophysiological recordings of induced neurons (iNs) cultured alone or in co-culture with SB-HEF-iAs obtained from the same fibroblast population. Electrophysiological recordings were performed on n=38-40 cells. (**H**) Immunofluorescence images of human neonatal, adult and aged fibroblasts 3 weeks after induction of SB. Data are presented as mean ± SEM, except for calcium traces (F) that are presented as mean ± SD, of n=3-6 independent induction experiments. Kruskal-Wallis tests (B, F), Wilcoxon matched-pairs rank test (E) and unpaired t-tests (G) were performed for statistical analysis. *p<0.05. ***p<0.001. Scale bars, 25 µm (A), 50 µm (C, F), 20 µm (I). AP=action potential.

Next, we observed that iAs obtained through *Sox9*, *Nfia* and *Nfib* overexpression in HEFs (SAB-HEF-iAs), expressed commonly used astrocytic markers and proteins involved in several crucial astrocytic functions. For instance, SAB-HEF-iAs expressed the astrocytic marker ALDH1L1, glutamate transporters (EAAT1 and EAAT2), an enzyme involved in glutamate-glutamine conversion in astrocytes (GS), and proteins related to astrocyte ion and water homeostatic functions (CX43,

AQP4, KIR4.1, ATP1B2). Importantly, functional assessment of iAs revealed that they took up glutamate at similar levels as human primary fetal astrocytes (Fig. 6B), and biocytin diffusion experiments showed formation of functional gap junctions.

At this time point, we had learned that combined overexpression of *Sox9* and *Nfib* was optimal to generate mature iAs from hPSCs (Paper I). In addition, several reports had indicated that NFIA was important in the gliogenic switch, the developmental step in which neural stem cells change from strictly producing neurons to generating astrocytes and oligodendrocytes, but not for astrocyte maturation (Tchieu et al., 2019; Tiwari et al., 2018). Thus, we performed a TF screen by transducing HEFs with *Sox9*, *Nfia* and *Nfib* alone, in pairs and all three in combination. Immunocytochemical analysis of S100B and GFAP, 3 weeks after induction of transgenes, revealed that overexpression of *Sox9* and *Nfib*, without *Nfia*, yielded most iAs with a 3-fold increase compared to the SAB condition. Furthermore, a larger portion of iAs obtained using *Sox9* and *Nfib* (SB-HEF-iAs) had a star-shaped morphology, characteristic for astrocytes (Fig. 6C and 6D). We repeated the marker expression analysis and could confirm that SB-HEF-iAs also expressed ALDH1L1, EAAT1, EAAT2, GS, CX43, AQP4, KIR4.1 and ATP1B2. Functional assessments revealed that SB-HEF-iAs took up glutamate at similar levels as SAB-HEF-iAs (Fig. 6E) and formed functional gap junctions to neighboring iAs. Furthermore, we performed electrophysical measurements, which showed that both SAB-HEF-iAs and SB-HEF-iAs have electrophysiological features characteristic for astrocytes. These results indicated that iAs obtained with either SB or SAB overexpression in HEFs are functionally equivalent. However, based on the increased efficiency and morphological improvements seen in the SB condition, we concluded that *Sox9* and *Nfib* was the optimal combination for direct conversion of human fibroblasts to iAs.

Next, we proceeded with a thorough functional characterization of SB-HEF-iAs. We performed live cell Ca^{2+} imaging experiments and found that 74% of SB-transduced cells had a clear intracellular Ca^{2+} response upon ATP stimulation that was distinct from control-transduced cells and even human primary fetal astrocytes, in which only 8% and 15% of cells responded, respectively. In addition, the amplitude of the response was higher in SB-HEF-iAs than in control cells (Fig. 6F). This suggested that SB-HEF-iAs had acquired intercellular calcium signaling properties that were clearly different from control-transduced HEFs, and more prominent than in primary fetal astrocytes. Next, we tested immunocompetence of SB-HEF-iAs by stimulating cells with C1q, tumor necrosis factor α (TNFα) and interleukin-1α (IL-1α) and observed an upregulation of cytokine and immune related transcripts as well as C3D protein, accompanied by rounding of the soma, similar to what has been described in mouse and hPSC-derived astrocytes (Barbar et al., 2020; Liddelow et al., 2017; Tchieu et al., 2019). To test if SB-HEF-iAs supported neuronal function, we set up a co-culture system with SB-HEF-iAs and iNs, obtained through direct conversion of HEFs using *Ascl1* and *Ngn2*

overexpression (Ladewig et al., 2012). We observed that iNs co-cultured with SB-HEF-iAs had clearly improved attachment to coverslips in which only 4% of coverslips detached between days 33 and 47 compared to 50% when iNs were cultured alone. Next, we performed electrophysiological recordings of iNs cultured alone or in co-culture with SB-HEF-iAs. Analysis of ability to fire action potentials (APs) in response to increasing depolarizing current steps revealed that a larger proportion of iNs in co-cultures could repetitively and sustainably fire more than 3 APs (41% in co-cultures vs 23% in iN alone). Furthermore, 29% of iNs in co-cultures with SB-HEF-iAs had spontaneous activity at resting membrane potential compared to only 6% when cultured alone. Analysis of AP characteristics revealed higher amplitudes and shorter half-widths of APs in iNs in co-cultures compared to cultures with only iNs, indicative of a more mature state (Fig. 6G).

Conversion of human postnatal fibroblasts to iAs is crucial to enable potential benefits of direct conversion such as preservation of genetic heterogeneity and age-related epigenetic components of patient fibroblasts. Thus, we applied our method on human neonatal, adult, and aged fibroblasts. By using high quality, non-frozen viral preparations to obtain high levels of transgene expression, we could obtain S100B+ and GFAP+ cells 3 weeks after induction of *Sox9* and *Nfib* (Fig. 6H). Functional assessment of iAs obtained from human adult fibroblasts revealed that they took up glutamate at similar levels as SB-HEF-iAs.

In conclusion, we have established an efficient method for direct conversion of human fibroblasts to functional induced astrocytes (iAs). Expression of the minimal combination of *Sox9* and *Nfib* generates iAs with molecular, phenotypic and functional properties resembling primary human astrocytes. We present for the first time a co-culture system of iAs and iNs obtained through direct conversion from the same fibroblast population and show that iAs support iN function and maturation. Furthermore, we show that our method can convert fibroblasts, covering the entire human lifespan, to iAs.

Generation of an AxD disease model using direct conversion of patient fibroblasts (Paper IV)

Current *in vitro* models to study AxD based on hPSC-derived cells have provided several important insights into the disease mechanisms (Jones et al., 2018; Li et al., 2018a). However, hiPSC-derived cells represent rejuvenated versions of patient cells. On the other hand, direct conversion better preserves epigenetic components and features of cellular aging (Mertens et al., 2016), which might be important for disease progression, in particular for AxD type II that arise in older children or during adulthood. Nevertheless, direct conversion of patient fibroblasts to astrocytes had so far not been shown to model human disease. Thus, we set out to generate a

novel model of AxD using our established method for direct conversion of human fibroblasts to iAs (Paper III).

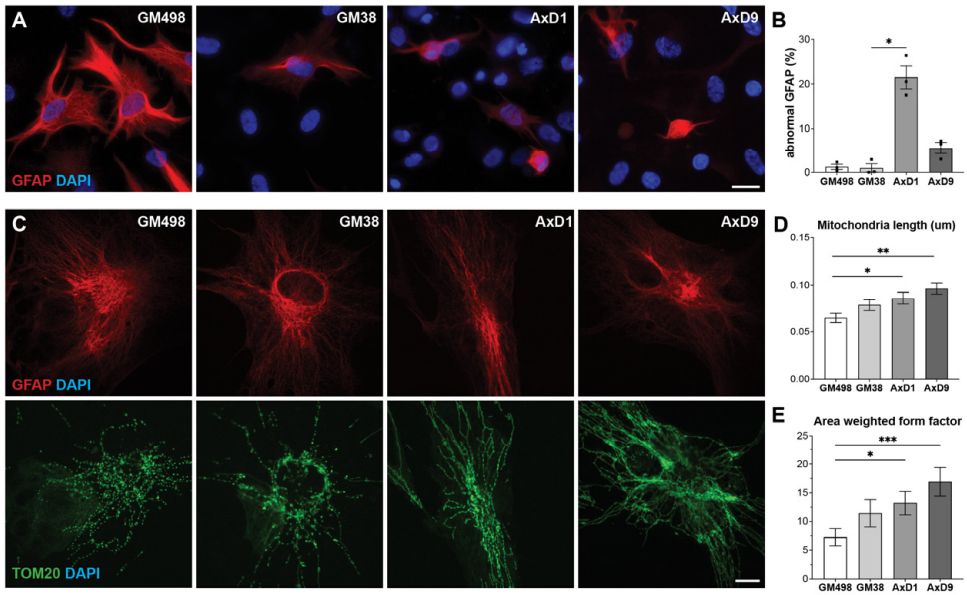


Fig. 7. Characterization of iAs obtained through direct conversion of AxD patient fibroblasts and age- and gender matched apparently healthy controls.
(**A**) Example immunofluorescence images of GFAP+ cells illustrating differences in GFAP expression pattern between control iAs (GM498, GM38) and AxD iAs, 3 weeks after induction of *Sox9* and *Nfib*. (**B**) Quantification of GFAP+ cells with abnormal GFAP expression pattern consisting of a stunted appearance, based on GFAP expression, and intense GFAP immunoreactivity, normalized to total number of GFAP+ cells. (**C**) Example immunofluorescence images of TOM20 and GFAP co-expressing cells, 5 weeks after induction of *Sox9* and *Nfib*. (**D**) Average length of mitochondria per cell based on TOM20 expression. (**E**) Area weighted form factor, a parameter used to measure complexity, of mitochondria based on TOM20 expression. Data are presented as mean ± SEM of n=3 independent viral transduction experiments. Kruskal-Wallis tests followed by Dunn's multiple comparison tests were performed for statistical analysis (B). One-way ANOVA tests followed by Tukey's multiple comparison tests (D, E) were performed on lognormal transformed data. *p<0.05. **p<0.01. ***p<0.001. Scale bars, 20 µm (A), 10 µm (C). GM498=GM00498, GM38=GM00038.

We show that 3 weeks after induction of *Sox9* and *Nfib* in two fibroblast lines derived from AxD patients, AxD1 and AxD9, and age- and gender matched apparently healthy control fibroblast lines, GM00498 and GM00038, result in 1-2% GFAP immunoreactive cells in all lines. Interestingly, we found that 20% and 5% of GFAP+ cells in AxD1 and AxD9 cultures, respectively, displayed an altered GFAP expression pattern with a stunted appearance, short processes, and intensive immunostaining for GFAP. In contrast, this phenotype was only observed in approximately 1% of control cells (Fig. 7A and 7B). By culturing the cells for an additional 2 weeks the number of cells expressing GFAP increased from 1-2% to approximately 10%. In addition, iAs appeared to have more mature morphologies

at 5 weeks as compared to 3 weeks, although some cells had a similar abnormal GFAP expression pattern as at 3 weeks. Immunocytochemical analysis of S100B revealed that approximately 10% of cells expressed S100B, without any obvious difference in expression pattern between lines.

Although we observed an abnormal expression pattern of GFAP in AxD lines, we did not observe any clear GFAP aggregates resembling Rosenthal fiber-like structures or difference in αB-crystallin or HSP27 expression, as assessed by immunocytochemistry.

AxD mutations have been suggested to alter mitochondrial transfer and function (Gao et al., 2019; Viedma-Poyatos et al., 2022). Therefore, we analyzed mitochondrial morphological parameters using the mitochondria marker TOM20 in 5-week-old GFAP+ iAs. Interestingly, we found that GFAP+ AxD iAs had longer mitochondria and that there was a tendency for mitochondria to both be larger and have more complex shapes compared to in control iAs (Fig. 7C-7E).

Finally, we show that it is possible to induce an oligodendrocytic cell fate directly in AxD patient fibroblasts through overexpression of *SOX10*, *OLIG2*, and *NKX6.2* according to a previously established method (Chanoumidou et al., 2021). The generation of iAs and induced oligodendrocytes (iOLs) performed here constitutes the first step to establish a platform, based on direct conversion to neurons, astrocytes, and oligodendrocytes, to study how AxD mutated astrocytes affect myelination.

Taken together, we show that iAs are obtained at similar levels from AxD patient and control fibroblasts. Although we did not detect any obvious Rosenthal fibers, we identified a phenotype in which AxD iAs display altered GFAP expression pattern and changed mitochondrial morphology.

Discussion and future perspectives

Studies of human astrocytes and their role in neurological disease have until recently been hindered by insufficient models. With the development of stem cell and genome engineering technologies, new tools to generate human astrocyte *in vitro* models have emerged. This field has accelerated rapidly during the years this thesis was conducted, and novel methods, including those developed in this thesis, have made it possible to model aspects of astrocyte biology in an exclusively human setting that previously could not be done.

In this thesis, we have developed methods to generate induced astrocytes (iAs) from human pluripotent stem cells (hPSCs) through transcription factor (TF) programming and by direct conversion of human fibroblasts. We have also performed differentiation of hPSCs as neural organoids. By using CRISPR/Cas9 genome editing and patient fibroblasts we have generated disease models of the leukodystrophies AxD and MLC that have the potential to be useful in elucidating the role of astrocytes in these genetic disabling white matter disorders.

Each of the methods to generate human astrocytes *in vitro* which were developed and used here, have unique qualities and limitations. Depending on the research question asked, or purpose for generating astrocytes, one method will be more informative or useful than another. This is discussed below and summarized in Figure 8.

Efficiency of generating astrocytes

At the time this thesis work was initiated, generation of astrocytes from hPSCs took months to obtain functional and mature astrocytes (Supplementary Table 1 in Canals et al., 2018) and direct conversion of human fibroblasts to iAs was inefficient and proof that the obtained cells were functional was lacking (Caiazzo et al., 2015). Cell culture and maintaining cell culture facilities are very expensive activities, thus a fast and efficient method to generate astrocytes *in vitro* allows for a larger number of experiments to be performed and more data acquired within a short timeframe. If resulting astrocytes have a high predictive value of human biology, this will accelerate our understanding of the roles of astrocytes in neurological disease, and facilitate discovery and development of novel CNS therapeutics.

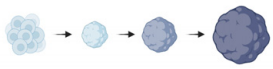
Neural organoids	TF programming	Direct conversion
		
Slow process to obtain mature astrocytes	Fast generation of mature astrocytes	Fast generation of astrocytes, functional maturity similar to hPSC-derived astrocytes?
Mimic development	Artifical, fast-forwarding development	Artificial
Mixed population of CNS cell types	Pure astrocyte cultures	Mix of astrocytes and fibroblasts
	Requires co-culture to study interactions between cell types	Requires co-culture to study interactions between cell types
Self-organizing 3D cytoarchitecture	3D cytoarchitectures can be built	3D cytoarchitectures can be built
Regional specificity can be controlled	Regional specificity not well-established	Regional specificity not well-established
Rejuventated	Rejuvenated	Age-related features preserved?
Well-suited for genome engineering	Well-suited for genome engineering	Limited suitability for genome engineering
Large quantities possible	Large quantities possible	Limited cell numbers

Fig. 8. Comparison of methods used in this thesis to generate human astrocytes from hPSCs and human fibroblasts.
Each method has its qualities and limitations such as efficiency to generate astrocytes, suitability to model development or aging, and for genome engineering. These needs to be considered when deciding which approach to use, and when interpreting obtained results. Created with BioRender.com.

TF programming protocols, using lineage-specific TFs, have been shown to rapidly and efficiently induce neuronal and oligodendrocyte cell fates in hPSCs (Canals et al., 2021). Inspired by this, we set out to establish a similar method for fast generation of induced astrocytes from hPSCs. The TF programming method developed in this thesis (Paper I), constitutes one of the fastest methods to generate functional and mature human astrocytes today (Kumar et al., 2022). Similar TF programming methods for rapid generation of functional astrocytes have been developed in parallel, but in contrast to ours, these are based on overexpression of *NFIA* in hiPSCs, or *NFIA* together with *SOX9* or *NFIB* alone in hiPSC-derived neural stem cells (NSCs) (Li et al., 2018b; Tchieu et al., 2019; Yeon et al., 2021). In addition, we have shown that overexpression of *Nfib* alone in hPSCs induce a similar astrocytic cell fate as *Sox9* and *Nfib*, although resulting cells are slightly less mature (Canals et al., 2018). Recently, overexpression of only *SOX9* was shown to induce an astrocytic cell fate in hPSC-derived NSCs (Neyrinck et al., 2021) but interestingly, *SOX9* alone has also been shown to generate myelinating oligodendrocytes when overexpressed in hiPSCs (Ng et al., 2021). These studies, including ours, confirm that all the transcription factors SOX9, NFIA and NFIB are involved in gliogenesis (Molofsky et al., 2012), but also that cell culture conditions and developmental stage at induction of transgenes are involved in determining cell fate. Noteworthy, we have performed TF screens by overexpressing *Sox9*, *Nfia*, and *Nfib* individually, in pairs and all three in combination in hPSCs, human fibroblasts

and human glioma cells. Collectively, these experiments have revealed that combined overexpression of *Sox9* and *Nfib* result in most iAs with a morphological appearance closest to primary astrocytes (Canals et al., 2018; Quist et al., 2022; Trovato et al., in press). Based on our current knowledge, we propose that overexpression of *Sox9* and *Nfib* is the optimal TF combination to induce an astrocytic cell fate in hPSCs and fibroblasts. However, a side-by-side comparison of iAs obtained through currently available TF programming protocols is required to fully scrutinize differences between resulting cells. Nevertheless, TF programming of hPSCs greatly reduce the time to generate functional astrocytes compared to traditional differentiation including neural organoid systems (Leventoux et al., 2020; Shaltouki et al., 2013; Sloan et al., 2017; Tcw et al., 2017)

The method for direct conversion of human fibroblasts to iAs developed in this thesis (Paper III), is less efficient compared to TF programming of hPSC to iAs. Despite this, it constitutes, to our knowledge, one of the most efficient methods available today to generate iAs directly from fibroblasts (Caiazzo et al., 2015; Tian et al., 2016). In addition, we have performed an extensive characterization to demonstrate that directly converted iAs have phenotypical and functional characteristics of astrocytes. Our thorough characterization, confirming an astrocytic identity, strengthens the use of our method compared to other available direct conversion methods to generate human astrocytes.

Despite the high efficacy of direct conversion of human fibroblasts to iAs, the resulting cultures consist of converted, partially converted and non-converted cells. This can be problematic when interpreting experimental read-outs performed on the entire cell population. Partially and non-converted fibroblasts might mask the effect of fully converted cells. Furthermore, observations might be due to an interplay between iAs and skin fibroblasts, a composition that is not found in the brain. Methods for direct conversion of human fibroblasts to iNs or iOLs have shown that complete conversion of all cells is rarely accomplished and a sorting step, using neuronal or oligodendrocyte specific markers, respectively, is required to achieve a population consisting of only converted cells (Chanoumidou et al., 2021; Drouin-Ouellet et al., 2017; Mertens et al., 2015). Thus, adaptation of the protocol to achieve close to complete conversion, or an enrichment or sorting strategy that separates fully converted iAs from other cells present in the culture, would be highly beneficial and increase the applicability of iAs obtained through direct conversion. Successful isolation of pure populations of converted iAs would allow for a more proper comparison of functionality and maturity level of directly converted iAs to astrocytes generated from hPSCs and primary human astrocytes. However, during the course of this thesis, a substantial effort was done to separate live iAs from non-converted fibroblasts but without success. Several markers reported to be specific for either astrocytes or fibroblasts have been tested but we, however, found that these markers were expressed by both cell types. Recently, a hALDH1L1-EGFP lentiviral reporter was cloned (Yeon et al., 2021). Thus, it would be interesting to

investigate if this reporter construct can be used to isolate a pure population of directly converted iAs.

Ability to replicate CNS complexity

Differentiation methods that aim to mimic normal development typically yield a mixture of astrocytes and neuronal cells that needs to be isolated if a pure culture is desired (Leventoux et al., 2020; Sloan et al., 2017; Tcw et al., 2017). However, cells are not working in isolation in the brain, thus mechanisms leading to dysfunction in the CNS are likely due to a complex interplay between several different CNS cell types. Neural organoids emerge by self-organization and are excellent platforms that allow for studies of mixed neural populations and cell-cell interaction (Marton and Pașca, 2020). Furthermore, recent differentiation protocols of neural organoids allow for generation of brain region-specific organoids by providing regional instructive molecules that can be combined into assembloids (Andersen et al., 2020; Sloan et al., 2018). This is an attractive approach for studies of neurological disorders that have a known regional preference such as Parkinson's disease, and to study interactions between different brain regions (Eichmüller and Knoblich, 2022; Smits et al., 2019).

In contrast, TF programming is an artificial method, fast forwarding developmental steps using ectopically high levels of transgenes (Yeon et al., 2021), and normally result in relative homogenous populations (Canals et al., 2021). Our TF programming method to generate iAs from hPSCs yields almost entirely pure iAs cultures. This allows to study astrocytes with little or no presence of other cells. When desired, interactions between CNS cells can be studied by combining iAs in co-cultures with iNs in predetermined proportions, similar to what has been done in this work and to study frontotemporal dementia (Canals et al., manuscript submitted for publication), or even in multi-cultures with other CNS cells. This allows for studies of interactions of astrocytes, neurons, and oligodendrocytes without requiring several months of differentiation (Marton et al., 2019; Sloan et al., 2017). Furthermore, 3D cytoarchitectures can be built by bioprinting or embedding iAs, iNs, and iOPCs in matrices in a controlled manner. However, as opposed to brain region specific organoids, the regional identity of iAs is currently not well established. With that said, that does not exclude the possibility to adapt our protocols and guide cells towards a certain region by adding regional instructive molecules.

Interestingly, it was recently shown that it is possible to induce a microglia cell fate in hPSCs through overexpression of the transcription factors *SPI1* and *CEBPA* (Chen et al., 2021), or *PU.1* (Sonn et al., 2022). This offers a possibility to generate quadruple cultures of the main CNS cells. Around the same time, overexpression

PU.1 was shown to induce microglial lineage cells in human cortical organoids (Cakir et al., 2022). In addition, by inducing or incorporating endothelial cells (Ham et al., 2020; Pham et al., 2018), or using organ-on-chip technology to mimic the blood-brain barrier and blood flow (Ahn et al., 2020; Herland et al., 2016), the similarity to a functional human brain and thus predictive value, will be further increased. The ability to mimic brain vasculature in cellular models is of particular interest for MLC, in which defects have been observed in perivascular astrocytes, or other neurological disorders with defective regulation of ion and water homeostasis such as other leukodystrophies (Min and van der Knaap, 2018).

As more complex *in vitro* models of the human CNS are developed, a parallel development of methods to interpret the data generated is required, as there is little use of a model unless we can understand it correctly.

Modeling of development and aging

Another parameter that needs to be considered when selecting which approach to use to generate astrocytes, is what developmental and maturation stage of cells will be most meaningful for the particular study. Traditional differentiation protocols, either in monolayer cultures, or in particular as neural organoids, capture *in vivo* developmental processes, and thus suitable for studies of diseases thought to arise during development (Lancaster et al., 2013; Paulsen et al., 2022). Astrocytes generated through differentiation of hPSCs in monolayer cultures rarely reach maturation and typically represent a fetal stage likely due to difficulties in maintaining healthy long-term cultures (Leventoux et al., 2020; Tcw et al., 2017). This can be circumvented by differentiating hPSCs as neural organoids, although at least 250 days of differentiation are required to reach full maturation of astrocytes (Sloan et al., 2017). On the other hand, our TF programming method of hPSCs yields iAs with mature properties within two to three weeks. Determination of maturation level of *in vitro* generated astrocytes is largely based on morphological observations and comparison of transcriptional profiles to primary fetal or postnatal human astrocytes using RNASeq data (Leventoux et al., 2020; Sloan et al., 2017; Tcw et al., 2017). Recently, principal component analysis of RNASeq data has shown that astrocytes differentiated from hPSCs in monolayer cultures, or astrocytes generated by overexpressing *NFIA* or *NFIB* alone in hPSC-derived NSCs, cluster in between primary human fetal and adult HEPACAM-immunopanned astrocytes with a profile slightly skewed towards fetal astrocytes (Yeon et al., 2021). We have not yet performed such thorough transcriptional analysis of iAs obtained with our TF programming method of hPSCs or direct conversion of fibroblasts. However, we have compared gene expression of a selected set of genes in hES-iAs to human primary adult astrocytes using single cell RT-qPCR, and based on these experiments, found that hES-iAs have a similar gene expression as adult astrocytes.

Moreover, hES-iAs have a morphological appearance of mature astrocytes, are post-mitotic and perform functions expected to be done by astrocytes in a postnatal brain (Zhang et al., 2016). In the case of iAs obtained through direct conversion, we have found that fibroblast-derived iAs have clearly more stellate morphologies and a more distinct ATP-induced Ca^{2+} response compared to astrocytes isolated from human fetal tissue, suggesting a more mature stage than that of fetal astrocytes (Yeon et al., 2021; Zhang et al., 2016).

To fully determine the maturation level of hES-iAs and fibroblasts-iAs, as well as to define the exact time required to reach the same maturation level compared to hPSC-derived astrocytes obtained through other protocols, RNASeq of hES-iAs and fibroblast-iAs would be required. However, based on the molecular, phenotypical and functional profile of hES-iAs, it is likely that our TF programming method greatly reduces the time to generate mature astrocytes as compared to neural organoid systems.

Direct conversion of fibroblasts to iAs has the potential to capture cellular age of fibroblasts, which cannot be done in hPSC-based systems. Studies of direct conversion of fibroblasts to iNs and iOLs have shown that resulting cells have a preserved epigenetic status and age-related features (Chanoumidou et al., 2021; Kim et al., 2018; Mertens et al., 2015; Tang et al., 2017). Furthermore, it was recently shown that astrocytes differentiated from induced neural progenitor cells (iNPCs) obtained from young and adult donors, display differences in gene expression and aging marks (Gatto et al., 2020). In contrast, reprogramming of fibroblasts, or other somatic cells, to hiPSCs, erases these molecular features and the resulting cells represent a rejuvenated state (Lapasset et al., 2011; Miller et al., 2013). In the case of hESCs, by nature, these represent an embryonic state. Thus, for diseases in which the initiating events occur later in life or develop during aging, hPSC-based models will not be able to accurately capture this additional layer of complexity. Furthermore, for many diseases, including AxD and MLC, it is currently unknown when the initiating events occur, during which maturation stage disease develops and if or to what extent, epigenetic components are involved in disease initiation and progression. This lack of knowledge is simply due to that it previously has not been possible to study. However, we show for the first time that human fibroblast obtained from the entire human lifespan can be directly converted to iAs. Thus, we now have the tools available to investigate if, or to what degree, epigenetic marks and age-related features are preserved during direct conversion of fibroblasts to iAs.

On the other hand, if the disease is caused by events occurring early in development, these have already taken place at a postnatal stage and thus, in patient fibroblasts. Furthermore, if early disease-initiating events are specific to the CNS, fibroblasts have likely not been affected. This limits the potential for studying underlying disease mechanisms occurring during development in cells obtained through direct conversion of patient fibroblasts. One can also speculate that TF programming of hPSCs is too fast and will not capture events occurring in neural stem cells or

progenitor populations. In these situations, traditional differentiation of hPSCs in monolayer cultures or as neural organoids would likely be more biologically relevant at the cost of substantial longer time to generate functional mature astrocytes.

Furthermore, for AxD and MLC, as well as other leukodystrophies, clinical manifestations are in some patients triggered or worsened by an inflammatory event or trauma (Benzoni et al., 2020; Bugiani et al., 2003; Riel-Romero et al., 2005; van der Knaap et al., 2006). Hence one would need to consider at what time point or maturation stage this challenge should be applied to obtain a similar situation to what occurs in patients. These triggering events for AxD and MLC patients have been reported to occur postnatally. However, it would be interesting to investigate if there has been a triggering event *in utero* for those patients that have not been exposed to a clear provocation after birth.

Applicability in disease modeling and drug discovery

For disease modeling using *in vitro* models, the use of proper controls is crucial to ensure that putative disease mechanisms are not due to normal variation. For diseases with a genetic link, CRISPR/Cas9 genome editing allows for generation of isogenic controls by either introducing mutations in hESCs or hiPSCs derived from apparently healthy donors, or correcting mutations in hiPSCs derived from patient fibroblasts. This is a powerful approach when attempting to dissect pathology of genetic diseases since it ensures that potential observed phenotypes are due to the mutation and not confounding factors such as differences in genetic background (Brooks et al., 2022; Soldner and Jaenisch, 2018).

CRISPR/Cas9 genome editing is also possible in human fibroblasts (Konstantinidis et al., 2022). Furthermore, it has been shown that CRISPR/Cas9-mediated gene inactivation, during direct conversion of human fibroblasts to neurons, can be accomplished (Rubio et al., 2016). Thus, isogenic controls could be generated through genome editing of fibroblasts. However, generation of clonal fibroblast lines is very difficult and constitutes a limitation for using direct conversion in modeling of genetic diseases. Alternatively, fibroblasts derived from a large number of different donors, are needed to ensure robust findings (Drouin-Ouellet et al., 2022; Traxler et al., 2022). However, this is challenging for studies of rare diseases, such as those studied here, as availability of patient material is very limited.

Furthermore, hPSCs have rapid cell division and large amounts of identical cells can be obtained relatively fast, which allows for many experiments to be performed. In contrast, the limited scalability and access to primary patient fibroblasts constitute a challenge to use cells obtained through direct conversion in drug discovery. Thus, to date, hPSC-based models, in which the hPSCs can be expanded

before subjected to differentiation or TF programming, are more feasible approaches.

Our iAs generation methods are not limited to study genetic diseases but can also be applied to model sporadic diseases. The direct conversion method is of particular interest for modeling sporadic diseases because copy number variations existing in fibroblasts are better represented in the final population (Mertens et al., 2016). In contrast, reprogramming to hiPSCs is based on clonal expansion and only one fibroblast will be represented in each hiPSC line. This can be circumvented by using many hiPSC clones from the same donor and building hiPSC libraries (Brooks et al., 2022; Mertens et al., 2016). However, modeling of sporadic disease requires substantial more differentiations of hiPSC lines or conversion experiments of fibroblasts, with matched controls, to distinguish disease-causing components from the normal genetic variation.

AxD and MLC astrocyte disease models

In this thesis astrocyte models of AxD and MLC were generated since these are two of the clearest examples of disease originating from dysfunctional astrocytes (van der Knaap and Bugiani, 2017). In addition, their monogenetic origin makes them attractive candidates for CRISPR/Cas9-mediated introduction of patient mutations. We decided to work with hESCs, as opposed to patient derived hiPSCs, due to the extreme difficulties in obtaining patient material needed for reprogramming to hiPSCs of rare diseases such as AxD and MLC. Instead, introduction of disease-associated mutation in hESCs enabled the generation of cellular models of AxD and MLC.

AxD modeling

Here, we introduced the R239C mutation in *GFAP* in hESCs through CRISPR/Cas9 genome engineering and subsequently applied our TF programming method on edited cells to generate AxD hES-iAs. We analyzed AxD hES-iAs for previously reported phenotypes and found GFAP inclusions, indicative of formation of Rosenthal fiber-like structures, as well as downregulation of gene transcripts involved in potassium buffering and altered ATP-induced Ca^{2+} response compared to isogenic controls. Interestingly, it has been suggested that compromised homeostatic functions of potassium by astrocytes lead to demyelination (Rash, 2010), a phenotype that is observed in AxD patients and would be of interest for further investigations using our AxD models.

We also show that it is possible to generate induced astrocytes through direct conversion of AxD patient fibroblasts. We found that AxD fibroblasts-iAs have altered mitochondrial morphology, in line with recent findings using astrocytoma cell lines that overexpressed mutant *GFAP* (Viedma-Poyatos et al., 2022).

Furthermore, we observed that AxD fibroblast-iAs had an altered expression pattern of GFAP. However, in contrast to our genetically engineered AxD hES-iAs model and other hPSCs-derived astrocytes with AxD GFAP mutations, no clear aggregates of GFAP were found (Kondo et al., 2016; Li et al., 2018a). On the other hand, the disorganized GFAP in AxD fibroblast-iAs had a similar appearance as in iAs obtained through direct conversion of mouse fibroblasts that overexpressed human *GFAP* with the R239C AxD mutation (Tian et al., 2016). By extending the time in culture of fibroblast-iAs, introducing a challenge to induce reactive astrogliosis, or by using high-resolution or electron microscopy, presence of Rosenthal fiber-like structures in fibroblast-iAs might be revealed. Another plausible explanation to the differences in GFAP appearance is that the AxD patient fibroblasts used here have the K228E and Q93P mutations, respectively, mutations that, to our knowledge, no other models have (Hagemann, 2022). Moreover, AxD can arise throughout life and has been classified into two types based on clinical manifestations (Prust et al., 2011). However, there is currently no clear correlation between genotype to disease onset and severity, and genetic modifiers have been suggested to be involved in pathology (Hagemann, 2022; Messing, 2018). For instance, the R239C mutation has been found in AxD patients with disease onset at all ages. Recently, it was shown that phosphorylation of GFAP at the Ser13 residue is associated with severe cases of AxD, suggesting that non-genetic components contribute to disease (Battaglia et al., 2019). Thus, the discrepancies in GFAP aggregation that we observe might be due to differences in the genetic background or posttranslational modifications of GFAP. Alternatively, we might model different types of AxD. One can speculate that AxD astrocytes derived from hPSCs, including AxD hES-iAs, represent type I that normally arise in early childhood. In contrast, iAs obtained through direct conversion might preserve currently unknown features that govern AxD subtype, thus having the potential to model type II that typically arise in older children and adults. However, this remains to be investigated.

Importantly, here we show, for the first time, that iAs can be generated directly from patient fibroblasts. This enables phenotypical, functional, and epigenetic characterization of AxD iAs obtained through direct conversion and from hiPSCs, derived from the same patient fibroblasts. Thus, we now have a unique possibility to first, investigate if epigenetic components or age are retained during direct conversion to iAs, and secondly, if so, if such features contribute to development of AxD and to different types of AxD. Knowledge gained from such studies might provide new insight in disease mechanisms leading to different clinical manifestations of AxD.

MLC modeling

Here we have generated two MLC hESC lines by introducing the S93L and S280L mutations, individually, in the *MLC1* gene using CRISPR/Cas9 genome editing. We show that these cell lines can be used to generate induced astrocytes (iAs), through

our TF programming method, and differentiated to human oligodendrocyte spheroids (hOLS).

We observed that iAs could be generated to a similar extent from MLC hESC lines as from isogenic control lines based on expression of S100B, VIMENTIN and GFAP. Interestingly, differentiation of MLC hESC lines to hOLS revealed that hOLS with *MLC1* mutations had increased expression of GFAP compared to isogenic controls. This suggests that there are either more astrocytes in MLC hOLS, or that GFAP is upregulated in present astrocytes, indicative of reactive astrogliosis, a hallmark for most neurological disorders and that has been observed in some MLC patients (Miles et al., 2009). Further investigation of markers for reactive astrocytes, and functional interrogation to assess a potential reactive state, will be required to better understand this observation (Escartin et al., 2021). Nevertheless, these results highlight that different observations can be done by using different models.

The function of MLC1 is not fully known but there are indications that it is associated with VRAC activity (Ridder et al., 2011). Reduced VRAC activity has been suggested to lead to accumulation of water intracellularly resulting in vacuolization observed in patients (Dubey et al., 2014; Lanciotti et al., 2012; Ridder et al., 2011). For these reasons, it would be very interesting, and important, to assess VRAC activity and cell volume response to osmotic changes in MLC hES-iAs and astrocytes in MLC hOLS.

Current MLC animal and *in vitro* models, based on primary rodent astrocytes, HeLa cells and human astrocytoma cell lines, overexpressing wildtype or mutant *MLC1* have indicated that mutated *MLC1*, or *HEPACAM*, result in mislocalization of MLC1 from the plasma membrane to intracellular compartments and is targeted for degradation (Duarri et al., 2008; Lanciotti et al., 2012; Lopez-Hernandez et al., 2011b). In line with these findings, overexpression studies in *Xenopus* oocytes have indicated a reduced stability of mutated MLC1 (Teijido et al., 2004). In contrast, based on qualitative assessments using immunocytochemistry, we did not observe any obvious difference in localization or expression of MLC1 in MLC hES-iAs compared to isogenic controls. There are to our knowledge no published studies showing that MLC1 mislocalization occurs in more biologically relevant models such as hPSC-derived neural cells. Furthermore, analyses of patient brain tissue have revealed reduced to undetectable levels of MLC1 at perivascular astrocyte endfeet and cell lysates from different brain regions, but not that MLC1 is mislocalized per se (Duarri et al., 2008; Lopez-Hernandez et al., 2011b; Min and van der Knaap, 2018). Thus, one can speculate if MLC1 is mislocalized in patients, or if it is a potential artifact due to external manipulation in an irrelevant cell background for human pathophysiology. Hence, it would be important to perform a quantitative assessment of MLC1 expression levels in MLC hES-iAs and hOLS to investigate if our models can capture this aspect of the disease seen in MLC patients.

Vacuolization of astrocytes have been described in MLC patients and MLC1- or HEPACAM-depleted primary rat astrocytes (Capdevila-Nortes et al., 2013; Duarri et al., 2011). However, we did not observe any obvious vacuoles in MLC hES-iAs or hOLS by visual inspection of cultures using bright-field microscopy. High-resolution imaging or staining with calcein-AM, a fluorescent dye that freely diffuses in cells used to ease visualization, might reveal the presence of vacuoles (Sirisi et al., 2014).

Furthermore, since symptoms in some patients arise, or are aggravated after mild trauma or fever, introducing a challenge such as inflammatory stress might be needed to induce more obvious phenotypes in our models.

In summary, we have generated and validated two homozygous MLC1 mutated hESC lines, and from these produced relevant cultures such as iAs and neural organoids containing astrocytes, neurons, and oligodendrocytes. These models constitute promising tools to model and study MLC disease mechanisms in human astrocytes and their interaction with other neural cell types. However, further characterization of previously known phenotypes described in MLC models and importantly, in MLC patients, of our models are needed to be confident that they can be useful for reliable disease modeling of MLC, and to determine if one model system is more informative than another.

Outlook

In this thesis, we have focused on generating astrocyte models of monogenetic diseases. However, studies on genetic disorders such as AxD and MLC can be informative for other diseases, both genetic and sporadic, with similar clinical manifestations, or to learn about fundamental astrocyte biology. For instance, neuronal and myelin degeneration is seen in multiple sclerosis (MS) but no clear genetic causes to MS have been identified. Thus, by using AxD as a model to study how astrocytes affect neurons, oligodendrocytes and myelination, new insights and potential novel therapeutic strategies for MS patients might be revealed. Furthermore, astrocytes in MLC patients fail to regulate ion and water homeostasis. Thus, using MLC as a model an improved understanding of astrocytic regulation of brain homeostasis might be achieved, which can be informative for other leukodystrophies with similar clinical manifestations (Min and van der Knaap, 2018). By extension, an improved knowledge about regulation of homeostatic functions by astrocytes, has the potential to be informative for studies on ALS in which dysregulation of ion and water homeostasis have been suggested to trigger motor neuron degeneration (Sirabella et al., 2018).

Excitingly, there is a growing interest from political authorities, pharmaceutical and biotech companies to develop diagnostic tools and therapeutics for rare diseases (Berry et al., 2020; Monaco et al., 2022). It has been estimated that 3.5-5.9% of the

entire global population have a rare disease, 70-80% have a monogenetic cause and the majority have pediatric onset (Condò, 2022; Nguengang Wakap et al., 2020). On this basis, a new initiative, the Bespoke Gene Therapy Consortium, a collaboration between the U.S. Food and Drug Administration (FDA), the National Institutes of Health (NIH), industry, and non-profit organizations, has recently been taken to establish standardized protocols that can be quickly adapted for the development and production of gene therapies to treat different rare monogenetic diseases. This is a very exciting initiative since both AxD and MLC, as well as other leukodystrophies that currently lack effective treatments, are monogenetic diseases and mainly affect children, making them suitable candidates to be included in such a pipeline.

Concluding remarks

Primary material from the human brain is and will remain very difficult to get hold of, hindering research on this exceptionally complex organ and its associated diseases. Thus, developing models that can accurately capture biological phenomena relevant for human CNS pathophysiology are highly warranted.

In this thesis, we have developed novel methods to generate human astrocytes through transcription factor programming from human pluripotent stem cells and through direct conversion of human fibroblasts. We envision that these methods have the potential to become useful tools in scrutinizing fundamental astrocyte biology and the role of astrocytes in a wide range of neurological disorders.

One remaining question is whether iAs obtained through direct conversion retain epigenetic profiles and age-related features that have pathological consequences. However, with the methods developed in this study, this is now possible to investigate by comparing iAs generated from hiPSCs derived from human fibroblast across the human lifespan and through direct conversion of the same fibroblasts, using the TFs *Sox9* and *Nfib.*

By using CRISPR/Cas9 genome editing and patient fibroblasts, we have generated astrocyte models of AxD and MLC. Although AxD and MLC are rare diseases, many leukodystrophies manifest in similar ways and share pathology. Thus, even though our studies have been focusing on a restricted number of genes and mutations, our models might reveal novel insights about the contribution of astrocytes in other leukodystrophies and their role in white matter integrity and function.

Taken together, the methods developed here, together with advanced neural organoid systems, constitute promising platforms to improve our understanding of the roles of astrocytes in health and disease. By using neural organoids representing early development, rejuvenated but mature astrocytes using TF programming of hPSCs, and astrocytes with putative preserved age and epigenetic status using direct conversion, different aspects of biology and disease can be captured. These methods have different qualities and limitations, which needs to be considered when selecting which model to use. In many situations, a combination of several model systems is likely the favorable option. For instance, it might be unknown when the initiating events occur, what developmental stage is involved in disease progression, if age is a contributing factor and whether, and if so when, a triggering event is

involved in disease initiation. However, there are now several sophisticated *in vitro* human astrocytes models available, including those developed in this thesis, that allows for dissecting the role of astrocytes in disease and identification of new targets for therapeutic interventions. Ultimately, I anticipate that knowledge gained from those studies will improve the lagging R&D productivity of CNS drugs and facilitate discovery of safe and efficient drugs.

Key methods

Cell culture

Human embryonic stem cells

H1 hESCs were cultured in hESC-qualified Matrigel-coated (Corning) 6-well plates in mTeSR1 media (STEMCELL Technologies). StemPro Accutase Cell Dissociation Reagent (Gibco) was used to dissociate cells. Cells were pelleted by centrifugation and seeded in mTeSR1 containing 10 μM ROCK inhibitor Y-27632 (STEMCELL Technologies).

Human fibroblasts

Human embryonic dermal fibroblasts (HEFs) were obtained from dead aborted human fetuses 6-9 weeks post-conception from Lund and Malmö University Hospitals according to guidelines approved by the Lund-Malmö Ethical Committee (6.1.8-2887/2017) and as described in Quist et al., 2022.

Human dermal neonatal (C-004-5C) and adult (C-013-5C) fibroblasts were from Invitrogen. Aged human fibroblast from an 82-year-old female (GM01706) and 96-year-old male (GM00731) were obtained from Coriell biorepository.

AxD fibroblasts were generated from skin biopsies from a 3-year-old male and an 8-year-old female with AxD that harbored K228E and Q93P mutations in *GFAP* respectively, at the Salhgrenska University Hospital (Gothenburg, Sweden) and used under local ethical approval (289-17). Age- and gender-matched, apparently healthy, control fibroblasts (GM00038 and GM00498) were obtained from NIGMS Human Genetic Cell Repository.

Fibroblasts were cultured in Fibroblast medium (DMEM, 10% Fetal Bovine Serum (FBS), GlutaMAX, Non-Essential Amino Acids (NEAA) and Sodium Pyruvate, all from Gibco) and passaged using Trypsin-EDTA (Sigma-Aldrich).

Primary human astrocytes

Human adult astrocytes were derived from fresh cortical tissue, obtained during resection surgery from patients who had pharmacologically intractable epilepsy, as described in Canals et al., 2018. The use of human brain tissue was approved by the local ethical committee in Lund (212/2007). Cells were cultured in Neurobasal medium (Gibco) supplemented with B-27 (Gibco), GlutaMAX, BDNF (Peprotech),

GDNF (Peprotech), and CNTF (Peprotech). After 3–4 weeks of cultivation, the medium was switched to DMEM:F12 1:1 supplemented with the N-2 and G5 supplements (all from Gibco).

Human fetal cortical astrocytes were derived according to guidelines approved by the Lund-Malmö Ethical Committee (6.1.8-2887/2017) and as previously described (Miskinyte et al., 2018) from fetuses 7-9 week post-conception. Human fetal astrocytes were cultures in media consisting of DMEM-F12, 10% FBS, GlutaMAX and N-2.

Cloning of transcription factor lentiviral vectors

Full-length cDNAs of mouse *Nfia*, *Nfib* or *Sox9* genes were amplified from plasmids available at Addgene (#64901, #64900, #41080 respectively) adding specific restriction sites to allow cloning in tetO-FUW lentiviral vectors carrying resistance genes for blasticidin, hygromycin (Addgene #97330) and puromycin (Addgene #97329) using EcoRI/BamHI (Nfia and Nfib) or EcoR1/XbaI (Sox9). Primers used in the cloning process are shown in Table 1. Resulting vectors were deposited to Addgene, tetO-FUW-Sox9-Puromycin (#117269), tetO-FUW-Nfia-Blasticidin (#117270) and tetO-FUW-Nfib-Hygromycin (#117271).

Table 1. Primers used for cloning of TFs into lentiviral vectors with selection genes.

Primer name	Primer sequence 5'-3'
Sox9 Forward	GAATTCATGAATCTCCTGGACCCCTTC
Sox9 Reverse	TCTAGAGGGTCTGGTGAGCTGTGTG
Nfia Forward	GAATTCCATGTATTCTCCGCTCTGTCTCAC
Nfia Reverse	GGATCCTTATCCCAGGTACCAGGACTG
Nfib Forward	GAATTCATGATGTATTCTCCCATCTGTCTCAC
Nfib Reverse	GGATCCCTAGCCCAGGTACCAGGAC

Lentiviral production

Third generation lentiviruses were produced using transient calcium transfection of HEK 293T cells and were collected by ultracentrifugation as previously described (Quist et al., 2021).

Transcription factor programming

Induced astrocytes (iAs) were in principle generated from hPSCs as described in Canals et al., 2018 and with minor changes as described in Quist et al., 2021. In brief, 450,000 hESCs were seeded in Growth factor reduced Matrigel (Corning)-coated 6-well in mTesSR1 containing 10 μM ROCK inhibitor and the day after,

transduced with FUW-rtTA (Addgene #20342) and doxycycline-inducible lentiviral vectors tetO-FUW-Sox9-Puromycin, and tetO-FUW-Nfib-Hygromycin. One day later (day 0), 2.5 µg/ml doxycycline (Sigma-Aldrich) was applied to initiate expression of *Sox9* and *Nfib* and was kept in the media throughout experiments. On day 1, mTeSR1 media was replaced by Expansion media consisting of DMEM/F12, 10% FBS, N-2 and supplemented with 1.25 µg/ml puromycin (Gibco) and 200 µg/ml hygromycin (Gibco) to enrich for cells expressing both transcription factors. Puromycin selection was performed during day 1-2 and hygromycin selection day 1-5. On day 3, Expansion media was gradually replaced by FGF medium consisting of Neurobasal, B-27, NEAA, GlutaMAX, 1% FBS, 8 ng/mL FGF2 (Peprotech), 5 ng/mL CNTF and 10 ng/mL BMP4 (Peprotech). On day 7, cells were detached using Accutase and seeded in poly-D-lysine:laminin or polyethylenimine:laminin-coated wells, petri dishes, or coverslips depending on the desired output. From day 10, iAs were maintained in Maturation media consisting of 1:1 DMEM/F12:Neurobasal, N-2, Sodium Pyruvate, 5 µg/mL N-acetyl cysteine (Sigma-Aldrich), 5 ng/mL Heparin-binding EGF-like Growth Factor (Sigma-Aldrich), 10 ng/mL CNTF, 10 ng/mL BMP4 and 1mM cAMP (Sigma-Aldrich), with half media change every 2-3 days. Media was supplemented with 10 µg/ml laminin every second media change to improve attachment of iAs.

Direct conversion

Human fibroblasts were induced to astrocytes as described in Quist et al., 2022. In brief, human embryonic or postnatal fibroblasts were seeded in Matrigel-coated 24-well plates (50,000 or 20,000 cells/well respectively) or 6-well plates (200,000 or 80,000 cells/well respectively) on day -2. The day after, fibroblasts were transduced with rtTA, tetO-Sox9-Puromycin and tetO-Nfib-Hygromycin, either with or without tetO-Nfia-Blasticidin, in Fibroblast medium containing 8 µg/mL Polybrene (Sigma). Transgene expression was induced by addition of 2.5 µg/ml doxycycline at day 0 and were kept throughout experiments. On day 1, a 3-day puromycin and a 5-day hygromycin selection period was started, during which medium was changed daily. In SAB-infected cultures, blasticidin (Gibco) selection was also performed for 5 days. On day 3, medium was changed to 3:4 Fibroblast medium:FGF medium (Neurobasal, B-27, 1% NEAA, 1% GlutaMAX, 0.2% FBS, 8 ng/ml FGF2, 5 ng/ml CNTF and 10 ng/ml BMP4. At day 4 medium was changed to 1:1 Fibroblast medium: FGF medium and at day 6 1:4 Fibroblast medium:FGF medium. At day 7 medium was changed to FGF medium and at day 9, half of the medium was changed. At day 11, half of the medium was changed to Serum-free medium (1:1 DMEM-F12, HEPES:Neurobasal, N-2, GlutaMAX, Sodium Pyruvate, 5 µg/ml N-Acetyl-L-cysteine and 5 µg/ml Heparin-binding EGF-like growth factor supplemented with 10 ng/ml CNTF, 10 ng/ml BMP4 and 500 µg/ml cAMP. Thereafter, half of the medium was changed every second or third day. If needed, cells were split 1:3.

For electrophysiological recordings, biocytin-injections and immunocytochemical analysis at day 37-42, cells were dissociated with Accutase and seeded on Matrigel-coated glass coverslips (5,000-20,000 cells/coverslip) 4-7 days before analysis.

CRISPR/Cas9 genome editing

sgRNA design and cloning

sgRNAs were designed around the target region using the online webtool http://crispr.mit.edu and cloned into pSpCas9(BB)-2A-Puro (pX459) V2.0 (Addgene #62988) and pSpCas9n(BB)-2A-Puro (pX462) V2.0 (Addgene #62987).

Transfection of hESCs

Transfections and cell line generation were performed as described in Canals and Ahlenius, 2021. In brief, 300,000 (AxD) or 400,000 (MLC) H1 hESCs were seeded and the day after, cells were transfected with 2 µg Cas9 vector containing sgRNA A/B or 3.0 µg Cas9n (50:50 sgRNA A:B) using Opti-MEM media (Gibco) and Lipofectamine Stem Transfection Reagent (Thermofisher Scientific). For cell line generation 1 µg ssODN repair template with phosphorothioate bond modifications (IDT) was included in the transfection mix. Details for genome editing components are found in Table 2.

Table 2. DNA sequences of gene editing components.

Gene editing component	Sequence (5'-3')
GFAP R239C sgRNA A	ACTGCGTGCGGATCTCTTTC
GFAP R239C sgRNA B	ACATGCATGAAGCCGAAGAG
MLC1 S93L sgRNA A	GGTGAAGCTCACAATTGCCG
MLC1 S93L sgRNA B	CTCCAGGAGGAACGCCAATG
MLC1 S280L sgRNA A	GCTGAATGACAGATATCCAG
MLC1 S280L sgRNA B	GAGAATCGTGGAGATGTTTA
GFAP R239C ssODN repair template	GTGGCCAAGCCAGACCTCACCGCAGCTCTGAAAG AGATCTGCACGCAGTATGAGGCAATGGCGTCCAG CAACATGCATGAAGCCGAAGAGTGGTACCGCTCC AAGGTAGCCCTGCCTGTG
MLC1 S93L ssODN repair template	CATGGGATTCCGGTTTCATTCCAGTGCATCCCATT GGCAATTGTGAGCTTCACCGTCTCCAGGAGGAAC GCCAATGTAGTGAGTCTAGCTTGCAGGCAGCCTCT GCCAGG
MLC1 S280L ssODN repair template	AGCAGCCTCACGTCTCCGCTGCTGTTCACAGCGTC TGGATATCTGTTATTCAGCATCATGAGAATCGTGG AGATGTTTAAAGATTACCCGCCAGCCATAAAAGTG AGTTGTAT

Analysis of gene editing

DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen). PCR was performed using Phusion High-Fidelity DNA Polymerase (Thermofisher Scientific). PCR primers were designed using Primer3 webtool and primer sequences are found in Table 3. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) before TOPO cloning and sequencing. TOPO cloning was done using Zero Blunt TOPO PCR Cloning Kit (Thermofisher Scientific) and plasmids isolated using MiniPrep Kit (Qiagen). For analysis of sgRNA efficiency, EcoRI digestion was performed to release the insert. For analysis of HDR using the provided ssODN repair templates, restriction enzyme digestions were carried out using enzymes that had recognitions sites introduced or removed upon introduction of the silent PAM mutation in the genome. The following restriction enzyme digestions were performed on PCR products: BglII and Hyp188I (*GFAP* R239C), BseDI and HphI (*MLC1* S93L), or DraI (*MLC1* S280L). All restriction enzymes were from Thermofisher Scientific and digestions were done according to manufacturer's instructions. Digestion products were loaded on 2% agarose gels together with undigested PCR product for each sample and 100 bp or 1 kb+ ladder (both from ThermoFisher Scientific).

Sanger DNA sequencing were performed by Eurofins Genomics. Sequencing of TOPO clones were done with M13 primer and sequencing of PCR products were done by providing the PCR primers used for amplification. Sequencing data was analyzed in SnapGene.

Table 3. PCR primer sequences for analysis of gene editing.

Primer	Sequence (5'-3')
GFAP R239C Forw	CTGGTACCGCTTCTCTCACC
GFAP R239C Rev	CAGCTTCTTCCACCCTCC
MLC1 S93L Forw	AAGTACCTGGGGGCCTTCTA
MLC1 S93L Rev	TGGGCAGGAGCTTTACTGTC
MLC1 S280L Forw	GAACCAGCTTGGGACTATGC
MLC1 S280L Rev	AGGAACAGCGGAGGAGGT

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References

Abyzov, A., Mariani, J., Palejev, D., Zhang, Y., Haney, M.S., Tomasini, L., Ferrandino, A.F., Rosenberg Belmaker, L.A., Szekely, A., Wilson, M., et al. (2012). Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells. Nature 492, 438-42. 10.1038/nature11629.

Adli, M. (2018). The CRISPR tool kit for genome editing and beyond. Nature Communications 9, 1911. 10.1038/s41467-018-04252-2.

Ahlenius, H. (2022). Past, Present, and Future of Direct Cell Reprogramming. Cellular Reprogramming 24, 205-11. 10.1089/cell.2022.0110.

Ahn, S.I., Sei, Y.J., Park, H.-J., Kim, J., Ryu, Y., Choi, J.J., Sung, H.-J., MacDonald, T.J., Levey, A.I., and Kim, Y. (2020). Microengineered human blood–brain barrier platform for understanding nanoparticle transport mechanisms. Nature Communications 11, 175. 10.1038/s41467-019-13896-7.

Allen, N.J., and Eroglu, C. (2017). Cell Biology of Astrocyte-Synapse Interactions. Neuron 96, 697-708. 10.1016/j.neuron.2017.09.056.

Almad, A., and Maragakis, N.J. (2018). A stocked toolbox for understanding the role of astrocytes in disease. Nat Rev Neurol. 10.1038/s41582-018-0010-2.

Andersen, J., Revah, O., Miura, Y., Thom, N., Amin, N.D., Kelley, K.W., Singh, M., Chen, X., Thete, M.V., Walczak, E.M., et al. (2020). Generation of Functional Human 3D Cortico-Motor Assembloids. Cell 183, 1913-29.e26. https://doi.org/10.1016/j.cell.2020.11.017.

Anzalone, A.V., Koblan, L.W., and Liu, D.R. (2020). Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors. Nature Biotechnology 38, 824-44. 10.1038/s41587-020-0561-9.

Barbar, L., Jain, T., Zimmer, M., Kruglikov, I., Sadick, J.S., Wang, M., Kalpana, K., Rose, I.V.L., Burstein, S.R., Rusielewicz, T., et al. (2020). CD49f Is a Novel Marker of Functional and Reactive Human iPSC-Derived Astrocytes. Neuron 107, 436-53 e12. 10.1016/j.neuron.2020.05.014.

Barker, A.J., and Ullian, E.M. (2010). Astrocytes and synaptic plasticity. Neuroscientist 16, 40-50. 10.1177/1073858409339215.

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., et al. (2019). Site-specific phosphorylation and caspase cleavage of GFAP are new markers of Alexander disease severity. Elife 8, e47789. 10.7554/eLife.47789.

Benfenati, V., and Ferroni, S. (2010). Water transport between CNS compartments: functional and molecular interactions between aquaporins and ion channels. Neuroscience 168, 926-40. https://doi.org/10.1016/j.neuroscience.2009.12.017.

Benzoni, C., Aquino, D., Di Bella, D., Sarto, E., Moscatelli, M., Pareyson, D., Taroni, F., and Salsano, E. (2020). Severe worsening of adult-onset Alexander disease after minor head trauma: Report of two patients and review of the literature. J Clin Neurosci 75, 221-3. 10.1016/j.jocn.2020.03.033.

Berry, S.A., Coughlin, C.R., McCandless, S., McCarter, R., Seminara, J., Yudkoff, M., and LeMons, C. (2020). Developing interactions with industry in rare diseases: lessonslearned and continuing challenges. Genetics in Medicine 22, 219-26. 10.1038/s41436-019-0616-9.

Bhaya, D., Davison, M., and Barrangou, R. (2011). CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. Annu Rev Genet 45, 273-97. 10.1146/annurev-genet-110410-132430.

Bonkowsky, J.L., Nelson, C., Kingston, J.L., Filloux, F.M., Mundorff, M.B., and Srivastava, R. (2010). The burden of inherited leukodystrophies in children. Neurology 75, 718-25. 10.1212/WNL.0b013e3181eee46b.

Borrett, D., and Becker, L.E. (1985). Alexander's disease. A disease of astrocytes. Brain 108 (Pt 2), 367-85. 10.1093/brain/108.2.367.

Brenner, M., Johnson, A.B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J.E., and Messing, A. (2001). Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. Nat Genet 27, 117-20. 10.1038/83679.

Briggs, R., and King, T.J. (1952). Transplantation of living nuclei from blastula cells into enucleated frogs' eggs *. Proceedings of the National Academy of Sciences 38, 455-63. 10.1073/pnas.38.5.455.

Brignone, M.S., Lanciotti, A., Macioce, P., Macchia, G., Gaetani, M., Aloisi, F., Petrucci, T.C., and Ambrosini, E. (2011). The β1 subunit of the Na,K-ATPase pump interacts with megalencephalic leucoencephalopathy with subcortical cysts protein 1 (MLC1) in brain astrocytes: new insights into MLC pathogenesis. Human Molecular Genetics 20, 90-103. 10.1093/hmg/ddq435.

Brooks, I.R., Garrone, C.M., Kerins, C., Kiar, C.S., Syntaka, S., Xu, J.Z., Spagnoli, F.M., and Watt, F.M. (2022). Functional genomics and the future of iPSCs in disease modeling. Stem Cell Reports 17, 1033-47. 10.1016/j.stemcr.2022.03.019.

Bruijn, L.I., Becher, M.W., Lee, M.K., Anderson, K.L., Jenkins, N.A., Copeland, N.G., Sisodia, S.S., Rothstein, J.D., Borchelt, D.R., Price, D.L., et al. (1997). ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. Neuron 18, 327-38. 10.1016/s0896-6273(00)80272-x.

Bugiani, M., Moroni, I., Bizzi, A., Nardocci, N., Bettecken, T., Gärtner, J., and Uziel, G. (2003). Consciousness disturbances in megalencephalic leukoencephalopathy with subcortical cysts. Neuropediatrics 34, 211-4. 10.1055/s-2003-42209.

Caiazzo, M., Giannelli, S., Valente, P., Lignani, G., Carissimo, A., Sessa, A., Colasante, G., Bartolomeo, R., Massimino, L., Ferroni, S., et al. (2015). Direct conversion of fibroblasts into functional astrocytes by defined transcription factors. Stem Cell Rep 4, 25-36. 10.1016/j.stemcr.2014.12.002.

Cakir, B., Tanaka, Y., Kiral, F.R., Xiang, Y., Dagliyan, O., Wang, J., Lee, M., Greaney, A.M., Yang, W.S., duBoulay, C., et al. (2022). Expression of the transcription factor PU.1 induces the generation of microglia-like cells in human cortical organoids. Nature Communications 13, 430. 10.1038/s41467-022-28043-y.

Camargo, N., Goudriaan, A., van Deijk, A.F., Otte, W.M., Brouwers, J.F., Lodder, H., Gutmann, D.H., Nave, K.A., Dijkhuizen, R.M., Mansvelder, H.D., et al. (2017). Oligodendroglial myelination requires astrocyte-derived lipids. PLoS Biol 15, e1002605. 10.1371/journal.pbio.1002605.

Canals, I., Comella-Bolla, A., Cepeda-Prado, E., Avaliani, N., Oburoglu, L., Bruzelius, A., King, N., Heuer, A., Rylander Ottosson, D., Soriano, J., and Ahlenius, H. Functional alterations in form of neuronal network hyperactivity in a CRISPR engineered pluripotent stem cell model of frontotemporal dementia. Manuscript submitted for publication.

Canals, I., and Ahlenius, H. (2021). CRISPR/Cas9 Genome Engineering in Human Pluripotent Stem Cells for Modeling of Neurological Disorders. Methods Mol Biol 2352, 237-51. 10.1007/978-1-0716-1601-7_16.

Canals, I., Ginisty, A., Quist, E., Timmerman, R., Fritze, J., Miskinyte, G., Monni, E., Hansen, M.G., Hidalgo, I., Bryder, D., et al. (2018). Rapid and efficient induction of functional astrocytes from human pluripotent stem cells. Nat Methods 15, 693-6. 10.1038/s41592-018-0103-2.

Canals, I., Quist, E., and Ahlenius, H. (2021). Transcription Factor-Based Strategies to Generate Neural Cell Types from Human Pluripotent Stem Cells. Cell Reprogram 23, 206-20. 10.1089/cell.2021.0045.

Capdevila-Nortes, X., López-Hernández, T., Apaja, P.M., López de Heredia, M., Sirisi, S., Callejo, G., Arnedo, T., Nunes, V., Lukacs, G.L., Gasull, X., et al. (2013). Insights into MLC pathogenesis: GlialCAM is an MLC1 chaperone required for proper activation of volume-regulated anion currents. Human Molecular Genetics 22, 4405-16. 10.1093/hmg/ddt290.

Chandrasekaran, A., Avci, H.X., Leist, M., Kobolak, J., and Dinnyes, A. (2016). Astrocyte Differentiation of Human Pluripotent Stem Cells: New Tools for Neurological Disorder Research. Front Cell Neurosci 10, 215. 10.3389/fncel.2016.00215.

Chanoumidou, K., Hernandez-Rodriguez, B., Windener, F., Thomas, C., Stehling, M., Mozafari, S., Albrecht, S., Ottoboni, L., Antel, J., Kim, K.P., et al. (2021). One-step Reprogramming of Human Fibroblasts into Oligodendrocyte-like Cells by SOX10, OLIG2, and NKX6.2. Stem Cell Reports 16, 771-83. 10.1016/j.stemcr.2021.03.001.

Chen, M.H., Hagemann, T.L., Quinlan, R.A., Messing, A., and Perng, M.D. (2013). Caspase cleavage of GFAP produces an assembly-compromised proteolytic fragment that promotes filament aggregation. ASN Neuro 5, e00125. 10.1042/an20130032.

Chen, S.W., Hung, Y.S., Fuh, J.L., Chen, N.J., Chu, Y.S., Chen, S.C., Fann, M.J., and Wong, Y.H. (2021). Efficient conversion of human induced pluripotent stem cells into microglia by defined transcription factors. Stem Cell Reports 16, 1363-80. 10.1016/j.stemcr.2021.03.010.

Colombo, J.A., and Reisin, H.D. (2004). Interlaminar astroglia of the cerebral cortex: a marker of the primate brain. Brain Res 1006, 126-31. 10.1016/j.brainres.2004.02.003.

Condò, I. (2022). Rare Monogenic Diseases: Molecular Pathophysiology and Novel Therapies. Int J Mol Sci 23. 10.3390/ijms23126525.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819-23. 10.1126/science.1231143.

Cook, D., Brown, D., Alexander, R., March, R., Morgan, P., Satterthwaite, G., and Pangalos, M.N. (2014). Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. Nature Reviews Drug Discovery 13, 419-31. 10.1038/nrd4309.

Cuevas-Diaz Duran, R., Wang, C.-Y., Zheng, H., Deneen, B., and Wu, J.Q. (2019). Brain Region-Specific Gene Signatures Revealed by Distinct Astrocyte Subpopulations Unveil Links to Glioma and Neurodegenerative Diseases. eneuro 6, ENEURO.0288-18.2019. 10.1523/ENEURO.0288-18.2019.

Di Giorgio, F.P., Carrasco, M.A., Siao, M.C., Maniatis, T., and Eggan, K. (2007). Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. Nat Neurosci 10, 608-14. 10.1038/nn1885.

Dobrindt, K., Zhang, H., Das, D., Abdollahi, S., Prorok, T., Ghosh, S., Weintraub, S., Genovese, G., Powell, S.K., Lund, A., et al. (2021). Publicly Available hiPSC Lines with Extreme Polygenic Risk Scores for Modeling Schizophrenia. Complex Psychiatry 6, 68-82. 10.1159/000512716.

Drouin-Ouellet, J., Lau, S., Brattas, P.L., Rylander Ottosson, D., Pircs, K., Grassi, D.A., Collins, L.M., Vuono, R., Andersson Sjoland, A., Westergren-Thorsson, G., et al. (2017). REST suppression mediates neural conversion of adult human fibroblasts via microRNA-dependent and -independent pathways. EMBO Mol Med 9, 1117-31. 10.15252/emmm.201607471.

Drouin-Ouellet, J., Legault, E.M., Nilsson, F., Pircs, K., Bouquety, J., Petit, F., Shrigley, S., Birtele, M., Pereira, M., Storm, P., et al. (2022). Age-related pathological impairments in directly reprogrammed dopaminergic neurons derived from patients with idiopathic Parkinson's disease. Stem Cell Reports 17, 2203-19. https://doi.org/10.1016/j.stemcr.2022.08.010.

Duarri, A., Lopez de Heredia, M., Capdevila-Nortes, X., Ridder, M.C., Montolio, M., López-Hernández, T., Boor, I., Lien, C.-F., Hagemann, T., Messing, A., et al. (2011). Knockdown of MLC1 in primary astrocytes causes cell vacuolation: A MLC disease cell model. Neurobiology of Disease 43, 228-38. https://doi.org/10.1016/j.nbd.2011.03.015.

Duarri, A., Teijido, O., López-Hernández, T., Scheper, G.C., Barriere, H., Boor, I., Aguado, F., Zorzano, A., Palacín, M., Martínez, A., et al. (2008). Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in MLC1 cause folding defects. Hum Mol Genet 17, 3728-39. 10.1093/hmg/ddn269.

Dubey, M., Bugiani, M., Ridder Margreet, C., Postma Nienke, L., Brouwers, E., Polder, E., Jacobs, J.G., Baayen Johannes, C., Klooster, J., Kamermans, M., et al. (2014). Mice with megalencephalic leukoencephalopathy with cysts: A developmental angle. Ann Neurol 77, 114-31. 10.1002/ana.24307.

Eichmüller, O.L., and Knoblich, J.A. (2022). Human cerebral organoids — a new tool for clinical neurology research. Nature Reviews Neurology 18, 661-80. 10.1038/s41582-022-00723-9.

Escartin, C., Galea, E., Lakatos, A., O'Callaghan, J.P., Petzold, G.C., Serrano-Pozo, A., Steinhäuser, C., Volterra, A., Carmignoto, G., Agarwal, A., et al. (2021). Reactive astrocyte nomenclature, definitions, and future directions. Nature Neuroscience. 10.1038/s41593-020-00783-4.

Feigin, V.L., Abajobir, A.A., Abate, K.H., Abd-Allah, F., Abdulle, A.M., Abera, S.F., Abyu, G.Y., Ahmed, M.B., Aichour, A.N., Aichour, I., et al. (2017). Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. The Lancet Neurology 16, 877-97. 10.1016/S1474-4422(17)30299-5.

Filley, C.M., and Fields, R.D. (2016). White matter and cognition: making the connection. Journal of Neurophysiology 116, 2093-104. 10.1152/jn.00221.2016.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nature Biotechnology 31, 822-6. 10.1038/nbt.2623.

Gao, L., Zhang, Z., Lu, J., and Pei, G. (2019). Mitochondria Are Dynamically Transferring Between Human Neural Cells and Alexander Disease-Associated GFAP Mutations Impair the Astrocytic Transfer. Front Cell Neurosci 13, 316. 10.3389/fncel.2019.00316.

Garcia-Leon, J.A., Kumar, M., Boon, R., Chau, D., One, J., Wolfs, E., Eggermont, K., Berckmans, P., Gunhanlar, N., de Vrij, F., et al. (2018). SOX10 Single Transcription Factor-Based Fast and Efficient Generation of Oligodendrocytes from Human Pluripotent Stem Cells. Stem Cell Reports 10, 655-72. 10.1016/j.stemcr.2017.12.014.

Gatto, N., Dos Santos Souza, C., Shaw, A.C., Bell, S.M., Myszczynska, M.A., Powers, S., Meyer, K., Castelli, L.M., Karyka, E., Mortiboys, H., et al. (2020). Directly converted astrocytes retain the ageing features of the donor fibroblasts and elucidate the astrocytic contribution to human CNS health and disease. Aging Cell 20, e13281. 10.1111/acel.13281.

Gilbert, A., Elorza-Vidal, X., Rancillac, A., Chagnot, A., Yetim, M., Hingot, V., Deffieux, T., Boulay, A.-C., Alvear-Perez, R., Cisternino, S., et al. (2021). Megalencephalic leukoencephalopathy with subcortical cysts is a developmental disorder of the gliovascular unit. Elife 10, e71379. 10.7554/eLife.71379.

Gurdon, J.B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morphol 10, 622-40.

Gurdon, J.B., Elsdale, T.R., and Fischberg, M. (1958). Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. Nature 182, 64-5. 10.1038/182064a0.

Guttenplan, K.A., and Liddelow, S.A. (2019). Astrocytes and microglia: Models and tools. J Exp Med 216, 71-83. 10.1084/jem.20180200.

Hagemann, T.L. (2022). Alexander disease: models, mechanisms, and medicine. Current Opinion in Neurobiology 72, 140-7. https://doi.org/10.1016/j.conb.2021.10.002.

Hagemann, T.L., Connor, J.X., and Messing, A. (2006). Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response. J Neurosci 26, 11162-73. 10.1523/jneurosci.3260-06.2006.

Hagemann, T.L., Gaeta, S.A., Smith, M.A., Johnson, D.A., Johnson, J.A., and Messing, A. (2005). Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction. Human Molecular Genetics 14, 2443-58. 10.1093/hmg/ddi248.

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., et al. (2021). Antisense therapy in a rat model of Alexander disease reverses GFAP pathology, white matter deficits, and motor impairment. Sci Transl Med 13, eabg4711. 10.1126/scitranslmed.abg4711.

Ham, O., Jin, Y.B., Kim, J., and Lee, M.O. (2020). Blood vessel formation in cerebral organoids formed from human embryonic stem cells. Biochem Biophys Res Commun 521, 84-90. 10.1016/j.bbrc.2019.10.079.

Hamilton, E.M.C., Tekturk, P., Cialdella, F., van Rappard, D.F., Wolf, N.I., Yalcinkaya, C., Çetinçelik, Ü., Rajaee, A., Kariminejad, A., Paprocka, J., et al. (2018). Megalencephalic leukoencephalopathy with subcortical cysts: Characterization of disease variants. Neurology 90, e1395-e403. 10.1212/wnl.0000000000005334.

Harikumar, K.B., Yester, J.W., Surace, M.J., Oyeniran, C., Price, M.M., Huang, W.-C., Hait, N.C., Allegood, J.C., Yamada, A., Kong, X., et al. (2014). K63-linked polyubiquitination of transcription factor IRF1 is essential for IL-1-induced production of chemokines CXCL10 and CCL5. Nature Immunology 15, 231-8. 10.1038/ni.2810.

Hastings, N., Kuan, W.-L., Osborne, A., and Kotter, M.R.N. (2022). Therapeutic Potential of Astrocyte Transplantation. Cell Transplantation 31, 09636897221105499. 10.1177/09636897221105499.

Hay, M., Thomas, D.W., Craighead, J.L., Economides, C., and Rosenthal, J. (2014). Clinical development success rates for investigational drugs. Nat Biotechnol 32, 40-51. 10.1038/nbt.2786.

Heaven, M.R., Flint, D., Randall, S.M., Sosunov, A.A., Wilson, L., Barnes, S., Goldman, J.E., Muddiman, D.C., and Brenner, M. (2016). Composition of Rosenthal Fibers, the Protein Aggregate Hallmark of Alexander Disease. Journal of Proteome Research 15, 2265-82. 10.1021/acs.jproteome.6b00316.

Heim, P., Claussen, M., Hoffmann, B., Conzelmann, E., Gärtner, J., Harzer, K., Hunneman, D.H., Köhler, W., Kurlemann, G., and Kohlschütter, A. (1997). Leukodystrophy incidence in Germany. American Journal of Medical Genetics 71, 475-8. https://doi.org/10.1002/(SICI)1096-8628(19970905)71:4<475::AID-AJMG20>3.0.CO;2-C.

Herland, A., van der Meer, A.D., FitzGerald, E.A., Park, T.E., Sleeboom, J.J., and Ingber, D.E. (2016). Distinct Contributions of Astrocytes and Pericytes to Neuroinflammation Identified in a 3D Human Blood-Brain Barrier on a Chip. PLoS One 11, e0150360. 10.1371/journal.pone.0150360.

Hilton, I.B., D'Ippolito, A.M., Vockley, C.M., Thakore, P.I., Crawford, G.E., Reddy, T.E., and Gersbach, C.A. (2015). Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nature Biotechnology 33, 510-7. 10.1038/nbt.3199.

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., et al. (2014). Disrupting MLC1 and GlialCAM and ClC-2 interactions in leukodystrophy entails glial chloride channel dysfunction. Nature Communications 5, 3475. 10.1038/ncomms4475.

Hughes, A.N. (2021). Glial Cells Promote Myelin Formation and Elimination. Front Cell Dev Biol 9, 661486. 10.3389/fcell.2021.661486.

Hurle, M.R., Nelson, M.R., Agarwal, P., and Cardon, L.R. (2016). Impact of genetically supported target selection on R&D productivity. Nature Reviews Drug Discovery 15, 596-7. 10.1038/nrd.2016.164.

Ilja Boor, P.K., de Groot, K., Mejaski-Bosnjak, V., Brenner, C., van der Knaap, M.S., Scheper, G.C., and Pronk, J.C. (2006). Megalencephalic leukoencephalopathy with subcortical cysts: an update and extended mutation analysis of MLC1. Hum Mutat 27, 505-12. 10.1002/humu.20332.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337, 816-21. 10.1126/science.1225829.

Jones, J.R., Kong, L., Hanna, M.G.I.V., Hoffman, B., Krencik, R., Bradley, R., Hagemann, T., Choi, J., Doers, M., Dubovis, M., et al. (2018). Mutations in GFAP Disrupt the Distribution and Function of Organelles in Human Astrocytes. Cell Reports 25, 947-58.e4. 10.1016/j.celrep.2018.09.083.

Kevelam, S.H., Steenweg, M.E., Srivastava, S., Helman, G., Naidu, S., Schiffmann, R., Blaser, S., Vanderver, A., Wolf, N.I., and van der Knaap, M.S. (2016). Update on Leukodystrophies: A Historical Perspective and Adapted Definition. Neuropediatrics 47, 349-54. 10.1055/s-0036-1588020.

Kim, Y., Zheng, X., Ansari, Z., Bunnell, M.C., Herdy, J.R., Traxler, L., Lee, H., Paquola, A.C.M., Blithikioti, C., Ku, M., et al. (2018). Mitochondrial Aging Defects Emerge in Directly Reprogrammed Human Neurons due to Their Metabolic Profile. Cell Rep 23, 2550-8. 10.1016/j.celrep.2018.04.105.

Klein, E.A., and Anzil, A.P. (1994). Prominent white matter cavitation in an infant with Alexander's disease. Clin Neuropathol 13, 31-8.

Kohlschütter, A., and Eichler, F. (2011). Childhood leukodystrophies: a clinical perspective. Expert Review of Neurotherapeutics 11, 1485-96. 10.1586/ern.11.135.

Kola, I., and Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? Nature Reviews Drug Discovery 3, 711-6. 10.1038/nrd1470.

Kondo, T., Funayama, M., Miyake, M., Tsukita, K., Era, T., Osaka, H., Ayaki, T., Takahashi, R., and Inoue, H. (2016). Modeling Alexander disease with patient iPSCs reveals cellular and molecular pathology of astrocytes. Acta Neuropathol Commun 4, 69. 10.1186/s40478-016-0337-0.

Konstantinidis, E., Molisak, A., Perrin, F., Streubel-Gallasch, L., Fayad, S., Kim, D.Y., Petri, K., Aryee, M.J., Aguilar, X., György, B., et al. (2022). CRISPR-Cas9 treatment partially restores amyloid-β 42/40 in human fibroblasts with the Alzheimer's disease PSEN 1 M146L mutation. Mol Ther Nucleic Acids 28, 450-61. 10.1016/j.omtn.2022.03.022.

Krencik, R., Weick, J.P., Liu, Y., Zhang, Z.J., and Zhang, S.C. (2011). Specification of transplantable astroglial subtypes from human pluripotent stem cells. Nat Biotechnol 29, 528-34. 10.1038/nbt.1877.

Kumar, M., Nguyen, N.T.P., Milanese, M., and Bonanno, G. (2022). Insights into Human-Induced Pluripotent Stem Cell-Derived Astrocytes in Neurodegenerative Disorders. Biomolecules 12, 344.

La Russa, M.F., and Qi, L.S. (2015). The New State of the Art: Cas9 for Gene Activation and Repression. Mol Cell Biol 35, 3800-9. 10.1128/mcb.00512-15.

Lancaster, M.A., Renner, M., Martin, C.-A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature 501, 373-9. 10.1038/nature12517.

Lanciotti, A., Brignone, M.S., Bertini, E., Petrucci, T.C., Aloisi, F., and Ambrosini, E. (2013). ASTROCYTES: EMERGING STARS IN LEUKODYSTROPHY PATHOGENESIS. Transl Neurosci 4. 10.2478/s13380-013-0118-1.

Lanciotti, A., Brignone, M.S., Camerini, S., Serafini, B., Macchia, G., Raggi, C., Molinari, P., Crescenzi, M., Musumeci, M., Sargiacomo, M., et al. (2010). MLC1 trafficking and membrane expression in astrocytes: Role of caveolin-1 and phosphorylation. Neurobiology of Disease 37, 581-95. https://doi.org/10.1016/j.nbd.2009.11.008.

Lanciotti, A., Brignone, M.S., Macioce, P., Visentin, S., and 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. 10.3390/ijms23010274.

Lanciotti, A., Brignone, M.S., Molinari, P., Visentin, S., De Nuccio, C., Macchia, G., Aiello, C., Bertini, E., Aloisi, F., Petrucci, T.C., et al. (2012). Megalencephalic leukoencephalopathy with subcortical cysts protein 1 functionally cooperates with the TRPV4 cation channel to activate the response of astrocytes to osmotic stress: dysregulation by pathological mutations. Human Molecular Genetics 21, 2166-80. 10.1093/hmg/dds032.

Lapasset, L., Milhavet, O., Prieur, A., Besnard, E., Babled, A., Aït-Hamou, N., Leschik, J., Pellestor, F., Ramirez, J.M., De Vos, J., et al. (2011). Rejuvenating senescent and centenarian human cells by reprogramming through the pluripotent state. Genes Dev 25, 2248-53. 10.1101/gad.173922.111.

Lee, H.G., Wheeler, M.A., and Quintana, F.J. (2022). Function and therapeutic value of astrocytes in neurological diseases. Nat Rev Drug Discov 21, 339-58. 10.1038/s41573-022-00390-x.

Leegwater, P.A.J., Yuan, B.Q., van der Steen, J., Mulders, J., Könst, A.A.M., Boor, P.K.I., Mejaski-Bosnjak, V., van der Maarel, S.M., Frants, R.R., Oudejans, C.B.M., et al. (2001). Mutations of MLC1 (KIAA0027), Encoding a Putative Membrane Protein, Cause Megalencephalic Leukoencephalopathy with Subcortical Cysts. The American Journal of Human Genetics 68, 831-8. https://doi.org/10.1086/319519.

Leventoux, N., Morimoto, S., Imaizumi, K., Sato, Y., Takahashi, S., Mashima, K., Ishikawa, M., Sonn, I., Kondo, T., Watanabe, H., et al. (2020). Human Astrocytes Model Derived from Induced Pluripotent Stem Cells. In Cells.

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., and Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduct Target Ther 5, 1. 10.1038/s41392-019-0089-y.

Li, J., Pan, L., Pembroke, W.G., Rexach, J.E., Godoy, M.I., Condro, M.C., Alvarado, A.G., Harteni, M., Chen, Y.-W., Stiles, L., et al. (2021). Conservation and divergence of vulnerability and responses to stressors between human and mouse astrocytes. Nat Commun 12, 3958. 10.1038/s41467-021-24232-3.

Li, L., Tian, E., Chen, X., Chao, J., Klein, J., Qu, Q., Sun, G., Sun, G., Huang, Y., Warden, C.D., et al. (2018a). GFAP Mutations in Astrocytes Impair Oligodendrocyte Progenitor Proliferation and Myelination in an hiPSC Model of Alexander Disease. Cell Stem Cell 23, 239-51.e6. 10.1016/j.stem.2018.07.009.

Li, X., Tao, Y., Bradley, R., Du, Z., Tao, Y., Kong, L., Dong, Y., Jones, J., Yan, Y., Harder, C.R.K., et al. (2018b). Fast Generation of Functional Subtype Astrocytes from Human Pluripotent Stem Cells. Stem Cell Reports 11, 998-1008. 10.1016/j.stemcr.2018.08.019.

Liddelow, S.A., Guttenplan, K.A., Clarke, L.E., Bennett, F.C., Bohlen, C.J., Schirmer, L., Bennett, M.L., Münch, A.E., Chung, W.-S., Peterson, T.C., et al. (2017). Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541, 481-7. 10.1038/nature21029.

Lin, Y.T., Seo, J., Gao, F., Feldman, H.M., Wen, H.L., Penney, J., Cam, H.P., Gjoneska, E., Raja, W.K., Cheng, J., et al. (2018). APOE4 Causes Widespread Molecular and Cellular Alterations Associated with Alzheimer's Disease Phenotypes in Human iPSC-Derived Brain Cell Types. Neuron 98, 1141-54 e7. 10.1016/j.neuron.2018.05.008.

Lopez-Hernandez, T., Ridder, M.C., Montolio, M., Capdevila-Nortes, X., Polder, E., Sirisi, S., Duarri, A., Schulte, U., Fakler, B., Nunes, V., et al. (2011a). Mutant GlialCAM causes megalencephalic leukoencephalopathy with subcortical cysts, benign familial macrocephaly, and macrocephaly with retardation and autism. Am J Hum Genet 88, 422-32. 10.1016/j.ajhg.2011.02.009.

Lopez-Hernandez, T., Sirisi, S., Capdevila-Nortes, X., Montolio, M., Fernandez-Duenas, V., Scheper, G.C., van der Knaap, M.S., Casquero, P., Ciruela, F., Ferrer, I., et al. (2011b). Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts. Hum Mol Genet 20, 3266-77. 10.1093/hmg/ddr238.

Magistretti, P.J., Pellerin, L., Rothman, D.L., and Shulman, R.G. (1999). Energy on demand. Science 283, 496-7. 10.1126/science.283.5401.496.

Marton, R.M., Miura, Y., Sloan, S.A., Li, Q., Revah, O., Levy, R.J., Huguenard, J.R., and Pașca, S.P. (2019). Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. Nature Neuroscience. 10.1038/s41593-018-0316-9.

Marton, R.M., and Pașca, S.P. (2020). Organoid and Assembloid Technologies for Investigating Cellular Crosstalk in Human Brain Development and Disease. Trends in Cell Biology 30, 133-43. https://doi.org/10.1016/j.tcb.2019.11.004.

McConnell, M.J., Lindberg, M.R., Brennand, K.J., Piper, J.C., Voet, T., Cowing-Zitron, C., Shumilina, S., Lasken, R.S., Vermeesch, J.R., Hall, I.M., et al. (2013). Mosaic copy number variation in human neurons. Science 342, 632-7. 10.1126/science.1243472.

Mertens, J., Marchetto, M.C., Bardy, C., and Gage, F.H. (2016). Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. Nat Rev Neurosci 17, 424-37. 10.1038/nrn.2016.46.

Mertens, J., Paquola, A.C.M., Ku, M., Hatch, E., Bohnke, L., Ladjevardi, S., McGrath, S., Campbell, B., Lee, H., Herdy, J.R., et al. (2015). Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects. Cell Stem Cell 17, 705-18. 10.1016/j.stem.2015.09.001.

Messing, A. (2018). Chapter 44 - Alexander disease. In Handb Clin Neurol, D.H. Geschwind, H.L. Paulson, and C. Klein, eds. (Elsevier), pp. 693-700.

Messing, A., Head, M.W., Galles, K., Galbreath, E.J., Goldman, J.E., and Brenner, M. (1998). Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice. Am J Pathol 152, 391-8.

Mignot, C., Delarasse, C., Escaich, S., Della Gaspera, B., Noé, E., Colucci-Guyon, E., Babinet, C., Pekny, M., Vicart, P., Boespflug-Tanguy, O., et al. (2007). Dynamics of mutated GFAP aggregates revealed by real-time imaging of an astrocyte model of Alexander disease. Exp Cell Res 313, 2766-79. 10.1016/j.yexcr.2007.04.035.

Miles, L., DeGrauw, T.J., Dinopoulos, A., Cecil, K.M., van der Knaap, M.S., and Bove, K.E. (2009). Megalencephalic leukoencephalopathy with subcortical cysts: a third confirmed case with literature review. Pediatr Dev Pathol 12, 180-6. 10.2350/08-06-0481.1.

Miller, Justine D., Ganat, Yosif M., Kishinevsky, S., Bowman, Robert L., Liu, B., Tu, Edmund Y., Mandal, P.K., Vera, E., Shim, J.-w., Kriks, S., et al. (2013). Human iPSC-Based Modeling of Late-Onset Disease via Progerin-Induced Aging. Cell Stem Cell 13, 691-705. https://doi.org/10.1016/j.stem.2013.11.006.

Min, R., and van der Knaap, M.S. (2018). Genetic defects disrupting glial ion and water homeostasis in the brain. Brain Pathol 28, 372-87. 10.1111/bpa.12602.

Minkel, H.R., Anwer, T.Z., Arps, K.M., Brenner, M., and Olsen, M.L. (2015). Elevated GFAP induces astrocyte dysfunction in caudal brain regions: A potential mechanism for hindbrain involved symptoms in type II Alexander disease. Glia 63, 2285-97. 10.1002/glia.22893.

Miskinyte, G., Gronning Hansen, M., Monni, E., Lam, M., Bengzon, J., Lindvall, O., Ahlenius, H., and Kokaia, Z. (2018). Transcription factor programming of human ES cells generates functional neurons expressing both upper and deep layer cortical markers. PLoS One 13, e0204688. 10.1371/journal.pone.0204688.

Molofsky, A.V., Krencik, R., Ullian, E.M., Tsai, H.H., Deneen, B., Richardson, W.D., Barres, B.A., and Rowitch, D.H. (2012). Astrocytes and disease: a neurodevelopmental perspective. Genes Dev 26, 891-907. 10.1101/gad.188326.112.

Monaco, L., Zanello, G., Baynam, G., Jonker, A.H., Julkowska, D., Hartman, A.L., O'Connor, D., Wang, C.M., Wong-Rieger, D., and Pearce, D.A. (2022). Research on rare diseases: ten years of progress and challenges at IRDiRC. Nat Rev Drug Discov 21, 319-20. 10.1038/d41573-022-00019-z.

Morgan, P., Brown, D.G., Lennard, S., Anderton, M.J., Barrett, J.C., Eriksson, U., Fidock, M., Hamren, B., Johnson, A., March, R.E., et al. (2018). Impact of a five-dimensional framework on R&D productivity at AstraZeneca. Nat Rev Drug Discov 17, 167-81. 10.1038/nrd.2017.244.

Nagai, M., Re, D.B., Nagata, T., Chalazonitis, A., Jessell, T.M., Wichterle, H., and Przedborski, S. (2007). Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. Nat Neurosci 10, 615-22. 10.1038/nn1876.

Nave, K.A., and Werner, H.B. (2014). Myelination of the nervous system: mechanisms and functions. Annu Rev Cell Dev Biol 30, 503-33. 10.1146/annurev-cellbio-100913-013101.

Nedergaard, M., Ransom, B., and Goldman, S.A. (2003). New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 26, 523-30. 10.1016/j.tins.2003.08.008.

Neyrinck, K., Van Den Daele, J., Vervliet, T., De Smedt, J., Wierda, K., Nijs, M., Vanbokhoven, T., D'hondt, A., Planque, M., Fendt, S.-M., et al. (2021). SOX9-induced Generation of Functional Astrocytes Supporting Neuronal Maturation in an All-human System. Stem Cell Reviews and Reports. 10.1007/s12015-021-10179-x.

Ng, A.H.M., Khoshakhlagh, P., Rojo Arias, J.E., Pasquini, G., Wang, K., Swiersy, A., Shipman, S.L., Appleton, E., Kiaee, K., Kohman, R.E., et al. (2021). A comprehensive library of human transcription factors for cell fate engineering. Nature Biotechnology 39, 510-9. 10.1038/s41587-020-0742-6.

Nguengang Wakap, S., Lambert, D.M., Olry, A., Rodwell, C., Gueydan, C., Lanneau, V., Murphy, D., Le Cam, Y., and Rath, A. (2020). Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database. European Journal of Human Genetics 28, 165-73. 10.1038/s41431-019-0508-0.

Oberheim, N.A., Takano, T., Han, X., He, W., Lin, J.H., Wang, F., Xu, Q., Wyatt, J.D., Pilcher, W., Ojemann, J.G., et al. (2009). Uniquely hominid features of adult human astrocytes. J Neurosci 29, 3276-87. 10.1523/JNEUROSCI.4707-08.2009.

Oberheim, N.A., Wang, X., Goldman, S., and Nedergaard, M. (2006). Astrocytic complexity distinguishes the human brain. Trends Neurosci 29, 547-53. 10.1016/j.tins.2006.08.004.

Pammolli, F., Righetto, L., Abrignani, S., Pani, L., Pelicci, P.G., and Rabosio, E. (2020). The endless frontier? The recent increase of R&D productivity in pharmaceuticals. J Transl Med 18, 162. 10.1186/s12967-020-02313-z.

Pang, Z.P., Yang, N., Vierbuchen, T., Ostermeier, A., Fuentes, D.R., Yang, T.Q., Citri, A., Sebastiano, V., Marro, S., Südhof, T.C., et al. (2011). Induction of human neuronal cells by defined transcription factors. Nature 476, 220-3. 10.1038/nature10202.

Paulsen, B., Velasco, S., Kedaigle, A.J., Pigoni, M., Quadrato, G., Deo, A.J., Adiconis, X., Uzquiano, A., Sartore, R., Yang, S.M., et al. (2022). Autism genes converge on asynchronous development of shared neuron classes. Nature 602, 268-73. 10.1038/s41586-021-04358-6.

Pekny, M., Pekna, M., Messing, A., Steinhäuser, C., Lee, J.M., Parpura, V., Hol, E.M., Sofroniew, M.V., and Verkhratsky, A. (2016). Astrocytes: a central element in neurological diseases. Acta Neuropathol 131, 323-45. 10.1007/s00401-015-1513-1.

Pham, M.T., Pollock, K.M., Rose, M.D., Cary, W.A., Stewart, H.R., Zhou, P., Nolta, J.A., and Waldau, B. (2018). Generation of human vascularized brain organoids. Neuroreport 29, 588-93. 10.1097/wnr.0000000000001014.

Pircs, K., Drouin-Ouellet, J., Horváth, V., Gil, J., Rezeli, M., Garza, R., Grassi, D.A., Sharma, Y., St-Amour, I., Harris, K., et al. (2022). Distinct subcellular autophagy impairments in induced neurons from patients with Huntington's disease. Brain 145, 3035-57. 10.1093/brain/awab473.

Prust, M., Wang, J., Morizono, H., Messing, A., Brenner, M., Gordon, E., Hartka, T., Sokohl, A., Schiffmann, R., Gordish-Dressman, H., et al. (2011). GFAP mutations, age at onset, and clinical subtypes in Alexander disease. Neurology 77, 1287-94. 10.1212/WNL.0b013e3182309f72.

Qian, K., Huang, H., Peterson, A., Hu, B., Maragakis, N.J., Ming, G.L., Chen, H., and Zhang, S.C. (2017). Sporadic ALS Astrocytes Induce Neuronal Degeneration In Vivo. Stem Cell Reports 8, 843-55. 10.1016/j.stemcr.2017.03.003.

Quist, E., Ahlenius, H., and Canals, I. (2021). Transcription Factor Programming of Human Pluripotent Stem Cells to Functionally Mature Astrocytes for Monocultures and Cocultures with Neurons. In Methods Mol Biol, H. Ahlenius, ed. (New York, NY: Humana), pp. 133-48.

Quist, E., Trovato, F., Avaliani, N., Zetterdahl, O.G., Gonzalez-Ramos, A., Hansen, M.G., Kokaia, M., Canals, I., and Ahlenius, H. (2022). Transcription factor-based direct conversion of human fibroblasts to functional astrocytes. Stem Cell Reports 17, 1620-35. 10.1016/j.stemcr.2022.05.015.

Rademakers, R., Baker, M., Nicholson, A.M., Rutherford, N.J., Finch, N., Soto-Ortolaza, A., Lash, J., Wider, C., Wojtas, A., DeJesus-Hernandez, M., et al. (2011). Mutations in the colony stimulating factor 1 receptor (CSF1R) gene cause hereditary diffuse leukoencephalopathy with spheroids. Nat Genet 44, 200-5. 10.1038/ng.1027.

Ran, F.A., Hsu, Patrick D., Lin, C.-Y., Gootenberg, Jonathan S., Konermann, S., Trevino, A.E., Scott, David A., Inoue, A., Matoba, S., Zhang, Y., et al. (2013). Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Cell 154, 1380-9. https://doi.org/10.1016/j.cell.2013.08.021.

Rash, J.E. (2010). Molecular disruptions of the panglial syncytium block potassium siphoning and axonal saltatory conduction: pertinence to neuromyelitis optica and other demyelinating diseases of the central nervous system. Neuroscience 168, 982-1008. https://doi.org/10.1016/j.neuroscience.2009.10.028.

Ren, B., and Dunaevsky, A. (2021). Modeling Neurodevelopmental and Neuropsychiatric Diseases with Astrocytes Derived from Human-Induced Pluripotent Stem Cells. Int J Mol Sci 22. 10.3390/ijms22041692.

Ridder, M.C., Boor, I., Lodder, J.C., Postma, N.L., Capdevila-Nortes, X., Duarri, A., Brussaard, A.B., Estevez, R., Scheper, G.C., Mansvelder, H.D., et al. (2011). Megalencephalic leucoencephalopathy with cysts: defect in chloride currents and cell volume regulation. Brain 134, 3342-54. 10.1093/brain/awr255.

Riel-Romero, R.M., Smith, C.D., and Pettigrew, A.L. (2005). Megalencephalic leukoencephalopathy with subcortical cysts in two siblings owing to two novel mutations: case reports and review of the literature. J Child Neurol 20, 230-4. 10.1177/088307380502000301.

Ringel, M.S., Scannell, J.W., Baedeker, M., and Schulze, U. (2020). Breaking Eroom's Law. Nat Rev Drug Discov 19, 833-4. 10.1038/d41573-020-00059-3.

Rowe, R.G., and Daley, G.Q. (2019). Induced pluripotent stem cells in disease modelling and drug discovery. Nature Reviews Genetics 20, 377-88. 10.1038/s41576-019-0100-z.

Rubio, A., Luoni, M., Giannelli, S.G., Radice, I., Iannielli, A., Cancellieri, C., Di Berardino, C., Regalia, G., Lazzari, G., Menegon, A., et al. (2016). Rapid and efficient CRISPR/Cas9 gene inactivation in human neurons during human pluripotent stem cell differentiation and direct reprogramming. Scientific Reports 6, 37540. 10.1038/srep37540.

Rutherford, H.A., and Hamilton, N. (2019). Animal models of leukodystrophy: a new perspective for the development of therapies. Febs j 286, 4176-91. 10.1111/febs.15060.

Saito, K., Shigetomi, E., Yasuda, R., Sato, R., Nakano, M., Tashiro, K., Tanaka, K.F., Ikenaka, K., Mikoshiba, K., Mizuta, I., et al. (2018). Aberrant astrocyte Ca(2+) signals "AxCa signals" exacerbate pathological alterations in an alexander disease model. Glia. 10.1002/glia.23300.

Sander, J.D., and Joung, J.K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nature Biotechnology 32, 347-55. 10.1038/nbt.2842.

Scannell, J.W., Blanckley, A., Boldon, H., and Warrington, B. (2012). Diagnosing the decline in pharmaceutical R&D efficiency. Nature Reviews Drug Discovery 11, 191-200. 10.1038/nrd3681.

Seifert, G., Carmignoto, G., and Steinhäuser, C. (2010). Astrocyte dysfunction in epilepsy. Brain Res Rev 63, 212-21. 10.1016/j.brainresrev.2009.10.004.

Serio, A., Bilican, B., Barmada, S.J., Ando, D.M., Zhao, C., Siller, R., Burr, K., Haghi, G., Story, D., Nishimura, A.L., et al. (2013). Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. PNAS 110, 4697-702. 10.1073/pnas.1300398110.

Shaltouki, A., Peng, J., Liu, Q., Rao, M.S., and Zeng, X. (2013). Efficient Generation of Astrocytes from Human Pluripotent Stem Cells in Defined Conditions. Stem Cells 31, 941-52. 10.1002/stem.1334.

Silva, M.C., and Haggarty, S.J. (2020). Human pluripotent stem cell–derived models and drug screening in CNS precision medicine. Annals of the New York Academy of Sciences 1471, 18-56. https://doi.org/10.1111/nyas.14012.

Simard, M., and Nedergaard, M. (2004). The neurobiology of glia in the context of water and ion homeostasis. Neuroscience 129, 877-96. 10.1016/j.neuroscience.2004.09.053.

Sirabella, R., Valsecchi, V., Anzilotti, S., Cuomo, O., Vinciguerra, A., Cepparulo, P., Brancaccio, P., Guida, N., Blondeau, N., Canzoniero, L.M.T., et al. (2018). Ionic Homeostasis Maintenance in ALS: Focus on New Therapeutic Targets. Frontiers in Neuroscience 12. 10.3389/fnins.2018.00510.

Sirisi, S., Folgueira, M., Lopez-Hernandez, T., Minieri, L., Perez-Rius, C., Gaitan-Penas, H., Zang, J., Martinez, A., Capdevila-Nortes, X., De La Villa, P., et al. (2014). Megalencephalic leukoencephalopathy with subcortical cysts protein 1 regulates glial surface localization of GLIALCAM from fish to humans. Hum Mol Genet 23, 5069-86. 10.1093/hmg/ddu231.

Sloan, S.A., Andersen, J., Paşca, A.M., Birey, F., and Paşca, S.P. (2018). Generation and assembly of human brain region–specific three-dimensional cultures. Nature Protocols 13, 2062-85. 10.1038/s41596-018-0032-7.

Sloan, S.A., and Barres, B.A. (2014). Mechanisms of astrocyte development and their contributions to neurodevelopmental disorders. Current Opinion in Neurobiology 27, 75-81. https://doi.org/10.1016/j.conb.2014.03.005.

Sloan, S.A., Darmanis, S., Huber, N., Khan, T.A., Birey, F., Caneda, C., Reimer, R., Quake, S.R., Barres, B.A., and Pasca, S.P. (2017). Human Astrocyte Maturation Captured in 3D Cerebral Cortical Spheroids Derived from Pluripotent Stem Cells. Neuron 95, 779-90. 10.1016/j.neuron.2017.07.035.

Smits, L.M., Reinhardt, L., Reinhardt, P., Glatza, M., Monzel, A.S., Stanslowsky, N., Rosato-Siri, M.D., Zanon, A., Antony, P.M., Bellmann, J., et al. (2019). Modeling Parkinson's disease in midbrain-like organoids. npj Parkinson's Disease 5, 5. 10.1038/s41531-019-0078-4.

Sofroniew, M.V., and Vinters, H.V. (2010). Astrocytes: biology and pathology. Acta Neuropathol 119, 7-35. 10.1007/s00401-009-0619-8.

Soldner, F., and Jaenisch, R. (2018). Stem Cells, Genome Editing, and the Path to Translational Medicine. Cell 175, 615-32. 10.1016/j.cell.2018.09.010.

Soldner, F., Stelzer, Y., Shivalila, C.S., Abraham, B.J., Latourelle, J.C., Barrasa, M.I., Goldmann, J., Myers, R.H., Young, R.A., and Jaenisch, R. (2016). Parkinson-associated risk variant in distal enhancer of alpha-synuclein modulates target gene expression. Nature 533, 95-9. 10.1038/nature17939.

Sonn, I., Honda-Ozaki, F., Yoshimatsu, S., Morimoto, S., Watanabe, H., and Okano, H. (2022). Single transcription factor efficiently leads human induced pluripotent stem cells to functional microglia. Inflammation and Regeneration 42, 20. 10.1186/s41232-022-00201-1.

Sosunov, A., Olabarria, M., and Goldman, J.E. (2018). Alexander disease: an astrocytopathy that produces a leukodystrophy. Brain Pathol 28, 388-98. 10.1111/bpa.12601.

Sugio, S., Tohyama, K., Oku, S., Fujiyoshi, K., Yoshimura, T., Hikishima, K., Yano, R., Fukuda, T., Nakamura, M., Okano, H., et al. (2017). Astrocyte-mediated infantile-onset leukoencephalopathy mouse model. Glia 65, 150-68. 10.1002/glia.23084.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-72. 10.1016/j.cell.2007.11.019.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-76. 10.1016/j.cell.2006.07.024.

Tanaka, K.F., Takebayashi, H., Yamazaki, Y., Ono, K., Naruse, M., Iwasato, T., Itohara, S., Kato, H., and Ikenaka, K. (2007). Murine model of Alexander disease: analysis of GFAP aggregate formation and its pathological significance. Glia 55, 617-31. 10.1002/glia.20486.

Tang, G., Perng, M.D., Wilk, S., Quinlan, R., and Goldman, J.E. (2010). Oligomers of mutant glial fibrillary acidic protein (GFAP) Inhibit the proteasome system in alexander disease astrocytes, and the small heat shock protein alphaB-crystallin reverses the inhibition. J Biol Chem 285, 10527-37. 10.1074/jbc.M109.067975.

Tang, Y., Liu, M.L., Zang, T., and Zhang, C.L. (2017). Direct Reprogramming Rather than iPSC-Based Reprogramming Maintains Aging Hallmarks in Human Motor Neurons. Front Mol Neurosci 10, 359. 10.3389/fnmol.2017.00359.

Tchieu, J., Calder, E.L., Guttikonda, S.R., Gutzwiller, E.M., Aromolaran, K.A., Steinbeck, J.A., Goldstein, P.A., and Studer, L. (2019). NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells. Nat Biotechnol 37, 267-75. 10.1038/s41587-019-0035-0.

Tcw, J., Wang, M., Pimenova, A.A., Bowles, K.R., Hartley, B.J., Lacin, E., Machlovi, S.I., Abdelaal, R., Karch, C.M., Phatnani, H., et al. (2017). An Efficient Platform for Astrocyte Differentiation from Human Induced Pluripotent Stem Cells. Stem Cell Reports 9, 600-14. 10.1016/j.stemcr.2017.06.018.

Teijido, O., Martínez, A., Pusch, M., Zorzano, A., Soriano, E., del Río, J.A., Palacín, M., and Estévez, R. (2004). Localization and functional analyses of the MLC1 protein involved in megalencephalic leukoencephalopathy with subcortical cysts. Human Molecular Genetics 13, 2581-94. 10.1093/hmg/ddh291.

Tian, E., Sun, G., Sun, G., Chao, J., Ye, P., Warden, C., Riggs, A.D., and Shi, Y. (2016). Small-Molecule-Based Lineage Reprogramming Creates Functional Astrocytes. Cell Rep 16, 781-92. 10.1016/j.celrep.2016.06.042.

Tian, R., Wu, X., Hagemann, T.L., Sosunov, A.A., Messing, A., McKhann, G.M., and Goldman, J.E. (2010). Alexander disease mutant glial fibrillary acidic protein compromises glutamate transport in astrocytes. J Neuropathol Exp Neurol 69, 335-45. 10.1097/NEN.0b013e3181d3cb52.

Tiwari, N., Pataskar, A., Péron, S., Thakurela, S., Sahu, S.K., Figueres-Oñate, M., Marichal, N., López-Mascaraque, L., Tiwari, V.K., and Berninger, B. (2018). Stage-Specific Transcription Factors Drive Astrogliogenesis by Remodeling Gene Regulatory Landscapes. Cell Stem Cell 23, 557-71. 10.1016/j.stem.2018.09.008.

Tomishima, M., and Kirkeby, A. (2021). Bringing Advanced Therapies for Parkinson's Disease to the Clinic: The Scientist's Perspective. J Parkinsons Dis 11, S135-s40. 10.3233/jpd-212685.

Tomokane, N., Iwaki, T., Tateishi, J., Iwaki, A., and Goldman, J.E. (1991). Rosenthal fibers share epitopes with alpha B-crystallin, glial fibrillary acidic protein, and ubiquitin, but not with vimentin. Immunoelectron microscopy with colloidal gold. Am J Pathol 138, 875-85.

Traiffort, E., Kassoussi, A., Zahaf, A., and Laouarem, Y. (2020). Astrocytes and Microglia as Major Players of Myelin Production in Normal and Pathological Conditions. Front Cell Neurosci 14, 79. 10.3389/fncel.2020.00079.

Traxler, L., Edenhofer, F., and Mertens, J. (2019). Next-generation disease modeling with direct conversion: a new path to old neurons. FEBS letters 593, 3316-37. 10.1002/1873-3468.13678.

Traxler, L., Herdy, J.R., Stefanoni, D., Eichhorner, S., Pelucchi, S., Szücs, A., Santagostino, A., Kim, Y., Agarwal, R.K., Schlachetzki, J.C.M., et al. (2022). Warburg-like metabolic transformation underlies neuronal degeneration in sporadic Alzheimer's disease. Cell Metabolism 34, 1248-63.e6. https://doi.org/10.1016/j.cmet.2022.07.014.

Trovato, F., Romana Stefani, F., Li, J., G. Zetterdahl, O., Canals, I., Ahlenius, H. and Bengzon, J. Transcription Factor Forced Astrocytic Differentiation Impairs Human Glioblastoma Growth In Vitro and In Vivo. In press.

Trudler, D., Ghatak, S., and Lipton, S.A. (2021). Emerging hiPSC Models for Drug Discovery in Neurodegenerative Diseases. Int J Mol Sci 22. 10.3390/ijms22158196.

Valori, C.F., Guidotti, G., Brambilla, L., and Rossi, D. (2019). Astrocytes: Emerging Therapeutic Targets in Neurological Disorders. Trends Mol Med 25, 750-9. 10.1016/j.molmed.2019.04.010.

van der Knaap, M.S., Barth, P.G., Stroink, H., van Nieuwenhuizen, O., Arts, W.F., Hoogenraad, F., and Valk, J. (1995a). Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. Ann Neurol 37, 324-34. 10.1002/ana.410370308.

van der Knaap, M.S., Barth, P.G., Vrensen, G.F., and Valk, J. (1996). Histopathology of an infantile-onset spongiform leukoencephalopathy with a discrepantly mild clinical course. Acta Neuropathol 92, 206-12. 10.1007/s004010050510.

van der Knaap, M.S., Boor, I., and Estévez, R. (2012). Megalencephalic leukoencephalopathy with subcortical cysts: chronic white matter oedema due to a defect in brain ion and water homoeostasis. The Lancet Neurology 11, 973-85. 10.1016/S1474-4422(12)70192-8.

van der Knaap, M.S., and Bugiani, M. (2017). Leukodystrophies: a proposed classification system based on pathological changes and pathogenetic mechanisms. Acta Neuropathol 134, 351-82. 10.1007/s00401-017-1739-1.

van der Knaap, M.S., Lai, V., Kohler, W., Salih, M.A., Fonseca, M.J., Benke, T.A., Wilson, C., Jayakar, P., Aine, M.R., Dom, L., et al. (2010). Megalencephalic leukoencephalopathy with cysts without MLC1 defect. Ann Neurol 67, 834-7. 10.1002/ana.21980.

van der Knaap, M.S., Pronk, J.C., and Scheper, G.C. (2006). Vanishing white matter disease. The Lancet Neurology 5, 413-23. https://doi.org/10.1016/S1474-4422(06)70440-9.

van der Knaap, M.S., Schiffmann, R., Mochel, F., and Wolf, N.I. (2019). Diagnosis, prognosis, and treatment of leukodystrophies. Lancet Neurol 18, 962-72. 10.1016/s1474-4422(19)30143-7.

van der Knaap, M.S., Valk, J., Barth, P.G., Smit, L.M., van Engelen, B.G., and Tortori Donati, P. (1995b). Leukoencephalopathy with swelling in children and adolescents: MRI patterns and differential diagnosis. Neuroradiology 37, 679-86. 10.1007/bf00593394.

Vanderver, A., Hussey, H., Schmidt, J.L., Pastor, W., and Hoffman, H.J. (2012). Relative incidence of inherited white matter disorders in childhood to acquired pediatric demyelinating disorders. Semin Pediatr Neurol 19, 219-23. 10.1016/j.spen.2012.10.001.

Verkhratsky, A., and Nedergaard, M. (2018). Physiology of Astroglia. Physiol Rev 98, 239-389. 10.1152/physrev.00042.2016.

Viedma-Poyatos, Á., González-Jiménez, P., Pajares, M.A., and 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.

Whiteley, J.T., Fernandes, S., Sharma, A., Mendes, A.P.D., Racha, V., Benassi, S.K., and Marchetto, M.C. (2022). Reaching into the toolbox: Stem cell models to study neuropsychiatric disorders. Stem Cell Reports 17, 187-210. 10.1016/j.stemcr.2021.12.015.

Yang, N., Chanda, S., Marro, S., Ng, Y.H., Janas, J.A., Haag, D., Ang, C.E., Tang, Y., Flores, Q., Mall, M., et al. (2017). Generation of pure GABAergic neurons by transcription factor programming. Nat Methods 14, 621-8. 10.1038/nmeth.4291.

Yang, N., Zuchero, J.B., Ahlenius, H., Marro, S., Ng, Y.H., Vierbuchen, T., Hawkins, J.S., Geissler, R., Barres, B.A., and Wernig, M. (2013). Generation of oligodendroglial cells by direct lineage conversion. Nat Biotechnol 31, 434-9. 10.1038/nbt.2564.

Yeon, G.B., Shin, W.H., Yoo, S.H., Kim, D., Jeon, B.M., Park, W.U., Bae, Y., Park, J.Y., You, S., Na, D., et al. (2021). NFIB induces functional astrocytes from human pluripotent stem cell-derived neural precursor cells mimicking in vivo astrogliogenesis. J Cell Physiol 236, 7625-41. 10.1002/jcp.30405.

Yoshida, T., Sasaki, M., Yoshida, M., Namekawa, M., Okamoto, Y., Tsujino, S., Sasayama, H., Mizuta, I., Nakagawa, M., and The Alexander Disease Study Group in, J. (2011). Nationwide survey of Alexander disease in Japan and proposed new guidelines for diagnosis. Journal of Neurology 258, 1998-2008. 10.1007/s00415-011-6056-3.

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., et al. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. Neuron 78, 785-98. 10.1016/j.neuron.2013.05.029.

Zhang, Y., Sloan, S.A., Clarke, L.E., Caneda, C., Plaza, C.A., Blumenthal, P.D., Vogel, H., Steinberg, G.K., Edwards, M.S., Li, G., et al. (2016). Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. Neuron 89, 37-53. 10.1016/j.neuron.2015.11.013.