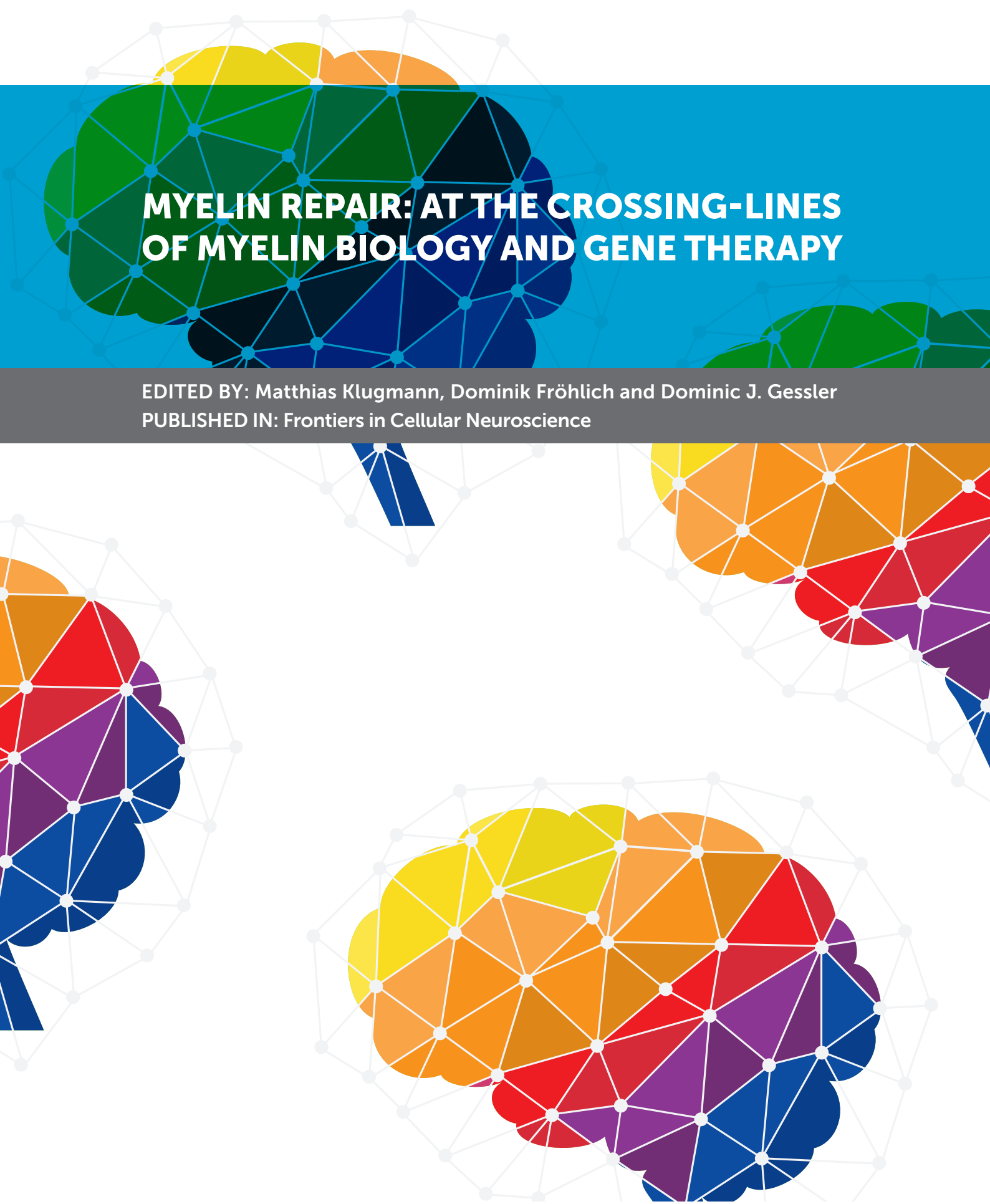
MYELIN REPAIR: AT THE CROSSING-LINES OF MYELIN BIOLOGY AND GENE THERAPY

EDITED BY: Matthias Klugmann, Dominik Fröhlich and Dominic J. Gessler
PUBLISHED IN: Frontiers in Cellular Neuroscience



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-618-7
DOI 10.3389/978-2-88974-618-7

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

MYELIN REPAIR: AT THE CROSSING-LINES OF MYELIN BIOLOGY AND GENE THERAPY

Topic Editors:
Matthias Klugmann, University of New South Wales, Australia
Dominik Fröhlich, University of New South Wales, Australia
Dominic J. Gessler, University of Massachusetts Medical School, United States

Citation: Klugmann, M., Fröhlich, D., Gessler, D. J., eds. (2022). Myelin Repair: At the Crossing-Lines of Myelin Biology and Gene Therapy.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-618-7

Table of Contents

frontiers in Cellular Neuroscience

EDITORIAL
published: 11 February 2022
doi: 10.3389/fncel.2022.853742

Editorial: Myelin Repair: At the Crossing-Lines of Myelin Biology and Gene Therapy

Dominik Fröhlich[1]*, *Dominic J. Gessler*[2,3,4] and *Matthias Klugmann*[1]

[1] Translational Neuroscience Facility, School of Medical Sciences, UNSW Sydney, Kensington, NSW, Australia, [2] Horae Gene Therapy Center, University of Massachusetts Chan Medical School, Worcester, MA, United States, [3] Department of Microbiology and Physiological Systems, University of Massachusetts Chan Medical School, Worcester, MA, United States, [4] Department of Neurosurgery, University of Minnesota, Minneapolis, MN, United States

Keywords: myelin, gene therapy, leukodystrophy, white matter, oligodendrocytes

Editorial on the Research Topic

Myelin Repair: At the Crossing-Lines of Myelin Biology and Gene Therapy

Leukodystrophies are genetic diseases characterized by impaired formation or maintenance of the brain's white matter (myelin). Although considered rare, over 50 different Leukodystrophies are known, amounting to a total population incidence of one in 7,600 births (Bonkowsky et al., 2010).

Premature demise is commonly the consequence of neurodevelopmental deficits caused by poor myelin formation. The high mortality, lack of treatment options, and monogenic nature of many white matter disorders make them particularly amenable to gene therapy.

In fact, gene therapy employing an *ex vivo* approach has shown remarkable clinical success for X-linked adrenoleukodystrophy (X-ALD) and metachromatic leukodystrophy (MLD) (Biffi et al., 2011). Intuitively, mutations of genes encoding proteins essential for oligodendrocyte functions underly many white matter disorders. However, *in vivo* gene therapy directed at oligodendrocytes has been notoriously difficult amid the lack of efficient gene delivery systems targeting glia.

Myelin dysfunction can also be secondary to cellular or metabolic pathologies of astroglia or neurons reflecting abnormal glia-neuronal interactions. To improve our understanding of the complex pathophysiologies and disease etiologies, leukodystrophy models are needed that foster the development of therapies and the design of clinical trials.

This Research Topic compiles original research and review articles from leading scientists in myelin biology and gene therapy, ranging from generating novel genetic cell and animal models to developing experimental gene therapy strategies for white matter disorders, for which some are currently in clinical trial.

In an original research article, Shaker et al. describe a fast and efficient new protocol to grow human brain organoids comprising myelinating oligodendrocytes, cortical neurons, and astrocytes in 42 days. These myelinated brain organoids are a valuable tool to study CNS disorders associated with myelin defects and will be instrumental for drug discovery and developing novel therapeutics.

The work by Fröhlich et al. characterizes humanized rodent models for the autosomal recessive disorder Hypomyelination with Brain stem and Spinal cord involvement and Leg spasticity (HBSL), which was first described in 2013 (Taft et al., 2013). Using CRISPR/Cas9, the authors introduced the HBSL-causing $Dars1^{D367Y}$ point mutation into the mouse genome. Homozygous $Dars1^{D367Y}$ mice were phenotypically normal, which was overcome by generating compound heterozygous $Dars1^{D367Y/null}$ mice. These mice showed neurological signs similar to HBSL patients with the corresponding missense mutation, enabling future therapeutic proof-of-concept studies.

OPEN ACCESS

Edited and reviewed by:
Dirk M. Hermann, University of Duisburg-Essen, Germany

***Correspondence:**
Dominik Fröhlich
d.frohlich@unsw.edu.au

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *13 January 2022*
Accepted: *19 January 2022*
Published: *11 February 2022*

Citation:
Fröhlich D, Gessler DJ and Klugmann M (2022) Editorial: Myelin Repair: At the Crossing-Lines of Myelin Biology and Gene Therapy. Front. Cell. Neurosci. 16:853742. doi: 10.3389/fncel.2022.853742

In addition, this Research Topic provides updates on the latest pathophysiology and therapy development for traumatic CNS injury, megalencephalic leukoencephalopathy, POLR3-related leukodystrophy, HBSL, LBSL, GM1 and GM2 gangliosidosis, and Canavan disease.

Traumatic brain and spinal cord injuries often result in demyelination and the failure to remyelinate. Huntemer-Silveira et al. review the effects of traumatic CNS injury on oligodendrocytes and the consequences of disrupted endogenous remyelination mechanisms. The authors highlight recent rodent and clinical studies aimed at enhancing remyelination through therapies involving small molecules, RNA interference, monoclonal antibodies, and cell replacement strategies.

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a vacuolating leukodystrophy characterized by megalencephaly, loss of motor function, epilepsy, mild mental decline, and no available treatment options. Bosch and Estevez provide a detailed overview of pathophysiology, established animal models, and potential therapeutic strategies, emphasizing preclinical adeno-associated virus (AAV)-MLC1 gene therapy in Mlc1 knockout mice from the same lab.

POLR3-related leukodystrophy is one of the most common types of hypomyelinating leukodystrophy, also known as 4H leukodystrophy, manifesting with hypomyelination, hypodontia, and hypogonadotropic hypogonadism. Perrier et al. explore in their review article the use of stem cell transplantation, gene replacement therapy, and gene editing as avenues for future treatment options.

The leukodystrophies Leukoencephalopathy with Brainstem and Spinal cord involvement and Lactate elevation (LBSL) and HBSL are both spectrum disorders characterized by a similar clinical presentation and the lack of curative treatment options. LBSL and HBSL are caused by mitochondrial and cytoplasmic aspartyl-tRNA synthetase mutations, respectively. Muthiah et al. highlight similarities and differences between the two leukodystrophies and summarize current knowledge of preclinical and clinical features, including neuroimaging, diagnosis, disease mechanisms, mouse models, and treatment options.

GM1 and GM2 gangliosidosis are devastating neurodegenerative lysosomal storage disorders with white matter manifestation. The development of gene therapies for GM1 and GM2 gangliosidosis has seen promising progress in recent preclinical studies. These and other findings are outlined in the review article by Maguire and Martin, which critically discusses current viewpoints on the origin of white matter deficits in gangliosidoses and potential obstacles for effective gene therapies.

An essential prerequisite for successfully treating leukodystrophies is to enable myelin repair in a time-dependent fashion to restore CNS homeostasis. Using Canavan disease as a model system, Lotun et al. review the role of N-acetylaspartate (NAA), one of the most abundant metabolites in the mammalian CNS, in the normal and diseased brain and discuss the involvement of astrocytes in both Canavan disease and other leukodystrophies.

To conclude this Research Topic, von Jonquieres et al. provide a comprehensive overview of emerging gene therapy concepts, specifically targeting glial cells. The authors review the latest advances in genetic and cellular treatment strategies for leukodystrophies, including *ex vivo* stem cell gene therapy and the use of AAV *in vivo*.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Biffi, A., Aubourg, P., and Cartier, N. (2011). Gene therapy for leukodystrophies. *Hum. Mol. Genet.* 20, R42–R53. doi: 10.1093/hmg/ddr142

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, 718–725. doi: 10.1212/WNL.0b013e3181eee46b

Taft, R. J., Vanderver, A., Leventer, R. J., Damiani, S. A., Simons, C., Grimmond, S. M., et al. (2013). Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am. J. Hum. Genet.* 92, 774–780. doi: 10.1016/j.ajhg.2013.04.006

Conflict of Interest: MK is affiliated with Research Beyond Borders, Boehringer-Ingelheim GmbH and Co KG. DG is affiliated with ASPA Therapeutics, Inc.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 11 January 2021
doi: 10.3389/fncel.2020.619707

Strategies for Oligodendrocyte and Myelin Repair in Traumatic CNS Injury

Anne Huntemer-Silveira[1], *Nandadevi Patil*[2], *Megan A. Brickner*[1] *and Ann M. Parr*[2]*

[1]Department of Neuroscience, University of Minnesota, Minneapolis, MN, United States, [2]Department of Neurosurgery, Stem Cell Institute, University of Minnesota, Minneapolis, MN, United States

A major consequence of traumatic brain and spinal cord injury is the loss of the myelin sheath, a cholesterol-rich layer of insulation that wraps around axons of the nervous system. In the central nervous system (CNS), myelin is produced and maintained by oligodendrocytes. Damage to the CNS may result in oligodendrocyte cell death and subsequent loss of myelin, which can have serious consequences for functional recovery. Demyelination impairs neuronal function by decelerating signal transmission along the axon and has been implicated in many neurodegenerative diseases. After a traumatic injury, mechanisms of endogenous remyelination in the CNS are limited and often fail, for reasons that remain poorly understood. One area of research focuses on enhancing this endogenous response. Existing techniques include the use of small molecules, RNA interference (RNAi), and monoclonal antibodies that target specific signaling components of myelination for recovery. Cell-based replacement strategies geared towards replenishing oligodendrocytes and their progenitors have been utilized by several groups in the last decade as well. In this review article, we discuss the effects of traumatic injury on oligodendrocytes in the CNS, the lack of endogenous remyelination, translational studies in rodent models promoting remyelination, and finally human clinical studies on remyelination in the CNS after injury.

Keywords: oligodendrocyte, myelin, remyelination, spinal cord injury, traumatic injury

OPEN ACCESS

Edited by:
Matthias Klugmann, University of New South Wales, Australia

Reviewed by:
Gregory W. J. Hawryluk, University of Manitoba, Canada
Jelena Skuljec, Essen University Hospital, Germany

***Correspondence:**
Ann M. Parr
amparr@umn.edu

***Specialty section**:*
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *20 October 2020*
Accepted: *07 December 2020*
Published: *11 January 2021*

Citation:
Huntemer-Silveira A, Patil N, Brickner MA and Parr AM (2021) Strategies for Oligodendrocyte and Myelin Repair in Traumatic CNS Injury. Front. Cell. Neurosci. 14:619707. doi: 10.3389/fncel.2020.619707

INTRODUCTION

Oligodendrocytes are the myelin-producing glial cells of the central nervous system (CNS). Myelin plays a critical role in neuronal communication by insulating the axon, enhancing the propagation of action potentials, and facilitating high-frequency firing (Nashmi and Fehlings, 2001; Hartline and Colman, 2007). Besides producing myelin, there is also evidence that oligodendrocytes provide the neurons they ensheath with important trophic support (Garbern et al., 2002; Fünfschilling et al., 2012; Nave and Werner, 2014; Duncan et al., 2020). Disruption of oligodendrocytes or the myelin sheath in injury and disease has severe consequences on neuronal function. Myelin loss decreases cell capacitance and exposes voltage-gated potassium channels, slowing signal transmission and decreasing the likelihood of action potential generation (Nashmi and Fehlings, 2001; Monje, 2018). Additionally, long term effects of oligodendrocyte death beyond demyelination include axonal atrophy and neuronal loss, giving rise

to a vicious cycle with axon damage further impacting oligodendrocyte function (Crowe et al., 1997; Nave, 2010). This may result in widespread motor and cognitive deficits and has been identified in several diseases.

Demyelination in the CNS may occur in response to autoimmune disease, genetic mutation, or trauma such as injury or stroke. Multiple sclerosis (MS) is one of the most well studied demyelinating diseases and is characterized by periods of neuroinflammation leading to degradation of myelin in both the gray and white matter of the CNS, with progressive neuronal loss and cognitive impairment in chronic stages (Nave, 2010; Inglese and Petracca, 2015). Leukodystrophies are a family of genetic diseases affecting white matter, a subset of which primarily affects myelin and oligodendrocytes (van der Knaap and Bugiani, 2017). Mutations have been linked to reduced oligodendrocyte differentiation and myelin formation, clinically manifesting in developmental delay, motor dysfunction, seizures, and more (Nave, 2010). Interestingly, substantial research in recent years has implicated myelin abnormality in several psychiatric disorders, highlighting its importance in neuronal transmission from sensorimotor to cognitive tasks (Fields, 2008; Inglese and Petracca, 2015).

Traumatic injury is another major cause of demyelination in the CNS and is the focus of this review article, with an emphasis on brain and spinal cord injuries (TBI and SCI, respectively). Following CNS trauma, damaged axons and oligodendrocytes may trigger demyelination that spreads well beyond the initial injury site, causing impairments in sensory, motor, cognitive, and autonomic function (Alizadeh et al., 2019; Fischer et al., 2020). The Centers for Disease Control and Prevention classify TBI as a serious public health concern, with upwards of 2 million cases per year in the United States alone (Centers for Disease Control and Prevention, 2019). Similarly, there are roughly 20,000 reported cases of SCI annually, with more than 50% resulting in paraplegia or tetraplegia (National Spinal Cord Injury Statistical Center, 2020). The leading causes of both TBI and SCI are preventable accidents such as vehicular collisions and falls (Centers for Disease Control and Prevention, 2019; National Spinal Cord Injury Statistical Center, 2020).

The incidence of demyelination in a variety of disease states makes it an applicable therapeutic target with widespread potential. However, this has also been a subject of controversy based on evidence that complete endogenous remyelination will occur with enough time (Duncan et al., 2020). Definitive reports of chronic demyelination are difficult to ascertain given the complex nature of human injuries and the relatively short time scale of animal models. Endogenous remyelination does occur in both the peripheral and central nervous systems, and in the CNS this mechanism is comparatively less efficient (Franklin and ffrench-Constant, 2008). While time may indeed be a key factor in allowing full remyelination, the consequences of acute demyelination as described above can ultimately lead to cell death before the system can regenerate. Additional processes occurring in tandem during injury and disease may also impair the endogenous response and prevent recovery. Thus, boosting the capacity for remyelination may serve as a way to minimize the long-term damage that follows demyelinating injuries and diseases. In this review article, we will discuss the mechanisms of demyelination and remyelination, with an emphasis on TBI/SCI, followed by an overview of current strategies targeting oligodendrocytes to promote remyelination in different animal models.

DEMYELINATION FOLLOWING TRAUMATIC INJURY

CNS trauma consists of both a primary and secondary injury, with distinct pathophysiology for each (**Figure 1**). The primary injury involves the direct mechanical impact on the CNS, causing compression, laceration, shearing, etc., and the immediate cellular and vascular damage at the injury site (Johnson et al., 2013; Alizadeh et al., 2019). The primary injury initiates a cellular cascade that gives rise to the secondary injury, the effects of which can last from minutes to years (Crowe et al., 1997; Buss et al., 2004; Ramlackhansingh et al., 2011; Walker and Tesco, 2013). This is distinct from primary and secondary demyelination. Primary demyelination refers to direct injury without axonal compromise, and secondary demyelination occurs indirectly as a result of axonal injury. Both of these can occur as a result of CNS trauma.

Traumatic brain and spinal cord injuries trigger a complex series of secondary events, activating pathways for reactive gliosis, necrosis and/or apoptosis, immune response, and degeneration. Many of the underlying mechanisms for these processes in both TBI and SCI are shared (Bramlett and Dietrich, 2007). Kakulas (2019) provides a clear and detailed description of the secondary cellular events occurring through each stage of SCI in humans. The acute phase occurs immediately after the injury and involves spinal cord swelling, due to hemorrhage and edema, and early necrosis of damaged tissue. The subacute phase begins hours after injury and is characterized by continued necrosis and debris clearing by macrophages. Neuroinflammation and demyelination are also observed. The chronic phase occurs weeks after injury and may persist for years. Phagocytosis of debris leaves behind a cavity at the injury site, and the wall of the lesion contains reactive glial cells that form a glial scar. There is also persisting demyelination and Wallerian degeneration of damaged axons, which progresses both rostral and caudal to the injury. Wallerian degeneration, a hallmark of axonal pathology, involves the catastrophic fragmentation of distal axons as a result of damage and loss of somatic support (Kakulas, 1999, 2019). The same pathologies follow TBI and are described in detail by Bramlett and Dietrich (2007) and Walker and Tesco (2013).

Demyelination has been observed as a result of both primary and secondary injury after TBI/SCI. Traumatic demyelination of damaged axons occurs at the lesion site in primary injury, while demyelination following secondary processes may extend to regions far from the initial insult (Crowe et al., 1997; Kakulas, 1999; Duncan et al., 2020). Slowed impulse conduction as a result of demyelination in the spinal cord contributes to the motor dysfunction and sensory impairment seen in SCI patients (Felts et al., 1997; Nashmi and Fehlings, 2001). Furthermore, axons left without oligodendrocyte support deteriorate over time, subject to extensive Wallerian degeneration, myelin

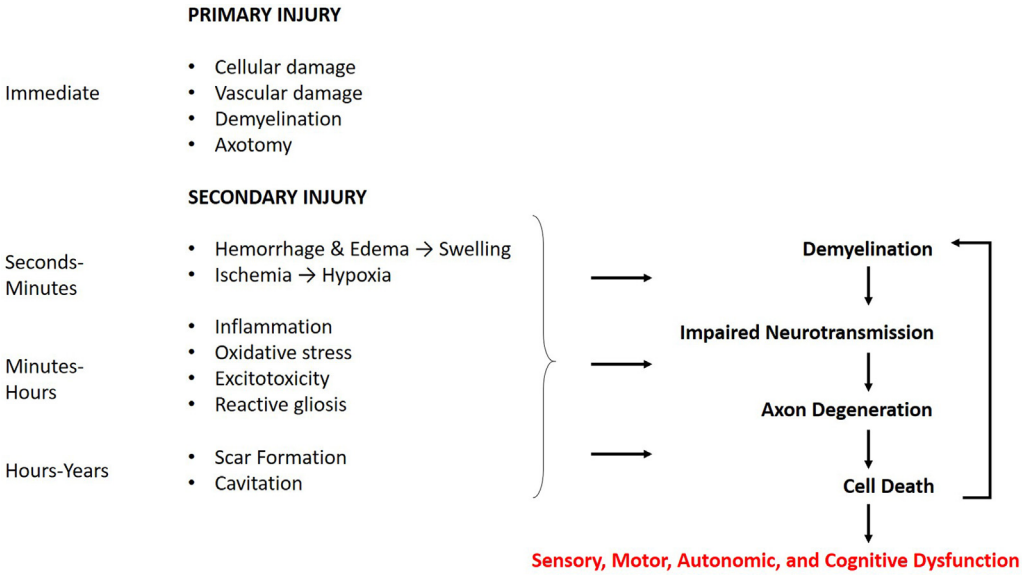


FIGURE 1 | Time course of the cellular response to central nervous system (CNS) trauma. The effects of the primary injury are immediate and directly contribute to secondary damage. The secondary injury begins within minutes of the primary injury and can last for years. The effects of the secondary injury further contribute to the demyelination, axon degeneration, and cell death caused by the primary injury. These mechanisms may manifest in sensory, motor, autonomic, and cognitive dysfunction, with the degree of severity related to that of the injury.

vacuolization, and pathology (Crowe et al., 1997; Oluich et al., 2012). In TBI, demyelination is believed to contribute to the long-term cognitive deficits reported in patients (Walker and Tesco, 2013; Armstrong et al., 2016). In the sections below, we will outline the effects of the primary and secondary injury on oligodendrocytes, the mechanisms behind demyelination and remyelination, and the limitations of endogenous remyelination following CNS trauma.

Oligodendrocytes and Injury Response

Oligodendrocytes, as generators of myelin, are key to understanding the mechanisms surrounding primary and secondary demyelination in the CNS. The primary injury may directly promote demyelination in two ways. First, the insult itself can strip the myelin from the axons and cause further damage, impairing conduction and downstream signaling. Oligodendrocytes may also be severely damaged from the injury, which can lead to demyelination of the axons they support (Pohl et al., 2011). Importantly, because oligodendrocytes myelinate multiple axons, their loss may affect axons that were spared by the initial injury, causing progressive or chronic demyelination extending beyond the lesion site (Conti et al., 1998; Emery et al., 1998; Buss et al., 2004). Myriad additional secondary processes provoke demyelination as well, however, the complex nature of the secondary injury poses an obstacle to discerning the relationship between oligodendrocytes, demyelination, and axonal degeneration.

The mechanisms of secondary injury are complex and have widespread effects. As such, some degree of compartmentalization is necessary for understanding the responses of different cell types. Previous work has uncovered several aspects of the lesion environment that increase the sensitivity and vulnerability of oligodendrocytes following injury. For example, excessive neurotransmitter release from necrotic tissue may cause glutamate and ATP mediated excitotoxicity in surrounding cells. Oligodendrocytes appear quite susceptible to this and often undergo excitotoxic cell death after injury (Xu et al., 2004; Butt, 2006; Domercq et al., 2010). Proteolytic enzymes released during necrosis may further potentiate damage. Free radical formation due to ischemia contributes to oxidative stress, to which oligodendrocytes demonstrate increased vulnerability (Thorburne and Juurlink, 1996; Giacci and Fitzgerald, 2018). The invasion of immune cells, release of inflammatory cytokines by microglia, and infiltrating neutrophils have also been associated with oligodendrocyte loss (Donnelly and Popovich, 2008; Satzer et al., 2015). These factors in turn increase the numbers of necrotic, apoptotic, and autophagic oligodendrocytes after CNS trauma (Kanno et al., 2009; Almad et al., 2011). It should be noted, however, that these responses are complex and serve both protective and harmful roles (Jones et al., 2005; Kotter et al., 2005; Ohri et al., 2018). Other cell types, such as neurons and astrocytes, also demonstrate varying degrees of sensitivity to these processes, and thus care should be taken to note the differential effects of targeting these mechanisms experimentally.

Apoptosis of oligodendrocytes has been reported in several TBI/SCI studies (Crowe et al., 1997; Conti et al., 1998; Emery et al., 1998; Lotocki et al., 2011; Flygt et al., 2013). As mentioned above, oligodendrocyte death may initiate demyelination in associated axons. However, axonal degeneration also appears to trigger oligodendrocyte apoptosis, as reported in

Crowe et al. (1997) where Wallerian degeneration preceded the emergence of adjacent apoptotic CC1/RIP positive cells. This corroborates other work demonstrating a dependence of oligodendrocytes on neuronal activity for survival and highlights the reciprocal quality of their relationship (Barres et al., 1993). A chain effect thus emerges, whereby damaged axons trigger apoptosis in oligodendrocytes, causing further demyelination of distal axons. This intricate relationship sheds light on the motivation behind utilizing oligodendrocyte replacement and myelin growth-promoting factors after CNS injury to prevent widespread deterioration. Multiple studies utilizing such approaches in acute and subacute models of SCI show promising evidence of functional recovery (Hwang et al., 2009; Erceg et al., 2010; Kawabata et al., 2016). Similar improvements in chronic SCI models have been more difficult to achieve, possibly because this progressive demyelination and degeneration have gone unchecked for much longer (Keirstead et al., 2005; Karimi-Abdolrezaee et al., 2006; Nishimura et al., 2013).

Mechanisms of Remyelination After Injury

The remyelination of axons following injury in many ways resembles the myelination of the developing CNS, with some key temporal and molecular differences (Gallo and Deneen, 2014; Almeida, 2018). Much of the attention on remyelination in the CNS has focused on oligodendrocyte progenitor cells (OPCs). Franklin and ffrench-Constant (2017) describe the three phases of OPC mediated remyelination in the CNS: activation, recruitment, and differentiation. During activation, OPCs undergo a change in gene expression allowing them to become proliferative (Moyon et al., 2015). During recruitment, OPCs originating both locally and from the subventricular zone (SVZ) migrate to the lesion site in response to injury (McTigue et al., 2001; Caillava et al., 2011; Flygt et al., 2013; Xing et al., 2014). Finally, OPCs exit the cell cycle and differentiate into myelin-producing oligodendrocytes. These processes involve a diverse array of mediators and molecules, varied by regional cellular context, that guide remyelination (Brosius-Lutz and Barres, 2014; Gallo and Deneen, 2014; Almeida, 2018). Pharmacological manipulation of identified receptors and signals is a common approach for promoting remyelination and will be discussed in more detail in later sections.

The capacity for mature oligodendrocytes to contribute to remyelination is a topic of debate. It has long been held and supported that because oligodendrocytes are post-mitotic and differentiated, they are both unable to migrate and also fail to remyelinate after damage (Blakemore and Keirstead, 1999; Crawford et al., 2016; Pukos et al., 2019). Indeed, it seems likely that OPCs provide the majority of remyelination after injury, as OPC transplantation greatly improves measures of myelination (Groves et al., 1993; Keirstead et al., 2005). However, recent reports suggest that mature, intact oligodendrocytes do engage in remyelination following injury (Yeung et al., 2014; Jeffries et al., 2016; Duncan I. D. et al., 2018; Macchi et al., 2020). The variability in findings likely relates to differences in cell lines, animal models, the type of demyelinating injury, and methods of evaluating myelination across studies. Additional work emphasizing consistency across approaches will be necessary to determine the validity of promoting oligodendrocyte vs. OPC survival as a strategy following CNS trauma.

The process of myelination itself is also quite complex. The successive wrapping of myelin layers is driven by actin dynamics and requires interactions between several adhesion molecules, actin-binding proteins, and, critically, myelin basic protein (Nawaz et al., 2015; Zuchero et al., 2015; Klingseisen et al., 2019). There is an activity-dependent component of myelination as well. Not only do OPCs preferentially activate in regions with elevated electrical activity, but oligodendrocytes themselves demonstrate a predilection to myelinate more active axons (Barres and Raff, 1993; Gibson et al., 2014; Hines et al., 2015). Though this is believed to underlie the specificity of myelination *in vivo*, the mechanisms behind it are unclear. Several growth factors and signaling molecules have been implicated thus far (Ronzano et al., 2020). For example, the trophic factor BDNF, secreted by neurons, astrocytes, oligodendrocytes, and Schwann cells, modulates myelination in an activity-dependent manner (McTigue et al., 1998; Ikeda et al., 2002; Lundgaard et al., 2013). This may have ramifications for the use of electrical stimulation as a treatment for peripheral nerve regeneration and SCI. For both of these pathologies, electrical stimulation has been shown to increase BDNF levels (McGregor and English, 2019; Ghorbani et al., 2020). Thus, BDNF-mediated remyelination may underlie some of the functional recovery seen in SCI patients receiving epidural stimulation (Pettigrew et al., 2017).

Schwann cell-mediated remyelination can also occur following SCI. Schwann cells, which are the myelinating cells of the peripheral nervous system, are known to infiltrate the spinal cord after SCI and in other demyelinating diseases (Duncan and Hoffman, 1997). Whether this process is beneficial or detrimental is also a subject of some controversy. It should be noted that, unlike oligodendrocytes, Schwann cells typically myelinate in a 1:1 ratio, making them potentially less efficient in comparison. However, Schwann cells have been used in transplantation studies to promote remyelination, and grafts of Schwann cells improve regeneration of CNS axons (Aguayo et al., 1981; Kanno et al., 2015). Furthermore, Schwann cells are being actively pursued in clinical trials for use in SCI, with phase I testing complete (Anderson et al., 2017).

Finally, it is important to note the extent to which remyelination alone contributes to functional recovery following TBI/SCI is not clearly discerned (Myers et al., 2016; Duncan G. J. et al., 2018). This lack of clarity arises because oligodendrocytes provide other forms of support to surrounding neurons, such as secretion of growth factors and immune modulators (Du and Dreyfus, 2002; Assinck et al., 2017). Oligodendrocytes may then be promoting recovery through other mechanisms of action besides or in addition to remyelination. This has been proposed as an alternate explanation for why recovery of function is seen in acute and subacute SCI but not chronic models. Furthermore, the early recovery pattern observed in some of these studies suggests that at least some functional recovery occurs before remyelination (Duncan G. J. et al., 2018). This is not to suggest that

remyelination is an inadequate target for repair strategies; rather it emphasizes the need for combined approaches that can act on multiple aspects of the repair process to achieve the best outcomes.

Barriers to Endogenous Remyelination

Endogenous remyelination in the CNS is limited and often fails to provide functional recovery (Franklin and ffrench-Constant, 2008; Boyd et al., 2013; Franklin and Goldman, 2015). The reasons for this are intricate and many components are poorly understood. **Figure 2** depicts the possible outcomes following demyelination. Evidence suggests that when the axon is preserved remyelination occurs naturally and may restore both signal conduction and sensorimotor function (Smith et al., 1979; Duncan et al., 2009). However, the newly formed myelin is thinner and conduction amplitude is lower than in normally myelinated axons, implying an intrinsic limitation in the capacity for remyelination (Smith et al., 1979; Nashmi and Fehlings, 2001; Franklin and ffrench-Constant, 2017). Furthermore, the Wallerian degeneration of damaged axons poses a major obstacle to remyelination. This loss of axons and the limited capacity of the CNS to regenerate hinders prospects for remyelination. Indeed, one study utilizing a demyelinating model of MS found that remyelination alone was not sufficient for sustained functional recovery when axonal degeneration persisted (Manrique-Hoyos et al., 2012). As such, therapies focused on remyelination must also consider pairing with strategies to boost axon growth.

As discussed previously, adult precursor cells occupying the CNS respond to demyelinating injuries. However, compared to other species, the contribution of these cells to mammalian regeneration is attenuated. There are three well-established barriers to axon regeneration in the CNS: the glial scar, myelin inhibitory molecules, and lack of trophic support. Though evidence exists in support of all of these factors, the extent to which each contributes overall to regeneration failure remains to be determined. For this review article, they will be discussed briefly.

The glial scar has been a subject of controversy for years, with conflicting claims demonstrating both protective and

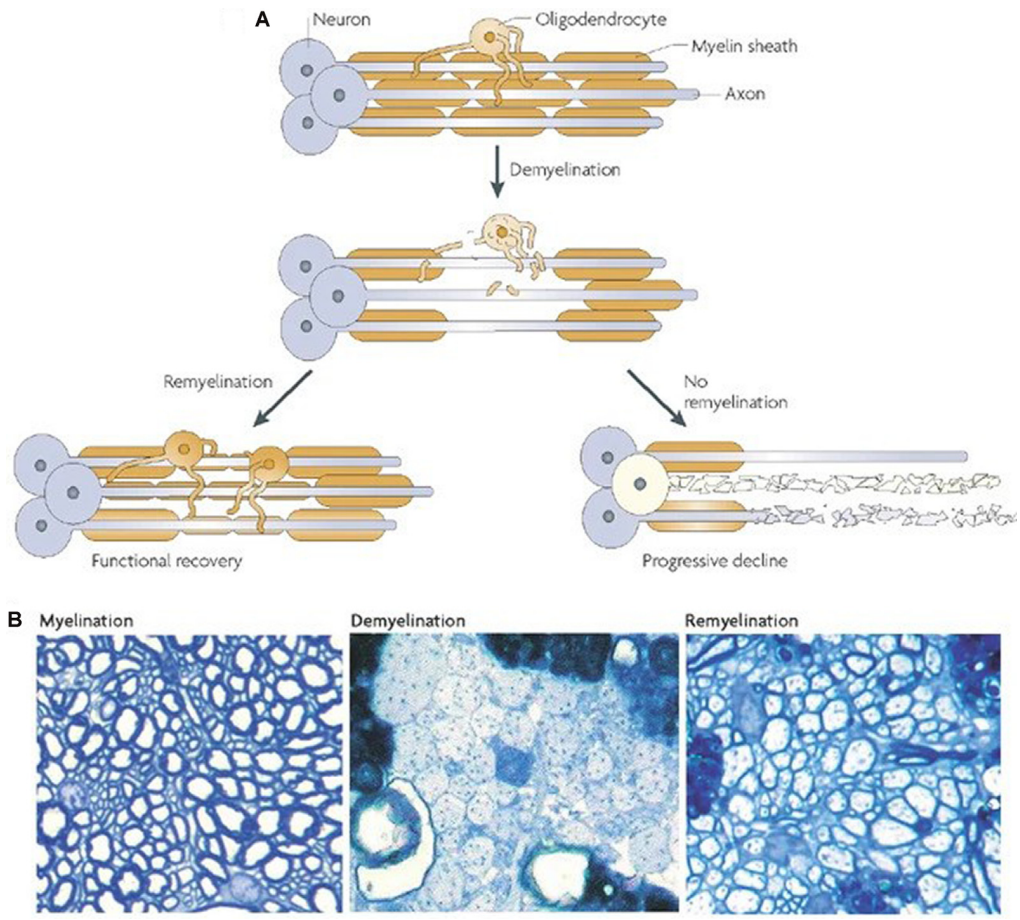


FIGURE 2 | Outcomes of demyelination. **(A)** A demyelinated axon will either be remyelinated, giving at least partial restoration of function, or it will undergo Wallerian degeneration. **(B)** Transverse sections from adult rat cerebellar white matter, fixed in resin and imaged with light microscopy. The left panel depicts normally myelinated axons. The middle panel shows demyelinated axons following injection with ethidium bromide. The right panel shows remyelinated axons 4 weeks after ethidium bromide injection. It should be noted that the new myelin is thinner and shorter (**A,B** from Franklin and ffrench-Constant, 2008).

detrimental effects to recovery following injury. The scar is the result of reactive gliosis extending into the chronic phase of trauma, leaving behind a dense layer of astrocytes surrounding the lesion cavity. First, this acts as a thick physical barrier, preventing axons that may otherwise regenerate from crossing the cavity. The extracellular matrix of the glial scar is also rich in chondroitin sulfate proteoglycans (CSPGs), some subtypes of which are inhibitory to axon growth (Mckeon et al., 1995; Barritt et al., 2006; Silver, 2016). On the other hand, CSPGs that stimulate axon growth and survival have also been identified in the glial scar, lending to arguments of its multifaceted function (Nichol et al., 1994; Nakanishi et al., 2006; Schäfer and Tegeder, 2018). This is further complicated by studies showing little to no effect of glial scar ablation on regeneration (Anderson et al., 2016; Patil et al., 2018; Haindl et al., 2019). Together, the current literature suggests that the glial scar initially serves to prevent healthy axons from entering a region of inflammation and necrosis. Once the debris from the injury has been cleared, however, its presence may impede regeneration and recovery (Rolls et al., 2009). It remains uncertain whether the glial scar affects oligodendrocyte migration and remyelination as well.

A major breakthrough in the study of axon regeneration came from the discovery of molecules within the myelin itself that inhibit axon growth (Caroni and Schwab, 1988). Many such molecules have now been identified. Among the most characterized are Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp; McKerracher et al., 1994; Kottis et al., 2002; Huebner and Strittmatter, 2009). These three molecules converge on the shared Nogo receptor (NgR1), which interacts with the transmembrane protein LINGO-1 to limit axon growth (Mi et al., 2004). While these molecules are enriched in CNS myelin, they are largely absent from PNS myelin. This is believed to contribute to the differences in the regeneration capacity of CNS and PNS axons (Huebner and Strittmatter, 2009). Further research is needed in this regard, as studies utilizing animal knockouts for these molecules show mixed results (Bartsch et al., 1995; Wong et al., 2003; Lee et al., 2010). This will be critical for the development of effective combination therapies, as remyelination strategies may be more beneficial if applied after treatments for neuronal regeneration. The optimal temporal relationship between regeneration and remyelination requires further elucidation.

CNS axons also receive diminished trophic support after injury compared to the peripheral nervous system. Following injury, Schwann cells in the PNS upregulate numerous neurotrophins and transcription factors, and impairing this process significantly increases cell death (Arthur-Farraj et al., 2012; Brosius-Lutz and Barres, 2014). Also, Schwann cells provide structural support to healthy, uninjured axons through the secretion of the basal lamina, which is prevalent throughout the PNS. The presence of the basal lamina is critical following peripheral nerve injury, as it provides a laminin-rich substrate for axon regrowth (Chen et al., 2007). Oligodendrocytes, on the other hand, do not supply equivalent aid in the CNS, and this is believed to account for some differences in regeneration between the CNS and PNS. Several therapeutic strategies have harnessed the use of neurotrophins and Schwann cell transplantation in models of CNS trauma to enhance regeneration (Aguayo et al., 1981; Kelamangalath and Smith, 2013; Kanno et al., 2015).

For both remyelination and axon regeneration, age is an unavoidable risk factor that diminishes the body's endogenous response (Sim et al., 2002; Franklin and ffrench-Constant, 2008; Hampton et al., 2012). This intrinsic decrease in capacity over time poses a major hurdle for clinical treatment, as falls in the elderly make up a substantial population of CNS trauma cases (Centers for Disease Control and Prevention, 2019).

While these barriers to remyelination and regeneration have been a source of consternation for scientists and clinicians alike, technological advances in the last decade have greatly improved prospects. One preprint from the Blackmore lab utilizing cell transplantation paired with pro-regenerative gene delivery and growth permissive grafting showed enhanced axon regeneration and functional recovery in a murine SCI model (Jayaprakash et al., 2019). Such combination therapies hold considerable promise in the treatment of TBI/SCI.

TRANSLATIONAL APPROACHES IN ANIMAL MODELS

Remyelinating therapies help restore metabolic support to degenerating axons and facilitate the restoration of proper conduction and function. Most available treatments target the prevention of progressive demyelination that contributes to chronic disability, but few can promote myelin repair. This section will discuss the translational methodologies utilized in animal models that have made progress towards clinical application.

Several species and models that have contributed to the study of demyelination in injury and disease will be highlighted below. Available models for traumatic injury range in type and severity, lending important insights into the capacity of the CNS for recovery based on the degree of damage. The most common TBI models include fluid percussion, weight drop, and cortical contusion injuries, which may be focal or diffuse (Albert-Weissenberger and Sirén, 2010). Similarly, SCI models range from mild compression or contusion to complete transection (Kjell and Olson, 2016). Rodent models are most commonly used for TBI/SCI, though large animal models are also utilized and are critical for moving toward clinical application. In addition to models of demyelinating diseases have not only contributed to the current understanding of demyelination in general but have also greatly informed the field of CNS trauma. The *shiverer* mouse model, possessing a mutation in the gene for myelin basic protein, is among the most common of these and is often used to study leukodystrophies (Nave, 2010). The Long Evans shaker rat model also has a mutation in the myelin basic protein gene, resulting in widespread loss of myelin (Kwiecien et al., 1998). Other demyelination models rely on the injection of toxins, such as ethidium bromide or lysolecithin, followed by irradiation (Kulbatski et al., 2008). Findings from each of

these models will be included in the sections that follow, and for simplicity will be referred to as either "TBI/SCI models" or "demyelination models."

Small Molecules

Remyelination is governed by several extrinsic and intrinsic factors that act either as inhibitors or stimulators of OPC differentiation (Kremer et al., 2016; Lopez Juarez et al., 2016; Gruchot et al., 2019). As discussed above, following demyelinating damage, endogenous OPCs from the SVZ migrate to the demyelinated area and differentiate into mature myelinating oligodendrocytes to aid in remyelination. This has been shown in several animal models of demyelination (Nait-Oumesmar et al., 2007; Kim and Szele, 2008; Mecha et al., 2013). However, remyelination mediated by this process is inefficient and ultimately incomplete in several disease states, such as MS, due in part to inadequate OPC differentiation. Small molecules can be used to enhance this endogenous process. Göttle et al. (2019) classified the small molecules that aid in remyelination into three major groups: (1) receptor/membrane-bound molecules; (2) physiologically occurring free molecules; (3) non-physiologically occurring free molecules. Here we will discuss the use of these small molecules as a therapeutic strategy to advance remyelination in pathologically demyelinated axons in the CNS.

Many receptor systems activate pathways that control myelination. G-protein coupled receptors (GPCRs) are among the most ubiquitous in this regard and thus offer a promising therapeutic target. Two specific GPCRs identified for their role in myelination are the muscarinic receptor 1 (M1R) and the kappa opioid receptor (KOR). Researchers have employed various strategies to develop small molecules that can target these pathways. One approach is to repurpose the US Food and Drug Administration (FDA) approved drugs such as benzatropine (anticholinergic), clemastine (antihistamine), and miconazole (antifungal). These show potential in enhancing oligodendrocyte formation *in vitro* and promoting functional remyelination in animal models of MS (Deshmukh et al., 2013; Najm et al., 2015; Hubler et al., 2018). Similarly, Mei et al. (2014, 2016a) identified a cluster of antimuscarinic molecules, which includes eight FDA-approved compounds, that enhance oligodendrocyte differentiation and remyelination. This group also identified a cluster of KOR agonists that significantly promotes oligodendrocyte differentiation and myelination in mice (Mei et al., 2016b). Furthermore, clemastine, a small molecule that stimulates significant OPC differentiation *in vitro* through the M1 muscarinic receptor, provides drug-induced repair and remyelination in multiple animal models (Li et al., 2015; Liu et al., 2016; Mei et al., 2016a; Zada et al., 2016). Clemastine is now available as an over-the-counter antihistamine that is being developed as a potential treatment for relapsing-remitting MS and has advanced into clinical trials (Green et al., 2017). Another family of GPCRs that has received increasing attention is the GPR17 receptors, which act as sensors of local damage to the myelin sheath. Modification of GPR17 activity promotes oligodendroglial maturation *in vitro* and in animal models (Merten et al., 2018; Dziedzic et al., 2020; Parravicini et al., 2020). Finally, the protective effects of pregnancy on MS relapse have led to the extensive focus on the estrogen receptor (ER) and its ligands as a means for promoting remyelination (Xiao et al., 2012; Moore et al., 2014; Najm et al., 2015; Itoh et al., 2017). Of particular note is the small molecule NDC1308, an estrogen receptor agonist that has been proposed as a remyelination therapy for several demyelinating and neurodegenerative diseases (Nye and Yarger, 2017). Whether these treatments can be applied to CNS trauma has yet to be tested.

The targeting of endogenous small molecules to promote remyelination specifically in CNS trauma has yielded some promising results. For instance, the RhoA family of GTPases are key regulators of neurite outgrowth, axon regeneration, and OPC migration. Acting on this pathway, the Vav family of GEFs poses a potential therapeutic target to improve and speed myelin repair (Ulc et al., 2017). Rac and Cdc-42 can also be used to promote OPC development, as they act in opposition to RhoA (Liang et al., 2004). Small molecules inhibiting Notch and Nogo-A signaling promote oligodendrocyte and myelin formation (Huebner and Strittmatter, 2009; Franklin and ffrench-Constant, 2017; Göttle et al., 2019). Also, Hubler et al. (2018) describe several pro-myelinating small molecules that function by directly inhibiting enzymes in the cholesterol biosynthesis pathway. The subsequent accumulation of the substrates of these enzymes promotes oligodendrocyte formation. These findings demonstrate the utility of enhancing the formation of oligodendrocytes as a target for the development of optimal remyelinating therapeutics.

In both traumatic brain and spinal cord injury, small molecules have been used to enhance the proliferation of endogenous stem cells. Multipotent stem cells from the SVZ, adjacent to the ventricles of the brain and central canal of the spinal cord, contribute to the formation of new oligodendrocytes following injury (Morshead and van der Kooy, 1990). Although early reports in amphibians and urodeles suggested these cells contribute to robust regeneration, in mammals this response is attenuated and does not produce significant recovery (Zhao et al., 2016). The use of growth factors such as FGF and EGF has been shown to stimulate this proliferative response and possibly improve physiological recovery in rodent models of SCI (Kojima and Tator, 2002; Parr and Tator, 2007).

A key difficulty in utilizing small molecules is determining the time point at which administration will be most beneficial. While many endogenous molecules have been implicated in remyelination, each pathway acts on different components of the process and thus requires specific temporal specification. Proper application of small molecule treatment following these temporal features ensures efficacy and will be especially important when applied in conjunction with other therapies.

Small molecules currently in ongoing clinical trials include Quetiapine, a nonselective GPCR antagonist, GSK239512, a histamine H3 receptor antagonist, Domperidone, a D2/D3 dopamine receptor antagonist, and Olesoxime, a cholesterol-like compound (Bothwell, 2017). In early 2020 Sanofi conducted a phase 2b study evaluating a BTK inhibitor, SAR442168, which reduced disease activity associated with

MS (Sanofi: Press Releases, 2020). Genentech also initiated a Phase III clinical trial for fenebrutinib, another BTK inhibitor found to be effective against multiple types of MS (Genentech: Press Releases, 2020).

Monoclonal Antibodies

Monoclonal antibodies (MAbs) refer to antibodies produced *in vitro* by a single clone of identical cells. Originally MAbs were used in research to study the mechanisms of antibody specificity and to generate reagents for pathogens (Lu et al., 2020). MAbs are now also utilized as therapeutic strategies for many disease conditions (Wang, 2011). Advancements have been particularly potent for cancer and autoimmune disorder therapies as well as the containment of infectious diseases (Walker et al., 2009; Lu et al., 2020). Recently, their application has expanded to that of CNS trauma.

While the literature on the use of MAbs specifically for remyelination post-injury is limited, there have been numerous studies that resulted in MAb-induced axonal regeneration and remyelination. Inflammation, which contributes to the widespread necrosis and apoptosis seen following injury, may be a valuable target for remyelination. For example, the inflammatory cytokine IL-20 is upregulated in the glial cells of rats after SCI and may play a role in the inhibition of axonal regeneration (Dumoutier et al., 2001). *In vivo* treatment of an anti-IL-20 MAb, 7E, following SCI in rats suppressed inflammatory responses, preserved myelin, reduced glial scar formation, and provoked improved motor and sensory function (Lee et al., 2020). Leukocytes, which release proinflammatory cytokines and free radicals that damage white and gray matter, are another potential target for remyelination (Blight, 1985; Carlson et al., 1998; Taoka and Okajima, 1998). Leukocytes are carried by the blood *via* interactions with endothelial cell adhesion molecules, and administration of antibodies blocking the CD11d/CD18 integrin involved in this process have improved motor function, preserved myelin, and reduced secondary damage following SCI (Gris et al., 2004; Hurtado et al., 2012).

MAbs have also been used to target neuroinflammation in TBI (Lenzlinger et al., 2001; Unterberg et al., 2004). Treatment with the anti-CD11d integrin MAb described above reduces levels of leukocyte infiltration, lessens neuroinflammation and neuronal loss, and is associated with increased behavioral functioning in multiple models of TBI (Utagawa et al., 2008; Bao et al., 2012; Shultz et al., 2013). High mobility group box-1 (HMGB1) protein is another molecule that mediates inflammation following injury (Scaffidi et al., 2002). An anti-HMGB1 MAb hindered the protein's translocation, reduced expression levels of inflammatory molecules, and improved motor function following a fluid percussion TBI (Okuma et al., 2012). The proinflammatory lysophosphatidic acid (LPA) is also upregulated after TBI in humans and mice (Frugier et al., 2011). Inhibiting LPA signaling *via* administration of B3, an anti-LPA MAb, following an injury can reduce lesion size and expression levels of inflammatory cytokines, as well as improve motor deficits (Crack et al., 2014). After mouse SCI, application of B3 suppresses the formation of a glial scar and enhances neuronal survival, neurite sprouting, and motor function (Goldshmit et al., 2012).

Treatments targeting growth cone function through the RhoA pathway have also been successful in promoting remyelination following CNS trauma. The IN-1 antibody, which neutralizes neurite growth inhibitors, has been utilized to infiltrate this signaling pathway (Mohammed et al., 2020). IN-1 MAb supports axonal regeneration following SCI (Schnell and Schwab, 1990; Brösamle et al., 2000). Another promising target is the repulsive guidance molecule A (RGMa), which accumulates in lesion sites of SCI (Schwab et al., 2005; Hata et al., 2006). RGMa is a protein-ligand that binds to the Neogenin receptor and is ultimately involved in neurite growth inhibition (Tassew et al., 2014). This mechanism depends on Rho kinase pathway activation. After SCI, RGMa can be neutralized by rat antibodies, which subsequently promotes axon regeneration and locomotor improvement (Hata et al., 2006). Administration of human anti-RGMa MAbs after rat SCI promotes neurobehavioral functioning, decreases neuronal apoptosis, and induces corticospinal tract axonal growth (Mothe et al., 2017, 2020). Induction of CST axonal sprouting and functional recovery has also been reported in primates (Nakagawa et al., 2019).

Similarly, MAbs for the myelin inhibitory molecules discussed earlier have shown improvement in promoting remyelination. Recent studies investigated combination treatments involving anti-Nogo-A MAbs in rat SCI. Combination therapy with methylprednisolone (MP) produced elevated axonal remyelination levels and greater locomotor function (Wu et al., 2014). Furthermore, an anti-MAG MAb treatment in a rat fluid percussion TBI model preserved hemispheric tissue volume and neuromotor function (Thompson et al., 2006).

The use of MAbs specifically for remyelination is better characterized in animal models of MS but may have translational potential to TBI/SCI as both result in demyelination. The main focus has been on the oligodendrocyte-reactive IgMκ subclass of MAbs because antibodies that do not bind to oligodendrocytes will not induce remyelination (Asakura et al., 1998; Bieber et al., 2002). Investigations of multiple IgMκ MAbs in Theiler's murine encephalomyelitis virus (TMEV) model of MS indicates they may prompt remyelination by directly binding to damaged oligodendrocytes, which initiates an immune effector mechanism (Asakura et al., 1998). Human IgM MAbs advanced remyelination efforts in the TMEV model at equal to higher degrees than the polyclonal human IgM (Warrington et al., 2000). Mice treated with a human serum MAb, sHIgM22, demonstrated an increase of axonal remyelination after CNS demyelination. Interestingly, the sHIgM22 treatment condition was associated with high levels of lesion adjacent macrophages. This suggests a potential role of antibody-induced macrophage recruitment in remyelination (Bieber et al., 2002). The recombinant form of human IgM MAb, rHIgM22, can also stimulate remyelination after TMEV-induced myelin loss in mice (Warrington et al., 2001). Assessment of TMEV infected mice before and after rHIgM22 treatment confirmed a decrease in spinal cord lesion tissue volume, mediated by MAb-induced remyelination within the spinal cord (Pirko et al., 2004). The

majority of lesions associated with TMEV are found in the spinal cord, which may provide translatability to SCI models and remyelination efforts. The evidence of IgM MAb-induced remyelination in models of MS provides a starting point for the potential efficacious treatment of TBI/SCI, especially for remyelination.

RNA Interference

RNA interference (RNAi) is both a physiological process and a therapeutic technique using microRNA (miRNA), short hairpin RNA (shRNA), and small interfering RNA (siRNA) to silence genes in a sequence-specific manner. Its development revolutionized gene therapy and has grown in the last few years, with two commercially available disease-targeting siRNA therapeutics approved since 2018 (Hu et al., 2020). Many more clinical and preclinical trials are in the works (Scherman et al., 2017). RNAi molecules bind the mRNA sequence of specific genes and cleave them, preventing translation into functional proteins. In contrast to small molecules and monoclonal antibodies, which target active proteins and pathways, RNAi interacts with the mRNA, allowing for greater specificity. Because it pairs with the sequence itself, this approach is ideal for diseases where a high-affinity molecule for a protein or receptor of interest has not been identified.

The research and development of RNAi have overcome many hurdles to achieve medical application. First, naked siRNAs are both unstable and subject to rapid degradation by nucleases, rendering them ineffective. Second, off-target effects are also possible, though rare, and physiological responses vary widely based on the tissue the drug is administered to. These concerns have been minimized by the production of chemically modified siRNAs, increasing their strength and precision. Additionally, numerous viral and nonviral delivery methods for RNAi have been refined, allowing for application to many types of organs and tissues (Grimm and Kay, 2007; Hu et al., 2020).

As of now, most treatments utilizing RNAi focus on heritable and neurodegenerative diseases, with little work in the realm of CNS trauma. However, these investigations are clearly translatable to the field of TBI/SCI. One example is the use of RNAi to manipulate cell death pathways for tumor suppression (Crnkovic-Mertens et al., 2003; Uchida et al., 2004; Kock et al., 2007). These successes in cancer research offer an encouraging way to minimize the widespread apoptosis seen for months following traumatic injury (Rink et al., 1995; Emery et al., 1998). Preliminary results using a cocktail of siRNAs to silence pro-apoptotic proteins following SCI showed evidence of improved myelination and decreased cell death (Michael et al., 2019). Further evidence comes from Niu et al. (2012) who used siRNA targeting the Wnt/Ca^{2+} pathway to prevent the pathological accumulation of calcium following TBI.

There is a small but growing pool of literature using RNAi in animal models of SCI and demyelinating disease that has shown promise. Studies employing siRNA to silence the expression of myelin inhibitory molecules, such as NoGo-A, demonstrate functional recovery in murine models of multiple sclerosis and SCI (Yang et al., 2010; Sun et al., 2012). Other laboratories have instead targeted cytoskeletal proteins and signaling molecules to improve motility and regeneration after SCI (Yu et al., 2010; Qu et al., 2014; Ding et al., 2017). Similarly, there are several secondary cascades affecting oligodendrocytes and remyelination after injury that may be feasible targets for RNAi. For instance, minimizing oxidative stress following SCI may decrease the vulnerability of neurons and oligodendrocytes to the lesion environment, lessening cell death and demyelination. A study by Gao and Li (2017) successfully silenced inducible nitric oxide synthase (iNOS) expression in M1 macrophages following acute SCI in rats, correlating with both a substantial decrease in the expression of apoptotic markers and an increase in anti-apoptotic markers. Functional testing was not performed in this experiment, but a similar study silencing IRF5 in macrophages in a mouse model of SCI found significant motor recovery as well as decreases in demyelination and inflammation (Li et al., 2016). Another experiment silencing nitric oxide synthase in a TBI model showed decreased neuronal degeneration in addition to improved working memory (Boone et al., 2017). As of now, most experiments using RNAi in TBI models focus on minimizing post-injury edema (Fukuda and Badaut, 2013; Xu et al., 2014; Guan et al., 2020).

While these findings have prompted excitement for the future of RNAi as a therapy for traumatic injury, several concerns must be kept in mind moving forward. First, the heterogeneous nature of the brain and spinal cord could result in differential uptake of siRNAs by different cell types with unintended consequences (Michael et al., 2019). However, the use of antibody-tagged siRNAs has been shown to increase the specificity of uptake to certain cell types (Liu, 2007). It is also important to note the key differences between experimental and clinical CNS injuries. Scientists take great precautions to ensure injuries are precise and consistent between animals. Human TBI/SCI cases, on the other hand, are each unique, with a great deal of variability from person to person. For developing translatable RNAi therapeutics, it is thus critical that treatments can be tailored to the individual to ensure the best outcomes.

Cell Replacement Strategies

Oligodendrocytes and OPCs are critical for promoting remyelination of the injured CNS. However, the death of oligodendrocytes and the failure of OPCs to adequately replenish them contributes significantly to the persistent neuronal demyelination and degeneration seen in trauma and disease (Franklin and ffrench-Constant, 2017). As a result, cell replacement therapy (CRT) offers an encouraging means of restoring oligodendrocyte and progenitor populations to improve remyelination (**Figure 3**). Both neural and non-neural cells have been used in CRT, though we will be focusing on neural cells and their potential to directly restore myelin. Additional benefits of CRT include neuroprotection, provision of regenerative molecules, modulated immune response, and neurological recovery (Assinck et al., 2017; Fischer et al., 2020). CRT has been tested in both injury and disease models with varying degrees of success.

Advancements in stem cell biology have greatly contributed to the prospects of CRT as a viable clinical treatment. Human pluripotent stem cells provide a highly responsive and

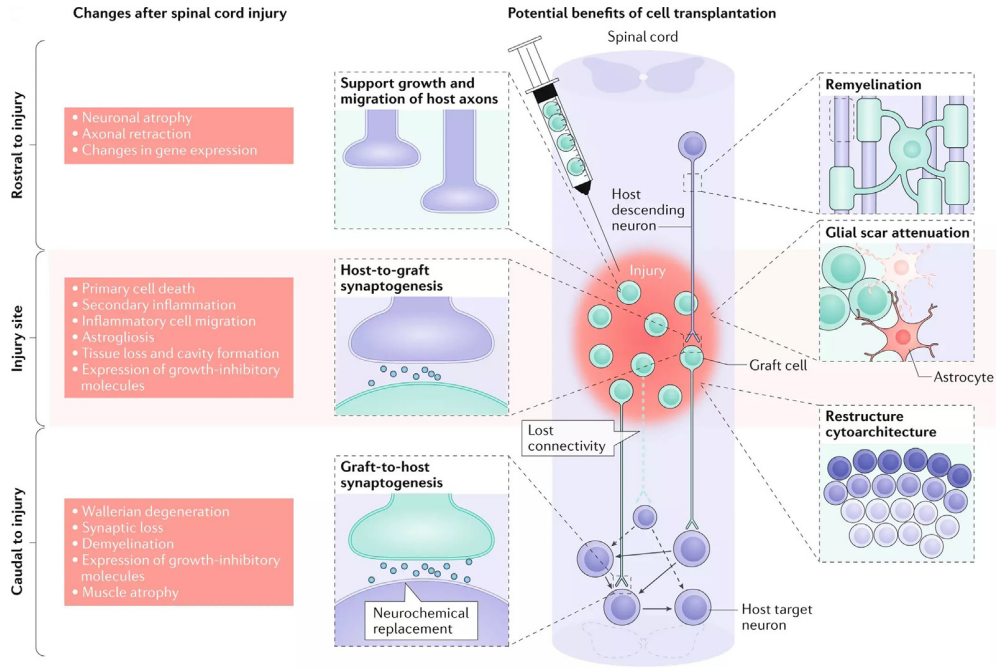


FIGURE 3 | Cellular response to spinal cord injury and the possible therapeutic benefits of cell replacement therapy (CRT). The left depicts the changes that occur in the spinal cord following injury, both rostral and caudal to the lesion site. The right details targeted outcomes of cell transplantation, including remyelination, synaptogenesis, axon regeneration, glial scar attenuation, and the restructuring of cytoarchitecture (adapted from Fischer et al., 2020).

programmable pool that can be differentiated into numerous cell types. Research to date has utilized stem cells obtained from several sources: fetal stem cells, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and neural stem cells (NSCs) derived from the adult nervous system. There is further diversity with regards to the differentiation protocols themselves as well as the stage at which these cells are transplanted. Cell transplantation protocols using NSCs, OPCs, mature oligodendrocytes, and stages in-between have all been documented to improve remyelination. While this demonstrates utility in stem cell application, there is now a need for a consolidation of methodologies as we move towards clinical translation.

An eventual goal of CRT is autologous transplantation, in which the patient's own cells are used to supplement the site of demyelination, however, many steps remain before this can be achieved. Early work laid the foundations towards this goal through the culture and transplantation of rodent ESCs into the rodent spinal cord (McDonald et al., 1999; Liu et al., 2000; Karimi-Abdolrezaee et al., 2006). These studies also reported increased myelination in the hosts, indicating at least some ES cells differentiated into myelinating oligodendrocytes after transplantation. Subsequent efforts have now shifted towards the transplantation of human stem cells into rodent models. Although this comes with the added difficulty of requiring substantial immune suppression of the animals to prevent rejection of the transplants, it also gives invaluable information regarding the safety of transplanting these cells (Forsberg and Hovatta, 2012).

Some of the earliest differentiation protocols direct cells toward a neural fate, allowing for the differentiation of different neural cell types at different stages (Cummings et al., 2005; Parr et al., 2007; Nishimura et al., 2013). Here, cells are considered NSCs or neural progenitor cells (NPCs), terms which have been used somewhat interchangeably in the literature. There are several benefits to transplanting at this early phase. First, because these cells are still highly responsive to exogenous signals, their differentiation can be tailored to the environment of the host nervous system. For instance, the increased expression of factors signaling the recruitment and differentiation of OPCs following injury may result in a higher proportion of donor cells forming oligodendrocytes than could be easily obtained from *in vitro* conditions (Karimi-Abdolrezaee et al., 2006). Post-transplant differentiation usually results in a heterogeneous population of cell types, consisting of oligodendrocytes as well as astrocytes and neurons, and occasionally even Schwann cells (McDonald et al., 1999; Akiyama et al., 2001). The extent to which this is a desirable outcome has been debated. If the goal is to strictly target remyelination, then the generation of pure populations of OPCs or oligodendrocytes is emphasized, however, this has rarely, if ever, been achieved. Furthermore, the transplantation of astrocytes and neurons has been linked to positive outcomes, such as the formation of neuronal relays and increasing neuroprotective effects (Erceg et al., 2010; Fischer et al., 2020). Diverse transplant populations may provide a more clinically relevant and combinatorial approach for treating a traumatic injury. One risk of NSC/NPC transplants comes with the propensity of undifferentiated cells for teratoma formation.

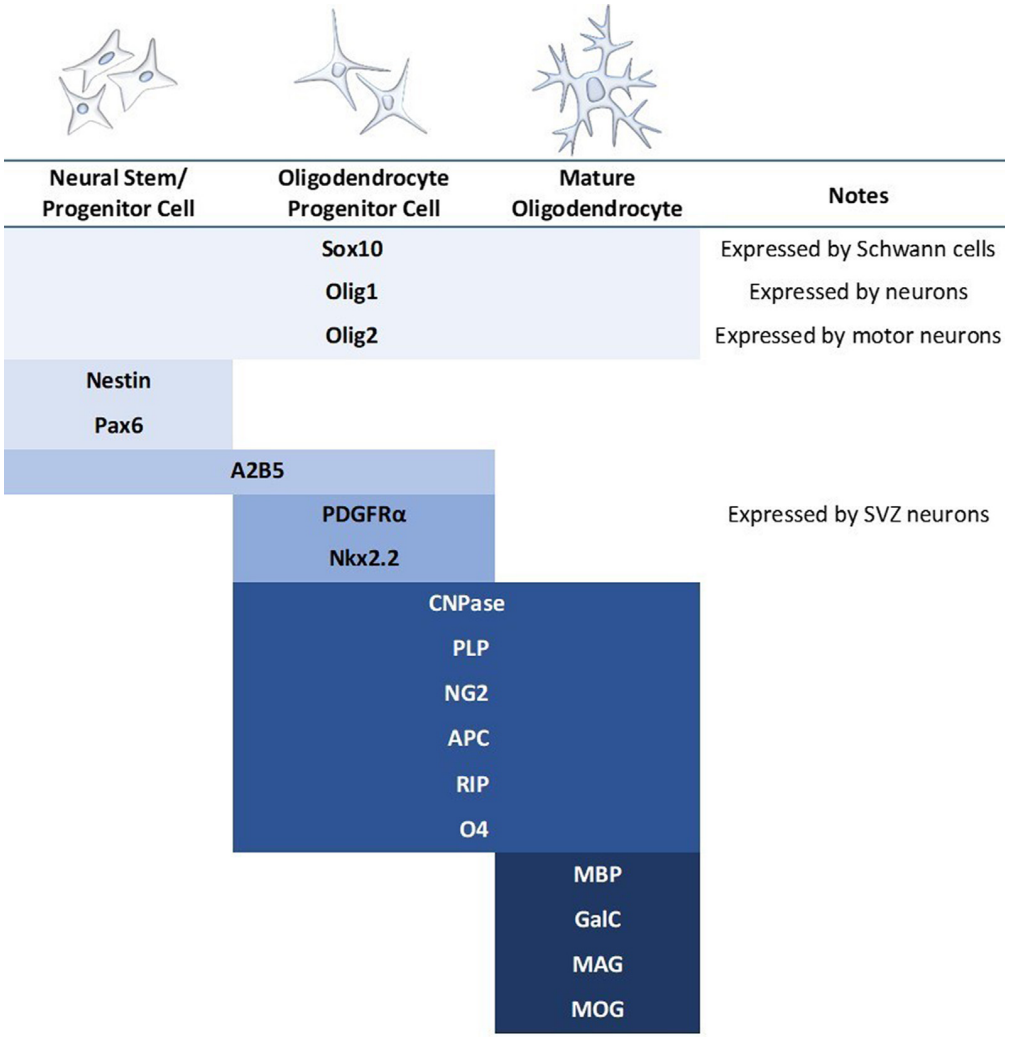


FIGURE 4 | Commonly used markers for the stages of mammalian oligodendrocyte differentiation. Blue bars indicate the stage at which the marker is expressed. A major concern in the literature is the use of only one marker to identify the oligodendrocyte stage when many of these markers express during multiple stages as well as in other cell types. It should also be noted that expression patterns differ based on regional contexts, such as brain vs. spinal cord.

Without an adequate differentiation protocol, there is a greater chance that some of the transplanted cells will retain their pluripotency and form tumors (Araki et al., 2013). It is thus critical that protocol development includes rigorous validation and documentation of cell lines to minimize this risk.

The differentiation and transplantation of OPCs, while one of the most promising areas of SCI research, has also proved to be quite difficult. Early studies went to great efforts to obtain pure OPC populations for transplantation, however, these protocols were lengthy and have been somewhat difficult to replicate (Keirstead et al., 2005; Goldman and Kuypers, 2015). Despite this, there have been several experiments utilizing OPC transplantation in the brain and spinal cord with fair success (Windrem et al., 2004; Hwang et al., 2009; Wang et al., 2013; Kawabata et al., 2016). These studies have reported increased remyelination and spared white matter, synaptogenesis, and locomotor recovery. However, the derivation of OPCs continues to be somewhat elusive. All cells develop along a spectrum, and therefore the "stages" of oligodendrocyte development are less a sequence of discrete subtypes and more a gradual blending from one stage to another. OPCs have been variably defined by a multitude of markers, and while expression levels can be informative about the progression of cell fate specification, there is not an established or reliable point at which a cell is definitively labeled an OPC (**Figure 4**). Most studies do not have pure OPC populations, but rather "enriched" OPC populations, and thus it is difficult to compare between studies or determine which cell type is providing the most benefit.

Because the role of the mature oligodendrocyte in myelination remains unclear, there are fewer transplantation protocols of fully differentiated oligodendrocytes. A landmark study by Nistor et al. (2005) demonstrated that when transplanted into the spinal cord of a dysmyelinated mouse model, differentiated oligodendrocytes survive, integrate into host tissue, and robustly

remyelinate bare axons. Similar results were reported by Liu et al. (2000) when transplanting cultured oligospheres. For the reasons mentioned above, though, it is likely these transplants contained a combination of OPCs and oligodendrocytes at different stages of maturation. Thus, the full contribution of mature oligodendrocytes to myelination has yet to be uncovered.

Though the literature on CRT in TBI is sparse, several recent reviews provide a thorough overview of the application of CRT following traumatic brain injury (Zhou et al., 2019; Zibara et al., 2019). Preclinical trials in rodent models have yielded promising results, and several clinical studies have been proposed in recent years. Reports have documented improved motor and cognitive measures as well as increased neuronal survival following transplantation (Weston and Sun, 2018). However, remyelination has rarely been a focus of cell replacement in TBI to date. Therefore, strong conclusions cannot yet be drawn regarding the effectiveness of oligodendrocytes and remyelination in TBI.

While there is strong therapeutic potential for CRT itself, combinatorial approaches continue to yield the best results in TBI/SCI. Cell transplantation has been paired with RNAi, electrical stimulation, as well as a variety of small molecules to promote neurophysiological recovery (Karimi-Abdolrezaee et al., 2006; Zhang et al., 2012; Jayaprakash et al., 2019). The use of combination therapies has been especially beneficial in developing treatments for chronic SCI. As mentioned above, CRT has proven fairly effective in acute and subacute models of SCI but has largely failed when used in chronic models (Keirstead et al., 2005; Parr et al., 2007; Nishimura et al., 2013). Remarkably, however, recent studies applying multiple strategies have found improvement in chronic SCI animals. Nori et al. (2018) paired OPC transplantation with chondroitinase treatment, reporting increases in remyelination and synaptogenesis giving rise to substantial locomotor improvement. Another team transplanted NPCs with a gamma-secretase inhibitor that promotes neuronal differentiation (Okubo et al., 2018). They also described significant remyelination, functional motor recovery, axon regeneration, and synaptogenesis, the latter likely due to the neural promoter. Given that the majority of SCI cases in the United States are chronic, these studies give hope to previously bleak odds of recovery. As protocols are refined, there is increased potential for combination therapeutics to improve outcomes for patients suffering from traumatic injury.

Additional concerns for the translatability of CRT have expanded the field into large animal models as well. Most TBI/SCI research has been conducted in mice and rats. However, rodents have a greater capacity for endogenous myelination compared to humans, and there is also variability in the size and location of key spinal tracts (Radtke et al., 2007). As such, preclinical trials in non-human primates and other large mammals will be helpful to ensure the safety and efficacy of transplants (Iwanami et al., 2005; Goldman, 2018).

While the large animal literature utilizing CRT for remyelination after SCI is still in its infancy, several key articles should be noted. Pig, dog, and primate models have examined the role of myelination in cell replacement strategies (McMahill et al., 2015; Kim et al., 2018; Rosenzweig et al., 2018). Pig and primate models are commonly used for preclinical trials (Iwanami et al., 2005; Santamaría et al., 2018). Naturally injured dog models are less prevalent but have served as an important bridge between experimentally injured animals and human cases (Granger et al., 2012). Testing the efficacy of treatments in large mammals decreases the risk of clinical trial failure, as differences in smaller animal models may confound the translatability of findings. To date, these models often report various measures of improvement in animals that receive cell transplants, ranging from functional recovery to electrophysiological and histological outcomes. The success of experiments in large mammals will in turn inform continued work in rodent models as protocols are refined and standardized.

Finally, besides TBI/SCI models, many of the studies discussed here also make use of murine models of demyelinating diseases. However, there are some important caveats in comparing the utility of CRT in CNS trauma and in disease. Researchers and clinicians have been optimistic in moving towards a goal of autologous transplants for cases of TBI/SCI, as this would lessen concerns for immune rejection of cells (Forsberg and Hovatta, 2012). This may not be an option for individuals with heritable demyelinating diseases, as the intrinsic capacity of these cells for remyelination may be impaired (Salewski et al., 2015). CRT for these patients then must either use cells from healthy individuals, raising the chances of immunoreactivity, or apply combination therapy with gene-editing technology. It is therefore of utmost importance that, as clinical practices are developed, the risks of immunosuppression are carefully weighed against patient profiles. Taken together, CRT offers a viable future for the treatment of CNS trauma and disease.

HUMAN CLINICAL TRIALS

There have been very few human clinical studies on either TBI or SCI with remyelination as a major or minor goal. We found no registered studies of neural or oligodendrocyte cell transplantation for TBI in human subjects. For remyelination after TBI, one Russian study (NCT02957123) is exploring whether patients with traumatic injury show functional improvement with intranasal inhalations of bioactive factors, produced by autologous M2 macrophages (Ostanin, 2018). This study is not strictly focused on remyelination but mentions it as a potential mechanism. Also, several newly launched clinical trials seek to evaluate the safety and efficacy of CRT in human TBI using mesenchymal stem cells (Schepici et al., 2020). Mesenchymal stem cells (MSCs) have not been discussed in this review article, however, they can be derived from several sources and differentiate into diverse cell types. There is currently no evidence of MSCs becoming myelinating glial cells *in vivo*, however, and thus the extent to which they contribute to remyelination following injury is not understood. Many of these early-phase clinical trials include white matter preservation as an outcome measure, thus when initial results are reported scientists may be able to glean more information about this relationship.

In the field of SCI, while there are several trials investigating ways to overcome the barrier of myelin breakdown products,

TABLE 1 | Summary of current and previous clinical trials utilizing cell replacement therapy in spinal cord injuries (SCI).

Trial	Cell source	Target population	Current status	Trial identifier
Yonsei University (Korea)	Fetal brain-derived NSCs	Acute and chronic cervical ASIA A/B	Completed Phase I/II 2008	NCT0000879
Geron/Asterias Biotherapeutics	ESC-derived OPCs	Acute thoracic ASIA A/B	Completed Phase I 2011	NCT01217008
"SCiStar" (USA)		Subacute cervical ASIA A/B	Completed Phase I/II 2017	NCT02302157
Stem Cells, Inc.	Fetal brain-derived NSCs	Subacute/chronic thoracic ASIA A-C	Completed Phase I/II 2015	NCT01321333
"Pathway" (USA)		Chronic cervical ASIA B/C	Completed Phase II 2016	NCT52163876
Miami Project (USA)	Autologous Schwann cells (sural nerve)	Subacute thoracic ASIA A	Completed Phase I 2016	NCT01739023
		Chronic cervical/thoracic ASIA A-C	Completed Phase I 2019	NCT02354625
Novagenesis Foundation (Russia)	Autologous NSCs +3D matrix	Acute/subacute/chronic, all levels, ASIA A	Completed Phase I 2018	NCT02326662
Neuroregen Scaffold (China)	NSCs + scaffold	Chronic cervical/thoracic ASIA A	Completed Phase I/II 2020	NCT02688049
Neuralstem (USA)	Fetal spinal cord-derived NSCs	Chronic cervical/thoracic ASIA A	Currently enrolling for Phase II	NCT01772810

there are no human trials specifically looking at endogenous remyelination. Several trials have investigated the potential of exogenous remyelination through CRT, either as a primary or secondary goal (**Table 1**). These trials have utilized a variety of neural cell types for a wide range of injuries and outcome measures. It is thus difficult to make broad conclusions about these studies, so they will be summarized below.

The first study occurred over 20 years ago at the University of Florida and involved the transplantation of human fetal spinal cord tissue into patients with chronic post-traumatic syringomyelia at the cervicothoracic junction (Thompson et al., 2001). This study demonstrated safety and feasibility in eight patients but was not advanced to further trials. One of the subsequent studies involves the transplantation of Schwann cells from the peripheral nervous system (Anderson et al., 2017). The remaining trials involve the transplantation of either fetal derived neural stem cells (Shin et al., 2015; StemCells, Inc., 2015; Neuralstem Inc., 2017), embryonic stem cell-derived oligodendrocyte progenitor cells (Chapman and Scala, 2012; Lineage Cell Therapeutics, Inc., 2020), or unclear source (Xiao et al., 2016). All trials injected cells directly into the spinal cord.

These trials had mixed results. The safety trials did not report any significant adverse effects and all reported feasibility of this approach (Boulis and Federici, 2011; Guest et al., 2013; Levi et al., 2018). For those that reported outcomes, they were modest and difficult to interpret. The Korean group reported, "moderate neurological benefit" with 5/19 transplanted patients improving at least one level in ASIA score compared to only one patient in the control group that demonstrated improvement (Shin et al., 2015). The SCiStar trial has also reported significant improvement in hand function in their cervical trial, however, this was in comparison to historical controls only, and a randomized controlled trial is planned (Lineage Cell Therapeutics, Inc., 2020). The Pathway trial, a randomized single-blind controlled trial, was terminated after an interim analysis. While there were some promising improvements observed, especially in sensory function, it was clear that they were not going to be sufficient to meet the primary endpoint (Levi et al., 2019). Thus, while these trials have shown promise, mechanisms remain elusive and the contribution of remyelination is not known.

CONCLUSION

In the last decade, there has been a steadily growing body of research highlighting the importance of the myelin sheath. This work began with demyelinating and white matter diseases and has since expanded to include CNS trauma and now developmental and psychiatric diseases. As such, treatments and therapies targeting oligodendrocytes and remyelination may have widespread potential for application. This review article has discussed some of the processes that contribute to oligodendrocyte and myelin damage in injury and disease as well as the strategies currently being utilized to promote recovery. Small molecules and monoclonal antibodies are among the most well-established therapeutics, with many commercial and clinical treatments currently available. Gene therapy and cell transplantation are newer approaches that have grown extensively in recent years. RNAi has proven beneficial for a variety of diseases, and it shows great potential for use as a combination therapy in TBI/SCI. Cell transplantation, especially of oligodendrocytes and their progenitors, improves remyelination in animal models and in some cases has been correlated with functional recovery. Although RNAi and CRT based approaches have led to clinical trials, the success of these trials has largely fallen short of expectations. To improve prospects moving forward, a standardization of molecular techniques, delivery methods, culture systems, cell lines, and differentiation protocols is necessary so that we may focus and fine-tune our methodology. This will be critical in ensuring the replicability of our research and the best outcomes for patients.

AUTHOR CONTRIBUTIONS

AP contributed to outlining the review and wrote sections of the manuscript. AH-S wrote the first draft of the manuscript. NP and MB wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

Our special thanks to Lucy Vulchanova for her feedback during the final revisions of this review article.

REFERENCES

Aguayo, A. J., David, S., and Bray, G. M. (1981). Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. *J. Exp. Biol.* 95, 231–240.

Akiyama, Y., Honmou, O., Kato, T., Uede, T., Hashi, K., and Kocsis, J. D. (2001). Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord. *Exp. Neurol.* 167, 27–39. doi: 10.1006/exnr.2000.7539

Albert-Weissenberger, C., and Sirén, A.-L. (2010). Experimental traumatic brain injury. *Exp. Transl. Stroke Med.* 2:16. doi: 10.1186/2040-7378-2-16

Alizadeh, A., Dyck, S. M., and Karimi-Abdolrezaee, S. (2019). Traumatic spinal cord injury: an overview of pathophysiology, models and acute injury mechanisms. *Front. Neurol.* 10:282. doi: 10.3389/fneur.2019.00282

Almad, A., Sahinkaya, F. R., and McTigue, D. M. (2011). Oligodendrocyte fate after spinal cord injury. *Neurotherapeutics* 8, 262–273. doi: 10.1007/s13311-011-0033-5

Almeida, R. G. (2018). The rules of attraction in central nervous system myelination. *Front. Cell. Neurosci.* 12:367. doi: 10.3389/fncel.2018.00367

Anderson, K. D., Guest, J. D., Dietrich, W. D., Bartlett Bunge, M., Curiel, R., Dididze, M., et al. (2017). Safety of autologous human schwann cell transplantation in subacute thoracic spinal cord injury. *J. Neurotrauma* 34, 2950–2963. doi: 10.1089/neu.2016.4895

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., et al. (2016). Astrocyte scar formation aids central nervous system axon regeneration. *Nature* 532, 195–200. doi: 10.1038/nature17623

Araki, R., Uda, M., Hoki, Y., Sunayama, M., Nakamura, M., Ando, S., et al. (2013). Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. *Nature* 494, 100–104. doi: 10.1038/nature11807

Armstrong, R. C., Mierzwa, A. J., Sullivan, G. M., and Sanchez, M. A. (2016). Myelin and oligodendrocyte lineage cells in white matter pathology and plasticity after traumatic brain injury. *Neuropharmacology* 110, 654–659. doi: 10.1016/j.neuropharm.2015.04.029

Arthur-Farraj, P. J., Latouche, M., Wilton, D. K., Quintes, S., Chabrol, E., Banerjee, A., et al. (2012). C-jun reprograms Schwann cells of injured nerves to generate a repair cell essential for regeneration. *Neuron* 75, 633–647. doi: 10.1016/j.neuron.2012.06.021

Asakura, K., Miller, D. J., Pease, L. R., and Rodriguez, M. (1998). Targeting of IgMκ antibodies to oligodendrocytes promotes CNS remyelination. *J. Neurosci.* 18, 7700–7708. doi: 10.1523/JNEUROSCI.18-19-07700.1998

Assinck, P., Duncan, G. J., Hilton, B. J., Plemel, J. R., and Tetzlaff, W. (2017). Cell transplantation therapy for spinal cord injury. *Nat. Neurosci.* 20, 637–647. doi: 10.1038/nn.4541

Bao, F., Shultz, S. R., Hepburn, J. D., Omana, V., Weaver, L. C., Cain, D. P., et al. (2012). A CD11d monoclonal antibody treatment reduces tissue injury and improves neurological outcome after fluid percussion brain injury in rats. *J. Neurotrauma* 29, 2375–2392. doi: 10.1089/neu.2012.2408

Barres, B. A., and Raff, M. C. (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature* 361, 258–260. doi: 10.1038/361258a0

Barres, B. A., Jacobson, M. D., Schmid, R., Sendtner, M., and Raff, M. C. (1993). Does oligodendrocyte survival depend on axons? *Curr. Biol.* 3, 489–497. doi: 10.1016/0960-9822(93)90039-q

Barritt, A. W., Davies, M., Marchand, F., Hartley, R., Grist, J., Yip, P., et al. (2006). Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. *J. Neurosci.* 26, 10856–10867. doi: 10.1523/JNEUROSCI.2980-06.2006

Bartsch, U., Bandtlow, C. E., Schnell, L., Bartsch, S., Spillmann, A. A., Rubin, B. P., et al. (1995). Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. *Neuron* 15, 1375–1381. doi: 10.1016/0896-6273(95)90015-2

Bieber, A. J., Warrington, A., Asakura, K., Ciric, B., Kaveri, S. V., Pease, L. R., et al. (2002). Human antibodies accelerate the rate of remyelination following lysolecithin-induced demyelination in mice. *Glia* 37, 241–249. doi: 10.1002/glia.10033

Blakemore, W. F., and Keirstead, H. S. (1999). The origin of remyelinating cells in the central nervous system. *J. Neuroimmunol.* 98, 69–76. doi: 10.1016/s0165-5728(99)00083-1

Blight, A. R. (1985). Delayed demyelination and macrophage invasion: a candidate for secondary cell damage in spinal cord injury. *Cent. Nerv. Syst. Trauma* 2, 299–315. doi: 10.1089/cns.1985.2.299

Boone, D. R., Leek, J. M., Falduto, M. T., Torres, K. E. O., Sell, S. L., Parsley, M. A., et al. (2017). Effects of AAV-mediated knockdown of nNOS and GPx-1 gene expression in rat hippocampus after traumatic brain injury. *PLoS One* 12:e0185943. doi: 10.1371/journal.pone.0185943

Bothwell, M. (2017). Mechanisms and medicines for remyelination. *Annu. Rev. Med.* 68, 431–443. doi: 10.1146/annurev-med-050715-104400

Boulis, N., and Federici, T. (2011). Surgical approach and safety of spinal cord stem cell transplantation. *Neurosurgery* 68, E599–E600. doi: 10.1227/NEU.0b013e3182095e2e

Boyd, A., Zhang, H., and Williams, A. (2013). Insufficient OPC migration into demyelinated lesions is a cause of poor remyelination in MS and mouse models. *Acta Neuropathol.* 125, 841–859. doi: 10.1007/s00401-013-1112-y

Bramlett, H. M., and Dietrich, W. D. (2007). Progressive damage after brain and spinal cord injury: pathomechanisms and treatment strategies. *Prog. Brain Res.* 161, 125–141. doi: 10.1016/S0079-6123(06)61009-1

Brösamle, C., Huber, A. B., Fiedler, M., Skerra, A., and Schwab, M. E. (2000). Regeneration of lesioned corticospinal tract fibers in the adult rat induced by a recombinant, humanized IN-1 antibody fragment. *J. Neurosci.* 20, 8061–8068. doi: 10.1523/JNEUROSCI.20-21-08061.2000

Brosius-Lutz, A., and Barres, B. A. (2014). Contrasting the glial response to axon injury in the central and peripheral nervous systems. *Dev. Cell* 28, 7–17. doi: 10.1016/j.devcel.2013.12.002

Buss, A., Brook, G. A., Kakulas, B., Martin, D., Franzen, R., Schoenen, J., et al. (2004). Gradual loss of myelin and formation of an astrocytic scar during Wallerian degeneration in the human spinal cord. *Brain* 127, 34–44. doi: 10.1093/brain/awh001

Butt, A. M. (2006). Neurotransmitter-mediated calcium signalling in oligodendrocyte physiology and pathology. *Glia* 54, 666–675. doi: 10.1002/glia.20424

Caillava, C., Vandenbosch, R., Jablonska, B., Deboux, C., Spigoni, G., Gallo, V., et al. (2011). Cdk2 loss accelerates precursor differentiation and remyelination in the adult central nervous system. *J. Cell Biol.* 193, 397–407. doi: 10.1083/jcb.201004146

Carlson, S. L., Parrish, M. E., Springer, J. E., Doty, K., and Dossett, L. (1998). Acute inflammatory response in spinal cord following impact injury. *Exp. Neurol.* 151, 77–88. doi: 10.1006/exnr.1998.6785

Caroni, P., and Schwab, M. E. (1988). Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.* 106, 1281–1288. doi: 10.1083/jcb.106.4.1281

Centers for Disease Control and Prevention. (2019). TBI: get the facts. Available online at: https://www.cdc.gov/traumaticbraininjury/get_the_facts.html. Accessed September 4, 2020.

Chapman, A. R., and Scala, C. C. (2012). Evaluating the first-in-human clinical trial of a human embryonic stem cell-based therapy. *Kennedy Inst. Ethics J.* 22, 243–261. doi: 10.1353/ken.2012.0013

Chen, Z.-L., Yu, W.-M., and Strickland, S. (2007). Peripheral regeneration. *Annu. Rev. Neurosci.* 30, 209–233. doi: 10.1146/annurev.neuro.30.051606.094337

Conti, A. C., Raghupathi, R., Trojanowski, J. Q., and McIntosh, T. K. (1998). Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period. *J. Neurosci.* 18, 5663–5672. doi: 10.1523/JNEUROSCI.18-15-05663.1998

Crack, P. J., Zhang, M., Morganti-Kossmann, M. C., Morris, A. J., Wojciak, J. M., Fleming, J. K., et al. (2014). Anti-lysophosphatidic acid antibodies improve traumatic brain injury outcomes. *J. Neuroinflammation* 11:37. doi: 10.1186/1742-2094-11-37

Crawford, A. H., Tripathi, R. B., Foerster, S., McKenzie, I., Kougioumtzidou, E., Grist, M., et al. (2016). Pre-existing mature oligodendrocytes do not contribute to remyelination following toxin-induced spinal cord demyelination. *Am. J. Pathol.* 186, 511–516. doi: 10.1016/j.ajpath.2015.11.005

Crnkovic-Mertens, I., Hoppe-Seyler, F., and Butz, K. (2003). Induction of apoptosis in tumor cells by siRNA-mediated silencing of the livin/ML-IAP/KIAP gene. *Oncogene* 22, 8330–8336. doi: 10.1038/sj.onc.1206973

Crowe, M. J., Bresnahan, J. C., Shuman, S. L., Masters, J. N., and Beattie, M. S. (1997). Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat. Med.* 3, 73–76. doi: 10.1038/nm0197-73

Cummings, B. J., Uchida, N., Tamaki, S. J., Salazar, D. L., Hooshmand, M., Summers, R., et al. (2005). Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc. Natl. Acad. Sci. U S A* 102, 14069–14074. doi: 10.1073/pnas.0507063102

Deshmukh, V. A., Tardif, V., Lyssiotis, C. A., Green, C. C., Kerman, B., Kim, H. J., et al. (2013). A regenerative approach to the treatment of multiple sclerosis. *Nature* 502, 327–332. doi: 10.1038/nature12647

Ding, Y.-M., Li, Y.-Y., Wang, C., Huang, H., Zheng, C.-C., Huang, S.-H., et al. (2017). Nischarin-siRNA delivered by polyethylenimine-alginate nanoparticles accelerates motor function recovery after spinal cord injury. *Neural Regen. Res.* 12, 1687–1694. doi: 10.4103/1673-5374.217348

Domercq, M., Perez-Samartin, A., Aparicio, D., Alberdi, E., Pampliega, O., and Matute, C. (2010). P2X7 receptors mediate ischemic damage to oligodendrocytes. *Glia* 58, 730–740. doi: 10.1002/glia.20958

Donnelly, D. J., and Popovich, P. G. (2008). Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp. Neurol.* 209, 378–388. doi: 10.1016/j.expneurol.2007.06.009

Du, Y., and Dreyfus, C. F. (2002). Oligodendrocytes as providers of growth factors. *J. Neurosci. Res.* 68, 647–654. doi: 10.1002/jnr.10245

Dumoutier, L., Leemans, C., Lejeune, D., Kotenko, S. V., and Renauld, J. C. (2001). Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types. *J. Immunol.* 167, 3545–3549. doi: 10.4049/jimmunol.167.7.3545

Duncan, G. J., Manesh, S. B., Hilton, B. J., Assinck, P., Liu, J., Moulson, A., et al. (2018). Locomotor recovery following contusive spinal cord injury does not require oligodendrocyte remyelination. *Nat. Commun.* 9:3066. doi: 10.1038/s41467-018-05473-1

Duncan, G. J., Manesh, S. B., Hilton, B. J., Assinck, P., Plemel, J. R., and Tetzlaff, W. (2020). The fate and function of oligodendrocyte progenitor cells after traumatic spinal cord injury. *Glia* 68, 227–245. doi: 10.1002/glia.23706

Duncan, I. D., and Hoffman, R. L. (1997). Schwann cell invasion of the central nervous system of the myelin mutants. *J. Anat.* 190, 35–49. doi: 10.1046/j.1469-7580.1997.19010035.x

Duncan, I. D., Brower, A., Kondo, Y., Curlee, J. F., and Schultz, R. D. (2009). Extensive remyelination of the CNS leads to functional recovery. *Proc. Natl. Acad. Sci. U S A* 106, 6832–6836. doi: 10.1073/pnas.0812500106

Duncan, I. D., Radcliff, A. B., Heidari, M., Kidd, G., August, B. K., and Wierenga, L. A. (2018). The adult oligodendrocyte can participate in remyelination. *Proc. Natl. Acad. Sci. U S A* 115, E11807–E11816. doi: 10.1073/pnas.1808064115

Dziedzic, A., Miller, E., Saluk-Bijak, J., and Bijak, M. (2020). The GPR17 receptor—a promising goal for therapy and a potential marker of the neurodegenerative process in multiple sclerosis. *Int. J. Mol. Sci.* 21:1852. doi: 10.3390/ijms21051852

Emery, E., Aldana, P., Bunge, M. B., Puckett, W., Srinivasan, A., Keane, R. W., et al. (1998). Apoptosis after traumatic human spinal cord injury. *J. Neurosurg.* 89, 911–920. doi: 10.3171/jns.1998.89.6.0911

Erceg, S., Ronaghi, M., Oria, M., Roselló, M. G., Aragó, M. A. P., Lopez, M. G., et al. (2010). Transplanted oligodendrocytes and motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. *Stem Cells* 28, 1541–1549. doi: 10.1002/stem.489

Felts, P. A., Baker, T. A., and Smith, K. J. (1997). Conduction in segmentally demyelinated mammalian central axons. *J. Neurosci.* 17, 7267–7277. doi: 10.1523/JNEUROSCI.17-19-07267.1997

Fields, R. D. (2008). White matter in learning, cognition and psychiatric disorders. *Trends Neurosci.* 31, 361–370. doi: 10.1016/j.tins.2008.04.001

Fischer, I., Dulin, J. N., and Lane, M. A. (2020). Transplanting neural progenitor cells to restore connectivity after spinal cord injury. *Nat. Rev. Neurosci.* 21, 366–383. doi: 10.1038/s41583-020-0314-2

Flygt, J., Djupsjö, A., Lenne, F., and Marklund, N. (2013). Myelin loss and oligodendrocyte pathology in white matter tracts following traumatic brain injury in the rat. *Eur. J. Neurosci.* 38, 2153–2165. doi: 10.1111/ejn.12179

Forsberg, M., and Hovatta, O. (2012). Challenges for the therapeutic use of pluripotent stem derived cells. *Front. Physiol.* 3:19. doi: 10.3389/fphys.2012.00019

Franklin, R. J. M., and ffrench-Constant, C. (2008). Remyelination in the CNS: from biology to therapy. *Nat. Rev. Neurosci.* 9, 839–855. doi: 10.1038/nrn2480

Franklin, R. J. M., and ffrench-Constant, C. (2017). Regenerating CNS myelin—from mechanisms to experimental medicines. *Nat. Rev. Neurosci.* 18, 753–769. doi: 10.1038/nrn.2017.136

Franklin, R. J. M., and Goldman, S. A. (2015). Glia disease and repair—remyelination. *Cold Spring Harb. Perspect. Biol.* 7:a020594. doi: 10.1101/cshperspect.a020594

Frugier, T., Crombie, D., Conquest, A., Tjhong, F., Taylor, C., Kulkarni, T., et al. (2011). Modulation of LPA receptor expression in the human brain following neurotrauma. *Cell. Mol. Neurobiol.* 31, 569–577. doi: 10.1007/s10571-011-9650-0

Fukuda, A. M., and Badaut, J. (2013). siRNA treatment: "a sword-in-the-stone" for acute brain injuries. *Genes* 4, 435–456. doi: 10.3390/genes4030435

Fünfschilling, U., Supplie, L. M., Mahad, D., Boretius, S., Saab, A. S., Edgar, J., et al. (2012). Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* 485, 517–521. doi: 10.1038/nature11007

Gallo, V., and Deneen, B. (2014). Glial development: the crossroads of regeneration and repair in the CNS. *Neuron* 83, 283–308. doi: 10.1016/j.neuron.2014.06.010

Gao, W., and Li, J. (2017). Targeted siRNA delivery reduces nitric oxide mediated cell death after spinal cord injury. *J. Nanobiotechnology* 15:38. doi: 10.1186/s12951-017-0272-7

Garbern, J., Yool, D., Moore, G., Wilds, I., Faulk, M., Klugmann, M., et al. (2002). Patients lacking the major CNS myelin protein, proteolipid protein 1, develop length-dependent axonal degeneration in the absence of demyelination and inflammation. *Brain* 125, 551–561. doi: 10.1093/brain/awf043

Genentech: Press Releases (2020). *Genentech Expands Its Multiple Sclerosis Portfolio With Investigational BTK Inhibitor Fenebrutinib and Initiates Novel Clinical Trials for Ocrevus (ocrelizumab)*. Available online at: https://www.gene.com/media/press-releases/14878/2020-09-08/genentech-expands-its-multiple-sclerosis. Accessed October 18, 2020.

Ghorbani, M., Shahabi, P., Karimi, P., Soltani-Zangbar, H., Morshedi, M., Bani, S., et al. (2020). Impacts of epidural electrical stimulation on Wnt signaling, FAAH and BDNF following thoracic spinal cord injury in rat. *J. Cell. Physiol.* 235, 9795–9805. doi: 10.1002/jcp.29793

Giacci, M., and Fitzgerald, M. (2018). Oligodendroglia are particularly vulnerable to oxidative damage after neurotrauma *in vivo*. *J. Exp. Neurosci.* 12:1179069518810004. doi: 10.1177/1179069518810004

Gibson, E. M., Purger, D., Mount, C. W., Goldstein, A. K., Lin, G. L., Wood, L. S., et al. (2014). Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. *Science* 344:1252304. doi: 10.1126/science.1252304

Goldman, S. A. (2018). Transplanted neural progenitors bridge gaps to benefit cord-injured monkeys. *Nat. Med.* 24, 388–390. doi: 10.1038/nm.4531

Goldman, S. A., and Kuypers, N. J. (2015). How to make an oligodendrocyte. *Development* 142, 3983–3995. doi: 10.1242/dev.126409

Goldshmit, Y., Matteo, R., Sztal, T., Ellett, F., Frisca, F., Moreno, K., et al. (2012). Blockage of lysophosphatidic acid signaling improves spinal cord injury outcomes. *Am. J. Pathol.* 181, 978–992. doi: 10.1016/j.ajpath.2012.06.007

Göttle, P., Förster, M., Weyers, V., Küry, P., Rejdak, K., Hartung, H.-P., et al. (2019). An unmet clinical need: roads to remyelination in MS. *Neurol. Res. Pract.* 1:21. doi: 10.1186/s42466-019-0026-0

Granger, N., Blamires, H., Franklin, R. J. M., and Jeffery, N. D. (2012). Autologous olfactory mucosal cell transplants in clinical spinal cord injury: a randomized double-blinded trial in a canine translational model. *Brain* 135, 3227–3237. doi: 10.1093/brain/aws268

Green, A. J., Gelfand, J. M., Cree, B. A., Bevan, C., Boscardin, W. J., Mei, F., et al. (2017). Clemastine fumarate as a remyelinating therapy for multiple sclerosis (ReBUILD): a randomised, controlled, double-blind, crossover trial. *Lancet* 390, 2481–2489. doi: 10.1016/S0140-6736(17)32346-2

Grimm, D., and Kay, M. A. (2007). RNAi and gene therapy: a mutual attraction. *Hematology Am. Soc. Hematol. Educ. Program* 2007, 473–481. doi: 10.1182/asheducation-2007.1.473

Gris, D., Marsh, D. R., Oatway, M. A., Chen, Y., Hamilton, E. F., Dekaban, G. A., et al. (2004). Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic and motor function. *J. Neurosci.* 24, 4043–4051. doi: 10.1523/JNEUROSCI.5343-03.2004

Groves, A. K., Barnett, S. C., Franklin, R. J., Crang, A. J., Mayer, M., Blakemore, W. F., et al. (1993). Repair of demyelinated lesions by transplantation of purified O-2A progenitor cells. *Nature* 362, 453–455. doi: 10.1038/362453a0

Gruchot, J., Weyers, V., Göttle, P., Förster, M., Hartung, H.-P., Küry, P., et al. (2019). The molecular basis for remyelination failure in multiple sclerosis. *Cells* 8:825. doi: 10.3390/cells8080825

Guan, Y., Li, L., Chen, J., and Lu, H. (2020). Effect of AQP4-RNAi in treating traumatic brain edema: multi-modal MRI and histopathological changes of early stage edema in a rat model. *Exp. Ther. Med.* 19, 2029–2036. doi: 10.3892/etm.2020.8456

Guest, J., Santamaria, A. J., and Benavides, F. D. (2013). Clinical translation of autologous Schwann cell transplantation for the treatment of spinal cord injury. *Curr. Opin. Organ Transplant.* 18, 682–689. doi: 10.1097/MOT.0000000000000026

Haindl, M. T., Köck, U., Zeitelhofer-Adzemovic, M., Fazekas, F., and Hochmeister, S. (2019). The formation of a glial scar does not prohibit remyelination in an animal model of multiple sclerosis. *Glia* 67, 467–481. doi: 10.1002/glia.23556

Hampton, D. W., Innes, N., Merkler, D., Zhao, C., Franklin, R. J. M., and Chandran, S. (2012). Focal immune-mediated white matter demyelination reveals an age-associated increase in axonal vulnerability and decreased remyelination efficiency. *Am. J. Pathol.* 180, 1897–1905. doi: 10.1016/j.ajpath.2012.01.018

Hartline, D. K., and Colman, D. R. (2007). Rapid conduction and the evolution of giant axons and myelinated fibers. *Curr. Biol.* 17, R29–R35. doi: 10.1016/j.cub.2006.11.042

Hata, K., Fujitani, M., Yasuda, Y., Doya, H., Saito, T., Yamagishi, S., et al. (2006). RGMa inhibition promotes axonal growth and recovery after spinal cord injury. *J. Cell Biol.* 173, 47–58. doi: 10.1083/jcb.200508143

Hines, J. H., Ravanelli, A. M., Schwindt, R., Scott, E. K., and Appel, B. (2015). Neuronal activity biases axon selection for myelination *in vivo*. *Nat. Neurosci.* 18, 683–689. doi: 10.1038/nn.3992

Hu, B., Zhong, L., Weng, Y., Peng, L., Huang, Y., Zhao, Y., et al. (2020). Therapeutic siRNA: state of the art. *Signal Transduct. Target. Ther.* 5:101. doi: 10.1038/s41392-020-0207-x

Hubler, Z., Allimuthu, D., Bederman, I., Elitt, M. S., Madhavan, M., Allan, K. C., et al. (2018). Accumulation of 8,9-unsaturated sterols drives oligodendrocyte formation and remyelination. *Nature* 560, 372–376. doi: 10.1038/s41586-018-0360-3

Huebner, E. A., and Strittmatter, S. M. (2009). Axon regeneration in the peripheral and central nervous systems. *Results Probl. Cell Differ.* 48, 339–351. doi: 10.1007/400_2009_19

Hurtado, A., Marcillo, A., Frydel, B., Bunge, M. B., Bramlett, H. M., and Dietrich, W. D. (2012). Anti-CD11d monoclonal antibody treatment for rat spinal cord compression injury. *Exp. Neurol.* 233, 606–611. doi: 10.1016/j.expneurol.2010.11.015

Hwang, D. H., Kim, B. G., Kim, E. J., Lee, S. I., Joo, I. S., Suh-Kim, H., et al. (2009). Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. *BMC Neurosci.* 10:117. doi: 10.1186/1471-2202-10-117

Ikeda, O., Murakami, M., Ino, H., Yamazaki, M., Koda, M., Nakayama, C., et al. (2002). Effects of brain-derived neurotrophic factor (BDNF) on compression-induced spinal cord injury: BDNF attenuates down-regulation of superoxide dismutase expression and promotes up-regulation of myelin basic protein expression. *J. Neuropathol. Exp. Neurol.* 61, 142–153. doi: 10.1093/jnen/61.2.142

Inglese, M., and Petracca, M. (2015). Therapeutic strategies in multiple sclerosis: a focus on neuroprotection and repair and relevance to schizophrenia. *Schizophr. Res.* 161, 94–101. doi: 10.1016/j.schres.2014.04.040

Itoh, N., Kim, R., Peng, M., DiFilippo, E., Johnsonbaugh, H., MacKenzie-Graham, A., et al. (2017). Bedside to bench to bedside research: estrogen receptor beta ligand as a candidate neuroprotective treatment for multiple sclerosis. *J. Neuroimmunol.* 304, 63–71. doi: 10.1016/j.jneuroim.2016.09.017

Iwanami, A., Kaneko, S., Nakamura, M., Kanemura, Y., Mori, H., Kobayashi, S., et al. (2005). Transplantation of human neural stem cells for spinal cord injury in primates. *J. Neurosci. Res.* 80, 182–190. doi: 10.1002/jnr.20436

Jayaprakash, N., Nowak, D., Eastwood, E., Krueger, N., Wang, Z., and Blackmore, M. G. (2019). Restoration of direct corticospinal communication across sites of spinal injury. *BioRxiv* 546374 [Preprint]. doi: 10.1101/546374

Jeffries, M. A., Urbanek, K., Torres, L., Wendell, S. G., Rubio, M. E., and Fyffe-Maricich, S. L. (2016). ERK1/2 Activation in preexisting oligodendrocytes of adult mice drives new myelin synthesis and enhanced CNS function. *J. Neurosci.* 36, 9186–9200. doi: 10.1523/JNEUROSCI.1444-16.2016

Johnson, V. E., Stewart, W., and Smith, D. H. (2013). Axonal pathology in traumatic brain injury. *Exp. Neurol.* 246, 35–43. doi: 10.1016/j.expneurol.2012.01.013

Jones, T. B., McDaniel, E. E., and Popovich, P. G. (2005). Inflammatory-mediated injury and repair in the traumatically injured spinal cord. *Curr. Pharm. Des.* 11, 1223–1236. doi: 10.2174/1381612053507468

Kakulas, B. A. (1999). The applied neuropathology of human spinal cord injury. *Spinal Cord* 37, 79–88. doi: 10.1038/sj.sc.3100807

Kakulas, B. A. (2019). Spinal cord injuries the facts of neuropathology: opportunities and limitations. *Curr. Neuropsychiatry Clin. Neurosci. Rep.* 1, 1–5. doi: 10.33702/cncnr.2019.1.1.1

Kanno, H., Ozawa, H., Sekiguchi, A., and Itoi, E. (2009). Spinal cord injury induces upregulation of Beclin 1 and promotes autophagic cell death. *Neurobiol. Dis.* 33, 143–148. doi: 10.1016/j.nbd.2008.09.009

Kanno, H., Pearse, D. D., Ozawa, H., Itoi, E., and Bunge, M. B. (2015). Schwann cell transplantation for spinal cord injury repair: its significant therapeutic potential and prospectus. *Rev. Neurosci.* 26, 121–128. doi: 10.1515/revneuro-2014-0068

Karimi-Abdolrezaee, S., Eftekharpour, E., Wang, J., Morshead, C. M., and Fehlings, M. G. (2006). Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. *J. Neurosci.* 26, 3377–3389. doi: 10.1523/JNEUROSCI.4184-05.2006

Kawabata, S., Takano, M., Numasawa-Kuroiwa, Y., Itakura, G., Kobayashi, Y., Nishiyama, Y., et al. (2016). Grafted human iPS cell-derived oligodendrocyte precursor cells contribute to robust remyelination of demyelinated axons after spinal cord injury. *Stem Cell Reports* 6, 1–8. doi: 10.1016/j.stemcr.2015.11.013

Keirstead, H. S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., et al. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J. Neurosci.* 25, 4694–4705. doi: 10.1523/JNEUROSCI.0311-05.2005

Kelamangalath, L., and Smith, G. M. (2013). Neurotrophin treatment to promote regeneration after traumatic CNS injury. *Front. Biol.* 8, 486–495. doi: 10.1007/s11515-013-1269-8

Kim, K.-T., Streijger, F., Manouchehri, N., So, K., Shortt, K., Okon, E. B., et al. (2018). Review of the UBC porcine model of traumatic spinal cord injury. *J. Korean Neurosurg. Soc.* 61, 539–547. doi: 10.3340/jkns.2017.0276

Kim, Y., and Szele, F. G. (2008). Activation of subventricular zone stem cells after neuronal injury. *Cell Tissue Res.* 331, 337–345. doi: 10.1007/s00441-007-0451-1

Kjell, J., and Olson, L. (2016). Rat models of spinal cord injury: from pathology to potential therapies. *Dis. Model. Mech.* 9, 1125–1137. doi: 10.1242/dmm.025833

Klingseisen, A., Ristoiu, A.-M., Kegel, L., Sherman, D. L., Rubio-Brotons, M., Almeida, R. G., et al. (2019). Oligodendrocyte neurofascin independently regulates both myelin targeting and sheath growth in the CNS. *Dev. Cell* 51, 730.e6–744.e6. doi: 10.1016/j.devcel.2019.10.016

Kock, N., Kasmieh, R., Weissleder, R., and Shah, K. (2007). Tumor therapy mediated by lentiviral expression of shBcl-2 and S-TRAIL. *Neoplasia* 9, 435–442. doi: 10.1593/neo.07223

Kojima, A., and Tator, C. H. (2002). Intrathecal administration of epidermal growth factor and fibroblast growth factor 2 promotes ependymal proliferation and functional recovery after spinal cord injury in adult rats. *J. Neurotrauma* 19, 223–238. doi: 10.1089/08977150252806974

Kotter, M. R., Zhao, C., van Rooijen, N., and Franklin, R. J. M. (2005). Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. *Neurobiol. Dis.* 18, 166–175. doi: 10.1016/j.nbd.2004.09.019

Kottis, V., Thibault, P., Mikol, D., Xiao, Z.-C., Zhang, R., Dergham, P., et al. (2002). Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. *J. Neurochem.* 82, 1566–1569. doi: 10.1046/j.1471-4159.2002.01146.x

Kremer, D., Göttle, P., Hartung, H.-P., and Küry, P. (2016). Pushing forward: remyelination as the new frontier in CNS diseases. *Trends Neurosci.* 39, 246–263. doi: 10.1016/j.tins.2016.02.004

Kulbatski, I., Mothe, A. J., Parr, A. M., Kim, H., Kang, C. E., Bozkurt, G., et al. (2008). Glial precursor cell transplantation therapy for neurotrauma and multiple sclerosis. *Prog. Histochem. Cytochem.* 43, 123–176. doi: 10.1016/j.proghi.2008.04.001

Kwiecien, J. M., O'Connor, L. T., Goetz, B. D., and Delaney, K. H. (1998). Morphological and morphometric studies of the dysmyelinating mutant, the Long Evans shaker rat. *J. Neurocytol.* 27, 581–591. doi: 10.1023/a:1006922227791

Lee, J. K., Geoffroy, C. G., Chan, A. F., Tolentino, K. E., Crawford, M. J., Leal, M. A., et al. (2010). Assessing spinal axon regeneration and sprouting in Nogo, MAG and OMgp deficient mice. *Neuron* 66, 663–670. doi: 10.1016/j.neuron.2010.05.002

Lee, J.-S., Hsu, Y.-H., Chiu, Y.-S., Jou, I.-M., and Chang, M.-S. (2020). Anti-IL-20 antibody improved motor function and reduced glial scar formation after traumatic spinal cord injury in rats. *J. Neuroinflammation* 17:156. doi: 10.1186/s12974-020-01814-4

Lenzlinger, P. M., Morganti-Kossmann, M.-C., Laurer, H. L., and McIntosh, T. K. (2001). The duality of the inflammatory response to traumatic brain injury. *Mol. Neurobiol.* 24, 169–181. doi: 10.1385/MN:24:1-3:169

Levi, A. D., Anderson, K. D., Okonkwo, D. O., Park, P., Bryce, T. N., Kurpad, S. N., et al. (2019). Clinical outcomes from a multi-center study of human neural stem cell transplantation in chronic cervical spinal cord injury. *J. Neurotrauma* 36, 891–902. doi: 10.1089/neu.2018.5843

Levi, A. D., Okonkwo, D. O., Park, P., Jenkins, A. L., Kurpad, S. N., Parr, A. M., et al. (2018). Emerging safety of intramedullary transplantation of human neural stem cells in chronic cervical and thoracic spinal cord injury. *Neurosurgery* 82, 562–575. doi: 10.1093/neuros/nyx250

Li, J., Liu, Y., Xu, H., and Fu, Q. (2016). Nanoparticle-delivered IRF5 siRNA facilitates M1 to M2 transition, reduces demyelination and neurofilament loss and promotes functional recovery after spinal cord injury in mice. *Inflammation* 39, 1704–1717. doi: 10.1007/s10753-016-0405-4

Li, Z., He, Y., Fan, S., and Sun, B. (2015). Clemastine rescues behavioral changes and enhances remyelination in the cuprizone mouse model of demyelination. *Neurosci. Bull.* 31, 617–625. doi: 10.1007/s12264-015-1555-3

Liang, X., Draghi, N. A., and Resh, M. D. (2004). Signaling from integrins to fyn to rho family gtpases regulates morphologic differentiation of oligodendrocytes. *J. Neurosci.* 24, 7140–7149. doi: 10.1523/JNEUROSCI.5319-03.2004

Lineage Cell Therapeutics, Inc. (2020). A phase 1/2a dose escalation study of AST-OPC1 in subjects with subacute cervical spinal cord injury. Available online at: https://clinicaltrials.gov/ct2/show/NCT02302157. Accessed October 19, 2020.

Liu, B. (2007). Exploring cell type-specific internalizing antibodies for targeted delivery of siRNA. *Brief. Funct. Genomics Proteomic.* 6, 112–119. doi: 10.1093/bfgp/elm015

Liu, J., Dupree, J. L., Gacias, M., Frawley, R., Sikder, T., Naik, P., et al. (2016). Clemastine enhances myelination in the prefrontal cortex and rescues behavioral changes in socially isolated mice. *J. Neurosci.* 36, 957–962. doi: 10.1523/JNEUROSCI.3608-15.2016

Liu, S., Qu, Y., Stewart, T. J., Howard, M. J., Chakrabortty, S., Holekamp, T. F., et al. (2000). Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc. Natl. Acad. Sci. U S A* 97, 6126–6131. doi: 10.1073/pnas.97.11.6126

Lopez Juarez, A., He, D., and Richard Lu, Q. (2016). Oligodendrocyte progenitor programming and reprogramming: toward myelin regeneration. *Brain Res.* 1638, 209–220. doi: 10.1016/j.brainres.2015.10.051

Lotocki, G., de Rivero Vaccari, J. P., Alonso, O., Molano, J. S., Nixon, R., Safavi, P., et al. (2011). Oligodendrocyte vulnerability following traumatic brain injury in rats. *Neurosci. Lett.* 499, 143–148. doi: 10.1016/j.neulet.2011.05.056

Lu, R.-M., Hwang, Y.-C., Liu, I.-J., Lee, C.-C., Tsai, H.-Z., Li, H.-J., et al. (2020). Development of therapeutic antibodies for the treatment of diseases. *J. Biomed. Sci.* 27:1. doi: 10.1186/s12929-019-0592-z

Lundgaard, I., Luzhynskaya, A., Stockley, J. H., Wang, Z., Evans, K. A., Swire, M., et al. (2013). Neuregulin and BDNF induce a switch to NMDA receptor-dependent myelination by oligodendrocytes. *PLoS Biol.* 11:e1001743. doi: 10.1371/journal.pbio.1001743

Macchi, M., Magalon, K., Zimmer, C., Peeva, E., El Waly, B., Brousse, B., et al. (2020). Mature oligodendrocytes bordering lesions limit demyelination and favor myelin repair *via* heparan sulfate production. *eLife* 9:e51735. doi: 10.7554/eLife.51735

Manrique-Hoyos, N., Jürgens, T., Grønborg, M., Kreutzfeldt, M., Schedensack, M., Kuhlmann, T., et al. (2012). Late motor decline after accomplished remyelination: impact for progressive multiple sclerosis. *Ann. Neurol.* 71, 227–244. doi: 10.1002/ana.22681

McDonald, J. W., Liu, X. Z., Qu, Y., Liu, S., Mickey, S. K., Turetsky, D., et al. (1999). Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat. Med.* 5, 1410–1412. doi: 10.1038/70986

McGregor, C. E., and English, A. W. (2019). The Role of BDNF in peripheral nerve regeneration: activity-dependent treatments and val66met. *Front. Cell. Neurosci.* 12:522. doi: 10.3389/fncel.2018.00522

Mckeon, R. J., Höke, A., and Silver, J. (1995). Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp. Neurol.* 136, 32–43. doi: 10.1006/exnr.1995.1081

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J., and Braun, P. E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* 13, 805–811. doi: 10.1016/0896-6273(94)90247-x

McMahill, B. G., Borjesson, D. L., Sieber-Blum, M., Nolta, J. A., and Sturges, B. K. (2015). Stem cells in canine spinal cord injury—promise for regenerative therapy in a large animal model of human disease. *Stem Cell Rev. Rep.* 11, 180–193. doi: 10.1007/s12015-014-9553-9

McTigue, D. M., Horner, P. J., Stokes, B. T., and Gage, F. H. (1998). Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. *J. Neurosci.* 18, 5354–5365. doi: 10.1523/JNEUROSCI.18-14-05354.1998

McTigue, D. M., Wei, P., and Stokes, B. T. (2001). Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. *J. Neurosci.* 21, 3392–3400. doi: 10.1523/JNEUROSCI.21-10-03392.2001

Mecha, M., Feliú, A., Carrillo-Salinas, F. J., Mestre, L., and Guaza, C. (2013). Mobilization of progenitors in the subventricular zone to undergo oligodendrogenesis in the theiler's virus model of multiple sclerosis: implications for remyelination at lesions sites. *Exp. Neurol.* 250, 348–352. doi: 10.1016/j.expneurol.2013.10.011

Mei, F., Fancy, S. P. J., Shen, Y.-A. A., Niu, J., Zhao, C., Presley, B., et al. (2014). Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis. *Nat. Med.* 20, 954–960. doi: 10.1038/nm.3618

Mei, F., Lehmann-Horn, K., Shen, Y.-A. A., Rankin, K. A., Stebbins, K. J., Lorrain, D. S., et al. (2016a). Accelerated remyelination during inflammatory demyelination prevents axonal loss and improves functional recovery. *Elife* 5:e18246. doi: 10.7554/eLife.18246

Mei, F., Mayoral, S. R., Nobuta, H., Wang, F., Desponts, C., Lorrain, D. S., et al. (2016b). Identification of the κ-opioid receptor as a therapeutic target for oligodendrocyte remyelination. *J. Neurosci.* 36, 7925–7935. doi: 10.1523/JNEUROSCI.1493-16.2016

Merten, N., Fischer, J., Simon, K., Zhang, L., Schröder, R., Peters, L., et al. (2018). Repurposing HAMI3379 to block GPR17 and promote rodent and human oligodendrocyte differentiation. *Cell Chem. Biol.* 25, 775.e5–786.e5. doi: 10.1016/j.chembiol.2018.03.012

Mi, S., Lee, X., Shao, Z., Thill, G., Ji, B., Relton, J., et al. (2004). LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat. Neurosci.* 7, 221–228. doi: 10.1038/nn1188

Michael, F. M., Chandran, P., Chandramohan, K., Iyer, K., Jayaraj, K., Sundaramoorthy, R., et al. (2019). Prospects of siRNA cocktails as tools for modifying multiple gene targets in the injured spinal cord. *Exp. Biol. Med.* 244, 1096–1110. doi: 10.1177/1535370219871868

Mohammed, R., Opara, K., Lall, R., Ojha, U., and Xiang, J. (2020). Evaluating the effectiveness of anti-nogo treatment in spinal cord injuries. *Neural Dev.* 15:1. doi: 10.1186/s13064-020-0138-9

Monje, M. (2018). Myelin plasticity and nervous system function. *Annu. Rev. Neurosci.* 41, 61–76. doi: 10.1146/annurev-neuro-080317-061853

Moore, S. M., Khalaj, A. J., Kumar, S., Winchester, Z., Yoon, J., Yoo, T., et al. (2014). Multiple functional therapeutic effects of the estrogen receptor β agonist indazole-Cl in a mouse model of multiple sclerosis. *Proc. Natl. Acad. Sci. U S A* 111, 18061–18066. doi: 10.1073/pnas.1411294111

Morshead, C. M., and van der Kooy, D. (1990). Separate blood and brain origins of proliferating cells during gliosis in adult brains. *Brain Res.* 535, 237–244. doi: 10.1016/0006-8993(90)91606-h

Mothe, A. J., Coelho, M., Huang, L., Monnier, P. P., Cui, Y.-F., Mueller, B. K., et al. (2020). Delayed administration of the human anti-RGMa monoclonal antibody elezanumab promotes functional recovery including spontaneous voiding after spinal cord injury in rats. *Neurobiol. Dis.* 143:104995. doi: 10.1016/j.nbd.2020.104995

Mothe, A. J., Tassew, N. G., Shabanzadeh, A. P., Penheiro, R., Vigouroux, R. J., Huang, L., et al. (2017). RGMa inhibition with human monoclonal antibodies promotes regeneration, plasticity and repair and attenuates neuropathic pain after spinal cord injury. *Sci. Rep.* 7:10529. doi: 10.1038/s41598-017-10987-7

Moyon, S., Dubessy, A. L., Aigrot, M. S., Trotter, M., Huang, J. K., Dauphinot, L., et al. (2015). Demyelination causes adult CNS progenitors to revert to an immature state and express immune cues that support their migration. *J. Neurosci.* 35, 4–20. doi: 10.1523/JNEUROSCI.0849-14.2015

Myers, S. A., Bankston, A. N., Burke, D. A., Ohri, S. S., and Whittemore, S. R. (2016). Does the preclinical evidence for functional remyelination following myelinating cell engraftment into the injured spinal cord support progression to clinical trials? *Exp. Neurol.* 283, 560–572. doi: 10.1016/j.expneurol.2016.04.009

Nait-Oumesmar, B., Picard-Riera, N., Kerninon, C., Decker, L., Seilhean, D., Höglinger, G. U., et al. (2007). Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors. *Proc. Natl. Acad. Sci. U S A* 104, 4694–4699. doi: 10.1073/pnas.0606835104

Najm, F. J., Madhavan, M., Zaremba, A., Shick, E., Karl, R. T., Factor, D. C., et al. (2015). Drug-based modulation of endogenous stem cells promotes functional remyelination *in vivo*. *Nature* 522, 216–220. doi: 10.1038/nature14335

Nakagawa, H., Ninomiya, T., Yamashita, T., and Takada, M. (2019). Treatment with the neutralizing antibody against repulsive guidance molecule-a promotes recovery from impaired manual dexterity in a primate model of spinal cord injury. *Cereb. Cortex* 29, 561–572. doi: 10.1093/cercor/bhx338

Nakanishi, K., Aono, S., Hirano, K., Kuroda, Y., Ida, M., Tokita, Y., et al. (2006). Identification of neurite outgrowth-promoting domains of neuroglycan c, a brain-specific chondroitin sulfate proteoglycan and involvement of phosphatidylinositol 3-kinase and protein kinase C signaling pathways in neuritogenesis. *J. Biol. Chem.* 281, 24970–24978. doi: 10.1074/jbc.M601498200

Nashmi, R., and Fehlings, M. G. (2001). Mechanisms of axonal dysfunction after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels. *Brain Res. Rev.* 38, 165–191. doi: 10.1016/s0165-0173(01)00134-5

National Spinal Cord Injury Statistical Center. (2020). Facts and figures at a glance. Available online at: https://www.nscisc.uab.edu/Public/Facts%20and%20Figures%202020.pdf. Accessed September 4, 2020.

Nave, K.-A. (2010). Myelination and the trophic support of long axons. *Nat. Rev. Neurosci.* 11, 275–283. doi: 10.1038/nrn2797

Nave, K.-A., and Werner, H. B. (2014). Myelination of the nervous system: mechanisms and functions. *Annu. Rev. Cell Dev. Biol.* 30, 503–533. doi: 10.1146/annurev-cellbio-100913-013101

Nawaz, S., Sánchez, P., Schmitt, S., Snaidero, N., Mitkovski, M., Velte, C., et al. (2015). Actin filament turnover drives leading edge growth during myelin sheath formation in the central nervous system. *Dev. Cell* 34, 139–151. doi: 10.1016/j.devcel.2015.05.013

Neuralstem Inc. (2017). A phase 1, open-label, single-site, safety study of human spinal cord-derived neural stem cell transplantation for the treatment of chronic SCI. Available online at: https://clinicaltrials.gov/ct2/show/NCT01772810. Accessed October 19, 2020.

Nichol, K. A., Everett, A. W., Schulz, M., and Bennett, M. R. (1994). Retinal ganglion cell survival *in vitro* maintained by a chondroitin sulfate proteoglycan from the superior colliculus carrying the HNK-1 epitope. *J. Neurosci. Res.* 37, 623–632. doi: 10.1002/jnr.490370509

Nishimura, S., Yasuda, A., Iwai, H., Takano, M., Kobayashi, Y., Nori, S., et al. (2013). Time-dependent changes in the microenvironment of injured spinal cord affects the therapeutic potential of neural stem cell transplantation for spinal cord injury. *Mol. Brain* 6:3. doi: 10.1186/1756-6606-6-3

Nistor, G. I., Totoiu, M. O., Haque, N., Carpenter, M. K., and Keirstead, H. S. (2005). Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia* 49, 385–396. doi: 10.1002/glia.20127

Niu, L.-J., Xu, R.-X., Zhang, P., Du, M.-X., and Jiang, X.-D. (2012). Suppression of frizzled-2-mediated Wnt/Ca^{2+} signaling significantly attenuates intracellular calcium accumulation *in vitro* and in a rat model of traumatic brain injury. *Neuroscience* 213, 19–28. doi: 10.1016/j.neuroscience.2012.03.057

Nori, S., Khazaei, M., Ahuja, C. S., Yokota, K., Ahlfors, J.-E., Liu, Y., et al. (2018). Human oligodendrogenic neural progenitor cells delivered with chondroitinase ABC facilitate functional repair of chronic spinal cord injury. *Stem Cell Reports* 11, 1433–1448. doi: 10.1016/j.stemcr.2018.10.017

Nye, S. H., and Yarger, J. G. (2017). A small molecule induces oligodendrogenesis leading to myelin sheath repair. *J. Clini. Endocri. Metabo. Disord.* 2017, 1–14. doi: 10.29011/JCMD-106.100006

Ohri, S. S., Bankston, A. N., Mullins, S. A., Liu, Y., Andres, K. R., Beare, J. E., et al. (2018). Blocking autophagy in oligodendrocytes limits functional recovery after spinal cord injury. *J. Neurosci.* 38, 5900–5912. doi: 10.1523/JNEUROSCI.0679-17.2018

Okubo, T., Nagoshi, N., Kohyama, J., Tsuji, O., Shinozaki, M., Shibata, S., et al. (2018). Treatment with a gamma-secretase inhibitor promotes functional recovery in human iPSC- derived transplants for chronic spinal cord injury. *Stem Cell Reports* 11, 1416–1432. doi: 10.1016/j.stemcr.2018.10.022

Okuma, Y., Liu, K., Wake, H., Zhang, J., Maruo, T., Date, I., et al. (2012). Anti-high mobility group box-1 antibody therapy for traumatic brain injury. *Ann. Neurol.* 72, 373–384. doi: 10.1002/ana.23602

Oluich, L.-J., Stratton, J. A. S., Xing, Y. L., Ng, S. W., Cate, H. S., Sah, P., et al. (2012). Targeted ablation of oligodendrocytes induces axonal pathology independent of overt demyelination. *J. Neurosci.* 32, 8317–8330. doi: 10.1523/JNEUROSCI.1053-12.2012

Ostanin, A. A. (2018). Safety/efficacy of intranasally-administered bioactive factors produced by autologous M2 macrophages in patients with organic brain syndrome. Available online at: https://clinicaltrials.gov/ct2/show/NCT02957123. Accessed October 14, 2020.

Parr, A. M., and Tator, C. H. (2007). Intrathecal epidermal growth factor and fibroblast growth factor-2 exacerbate meningeal proliferative lesions associated with intrathecal catheters. *Neurosurgery* 60, 926–933. doi: 10.1227/01.NEU.0000255441.59612.98

Parr, A. M., Kulbatski, I., and Tator, C. H. (2007). Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury. *J. Neurotrauma* 24, 835–845. doi: 10.1089/neu.2006.3771

Parravicini, C., Lecca, D., Marangon, D., Coppolino, G. T., Daniele, S., Bonfanti, E., et al. (2020). Development of the first *in vivo* GPR17 ligand through an iterative drug discovery pipeline: a novel disease-modifying strategy for multiple sclerosis. *PLoS One* 15:e0231483. doi: 10.1371/journal.pone.0231483

Patil, N., Truong, V., Holmberg, M. H., Lavoie, N. S., McCoy, M. R., Dutton, J. R., et al. (2018). Safety and efficacy of rose bengal derivatives for glial scar ablation in chronic spinal cord injury. *J. Neurotrauma* 35, 1745–1754. doi: 10.1089/neu.2017.5398

Pettigrew, R. I., Heetderks, W. J., Kelley, C. A., Peng, G. C. Y., Member, I., Krosnick, S. H., et al. (2017). Epidural spinal stimulation to improve

bladder, bowel and sexual function in individuals with spinal cord injuries: a framework for clinical Research. *IEEE Trans. Biomed. Eng.* 64, 253–262. doi: 10.1109/TBME.2016.2637301

Pirko, I., Ciric, B., Gamez, J., Bieber, A. J., Warrington, A. E., Johnson, A. J., et al. (2004). A human antibody that promotes remyelination enters the CNS and decreases lesion load as detected by T2-weighted spinal cord MRI in a virus-induced murine model of MS. *FASEB J.* 18, 1577–1579. doi: 10.1096/fj.04-2026fje

Pohl, H. B. F., Porcheri, C., Mueggler, T., Bachmann, L. C., Martino, G., Riethmacher, D., et al. (2011). Genetically induced adult oligodendrocyte cell death is associated with poor myelin clearance, reduced remyelination and axonal damage. *J. Neurosci.* 31, 1069–1080. doi: 10.1523/JNEUROSCI.5035-10.2011

Pukos, N., Goodus, M. T., Sahinkaya, F. R., and McTigue, D. M. (2019). Myelin status and oligodendrocyte lineage cells over time after spinal cord injury: what do we know and what still needs to be unwrapped? *Glia* 67, 2178–2202. doi: 10.1002/glia.23702

Qu, Y., Zhao, J., Wang, Y., and Gao, Z. (2014). Silencing ephrinB3 improves functional recovery following spinal cord injury. *Mol. Med. Rep.* 9, 1761–1766. doi: 10.3892/mmr.2014.2019

Radtke, C., Spies, M., Sasaki, M., Vogt, P. M., and Kocsis, J. D. (2007). Demyelinating diseases and potential repair strategies. *Int. J. Dev. Neurosci.* 25, 149–153. doi: 10.1016/j.ijdevneu.2007.02.002

Ramlackhansingh, A. F., Brooks, D. J., Greenwood, R. J., Bose, S. K., Turkheimer, F. E., Kinnunen, K. M., et al. (2011). Inflammation after trauma: microglial activation and traumatic brain injury. *Ann. Neurol.* 70, 374–383. doi: 10.1002/ana.22455

Rink, A., Fung, K. M., Trojanowski, J. Q., Lee, V. M., Neugebauer, E., and McIntosh, T. K. (1995). Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. *Am. J. Pathol.* 147, 1575–1583.

Rolls, A., Shechter, R., and Schwartz, M. (2009). The bright side of the glial scar in CNS repair. *Nat. Rev. Neurosci.* 10, 235–241. doi: 10.1038/nrn2591

Ronzano, R., Thetiot, M., Lubetzki, C., and Desmazieres, A. (2020). Myelin plasticity and repair: neuro-glial choir sets the tuning. *Front. Cell. Neurosci.* 14:42. doi: 10.3389/fncel.2020.00042

Rosenzweig, E. S., Brock, J. H., Lu, P., Kumamaru, H., Salegio, E. A., Kadoya, K., et al. (2018). Restorative effects of human neural stem cell grafts to the primate spinal cord. *Nat. Med.* 24, 484–490. doi: 10.1038/nm.4502

Salewski, R. P., Mitchell, R. A., Li, L., Shen, C., Milekovskaia, M., Nagy, A., et al. (2015). Transplantation of induced pluripotent stem cell-derived neural stem cells mediate functional recovery following thoracic spinal cord injury through remyelination of axons. *Stem Cells Transl. Med.* 4, 743–754. doi: 10.5966/sctm.2014-0236

Sanofi brain-penetrant BTK inhibitor significantly reduced disease activity in Phase 2 trial in relapsing multiple sclerosis. Available online at: https://www.sanofi.com/media-room/pressreleases/2020/2020-04-2307-00-002020541. Accessed October 18, 2020.

Santamaría, A. J., Solano, J. P., Benavides, F. D., and Guest, J. D. (2018). "Intraspinal delivery of schwann cells for spinal cord injury," in *Schwann Cells: Methods and Protocols Methods in Molecular Biology*, eds P. V. Monje and H. A. Kim (New York, NY: Springer), 467–484.

Satzer, D., Miller, C., Maxon, J., Voth, J., DiBartolomeo, C., Mahoney, R., et al. (2015). T cell deficiency in spinal cord injury: altered locomotor recovery and whole-genome transcriptional analysis. *BMC Neurosci.* 16:74. doi: 10.1186/s12868-015-0212-0

Scaffidi, P., Misteli, T., and Bianchi, M. E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 418, 191–195. doi: 10.1038/nature00858

Schäfer, M. K. E., and Tegeder, I. (2018). NG2/CSPG4 and progranulin in the posttraumatic glial scar. *Matrix Biol.* 68-69, 571–588. doi: 10.1016/j.matbio.2017.10.002

Schepici, G., Silvestro, S., Bramanti, P., and Mazzon, E. (2020). Traumatic brain injury and stem cells: an overview of clinical trials, the current treatments and future therapeutic approaches. *Medicina* 56:137. doi: 10.3390/medicina56030137

Scherman, D., Rousseau, A., Bigey, P., and Escriou, V. (2017). Genetic pharmacology: progresses in siRNA delivery and therapeutic applications. *Gene Ther.* 24, 151–156. doi: 10.1038/gt.2017.6

Schnell, L., and Schwab, M. E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* 343, 269–272. doi: 10.1038/343269a0

Schwab, J. M., Conrad, S., Monnier, P. P., Julien, S., Mueller, B. K., and Schluesener, H. J. (2005).Spinal cord injury-induced lesional expression of the repulsive guidance molecule (RGM). *Eur. J. Neurosci.* 21, 1569–1576. doi: 10.1111/j.1460-9568.2005.03962.x

Shin, J. C., Kim, K. N., Yoo, J., Kim, I.-S., Yun, S., Lee, H., et al. (2015). Clinical trial of human fetal brain-derived neural stem/progenitor cell transplantation in patients with traumatic cervical spinal cord injury. *Neural Plast.* 2015:630932. doi: 10.1155/2015/630932

Shultz, S. R., Bao, F., Weaver, L. C., Cain, D. P., and Brown, A. (2013). Treatment with an anti-CD11d integrin antibody reduces neuroinflammation and improves outcome in a rat model of repeated concussion. *J. Neuroinflammation* 10:26. doi: 10.1186/1742-2094-10-26

Silver, J. (2016). The glial scar is more than just astrocytes. *Exp. Neurol.* 286, 147–149. doi: 10.1016/j.expneurol.2016.06.018

Sim, F. J., Zhao, C., Penderis, J., and Franklin, R. J. M. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *J. Neurosci.* 22, 2451–2459. doi: 10.1523/JNEUROSCI.22-07-02451.2002

Smith, K. J., Blakemore, W. F., and Mcdonald, W. I. (1979). Central remyelination restores secure conduction. *Nature* 280, 395–396. doi: 10.1038/280395a0

StemCells, Inc. (2015). A phase I/II study of the safety and preliminary efficacy of intramedullary spinal cord transplantation of human central nervous system (CNS) stem cells (HuCNS-SC®) in subjects with thoracic (T2-T11) spinal cord trauma. Available online at: https://clinicaltrials.gov/ct2/show/NCT01321333. Accessed October 19, 2020.

Sun, H.-H., Gao, F., Liu, B., Yu, H.-T., Kong, N., and Liu, G.-M. (2012). Inhibition of nogo expression to promote repair after spinal cord injury. *Chin. Med. J.* 125, 4044–4048. doi: 10.3760/cma.j.issn.0366-6999.2012.22.024

Taoka, Y., and Okajima, K. (1998). Spinal cord injury in the rat. *Prog. Neurobiol.* 56, 341–358. doi: 10.1016/s0301-0082(98)00049-5

Tassew, N. G., Mothe, A. J., Shabanzadeh, A. P., Banerjee, P., Koeberle, P. D., Bremner, R., et al. (2014). Modifying lipid rafts promotes regeneration and functional recovery. *Cell Rep.* 8, 1146–1159. doi: 10.1016/j.celrep.2014.06.014

Thompson, F. J., Reier, P. J., Uthman, B., Mott, S., Fessler, R. G., Behrman, A., et al. (2001). Neurophysiological assessment of the feasibility and safety of neural tissue transplantation in patients with syringomyelia. *J. Neurotrauma* 18, 931–945. doi: 10.1089/089771501750451848

Thompson, H. J., Marklund, N., LeBold, D. G., Morales, D. M., Keck, C. A., Vinson, M., et al. (2006). Tissue sparing and functional recovery following experimental traumatic brain injury is provided by treatment with an anti-myelin-associated glycoprotein antibody. *Eur. J. Neurosci.* 24, 3063–3072. doi: 10.1111/j.1460-9568.2006.05197.x

Thorburne, S. K., and Juurlink, B. H. J. (1996). Low glutathione and high iron govern the susceptibility of oligodendroglial precursors to oxidative stress. *J. Neurochem.* 67, 1014–1022. doi: 10.1046/j.1471-4159.1996.67031014.x

Uchida, H., Tanaka, T., Sasaki, K., Kato, K., Dehari, H., Ito, Y., et al. (2004). Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth *in vitro* and *in vivo*. *Mol. Ther.* 10, 162–171. doi: 10.1016/j.ymthe.2004.05.006

Ulc, A., Gottschling, C., Schäfer, I., Wegrzyn, D., Leeuwen, S., van Luft, V., et al. (2017). Involvement of the guanine nucleotide exchange factor Vav3 in central nervous system development and plasticity. *Biol. Chem.* 398, 663–675. doi: 10.1515/hsz-2016-0275

Unterberg, A. W., Stover, J., Kress, B., and Kiening, K. L. (2004). Edema and brain trauma. *Neuroscience* 129, 1021–1029. doi: 10.1016/j.neuroscience.2004.06.046

Utagawa, A., Bramlett, H. M., Daniels, L., Lotocki, G., Dekaban, G., Weaver, L. C., et al. (2008). Transient blockage of the CD11d/CD18 integrin reduces contusion volume and macrophage infiltration after traumatic brain injury in rats. *Brain Res.* 1207, 155–163. doi: 10.1016/j.brainres.2008.02.057

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

Walker, K. R., and Tesco, G. (2013). Molecular mechanisms of cognitive dysfunction following traumatic brain injury. *Front. Aging Neurosci.* 5:29. doi: 10.3389/fnagi.2013.00029

Walker, L. M., Phogat, S. K., Chan-Hui, P.-Y., Wagner, D., Phung, P., Goss, J. L., et al. (2009). Broad and potent neutralizing antibodies from an african donor reveal a new HIV-1 vaccine target. *Science* 326, 285–289. doi: 10.1126/science.1178746

Wang, S. (2011). Advances in the production of human monoclonal antibodies. *Antib. Tech. J.* 2011, 1–4. doi: 10.2147/ANTI.S20195

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., et al. (2013). Human iPSC-derived oligodendrocyte progenitors can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell* 12, 252–264. doi: 10.1016/j.stem.2012.12.002

Warrington, A. E., Asakura, K., Bieber, A. J., Ciric, B., Keulen, V. V., Kaveri, S. V., et al. (2000). Human monoclonal antibodies reactive to oligodendrocytes promote remyelination in a model of multiple sclerosis. *Proc. Natl. Acad. Sci. U S A* 97, 6820–6825. doi: 10.1073/pnas.97.12.6820

Warrington, A. E., Bieber, A. J., Ciric, B., Keulen, V. V., Pease, L. R., Mitsunaga, Y., et al. (2001). Immunoglobulin-mediated CNS repair. *J. Allergy Clin. Immunol.* 108, S121–S125. doi: 10.1067/mai.2001.118301

Weston, N. M., and Sun, D. (2018). The potential of stem cells in treatment of traumatic brain injury. *Curr. Neurol. Neurosci. Rep.* 18:1. doi: 10.1007/s11910-018-0812-z

Windrem, M. S., Nunes, M. C., Rashbaum, W. K., Schwartz, T. H., Goodman, R. A., McKhann, G., et al. (2004). Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. *Nat. Med.* 10, 93–97. doi: 10.1038/nm974

Wong, E. V., David, S., Jacob, M. H., and Jay, D. G. (2003). Inactivation of myelin-associated glycoprotein enhances optic nerve regeneration. *J. Neurosci.* 23, 3112–3117. doi: 10.1523/JNEUROSCI.23-08-03112.2003

Wu, J., Zhao, Z., Sabirzhanov, B., Stoica, B. A., Kumar, A., Luo, T., et al. (2014). Spinal cord injury causes brain inflammation associated with cognitive and affective changes: role of cell cycle pathways. *J. Neurosci.* 34, 10989–11006. doi: 10.1523/JNEUROSCI.5110-13.2014

Xiao, L., Guo, D., Hu, C., Shen, W., Shan, L., Li, C., et al. (2012). Diosgenin promotes oligodendrocyte progenitor cell differentiation through estrogen receptor-mediated ERK1/2 activation to accelerate remyelination. *Glia* 60, 1037–1052. doi: 10.1002/glia.22333

Xiao, Z., Tang, F., Tang, J., Yang, H., Zhao, Y., Chen, B., et al. (2016). One-year clinical study of neuroregen scaffold implantation following scar resection in complete chronic spinal cord injury patients. *Sci. China Life Sci.* 59, 647–655. doi: 10.1007/s11427-016-5080-z

Xing, Y. L., Röth, P. T., Stratton, J. A. S., Chuang, B. H. A., Danne, J., Ellis, S. L., et al. (2014). Adult neural precursor cells from the subventricular zone contribute significantly to oligodendrocyte regeneration and remyelination. *J. Neurosci.* 34, 14128–14146. doi: 10.1523/JNEUROSCI.3491-13.2014

Xu, B., Yu, D.-M., and Liu, F.-S. (2014). Effect of siRNA-induced inhibition of IL-6 expression in rat cerebral gliocytes on cerebral edema following traumatic brain injury. *Mol. Med. Rep.* 10, 1863–1868. doi: 10.3892/mmr.2014.2462

Xu, G.-Y., Hughes, M. G., Ye, Z., Hulsebosch, C. E., and McAdoo, D. J. (2004). Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. *Exp. Neurol.* 187, 329–336. doi: 10.1016/j.expneurol.2004.01.029

Yang, Y., Liu, Y., Wei, P., Peng, H., Winger, R., Hussain, R. Z., et al. (2010). Silencing nogo-a promotes functional recovery in demyelinating disease. *Ann. Neurol.* 67, 498–507. doi: 10.1002/ana.21935

Yeung, M. S. Y., Zdunek, S., Bergmann, O., Bernard, S., Salehpour, M., Alkass, K., et al. (2014). Dynamics of oligodendrocyte generation and myelination in the human brain. *Cell* 159, 766–774. doi: 10.1016/j.cell.2014.10.011

Yu, C.-G., Yezierski, R. P., Joshi, A., Raza, K., Li, Y., and Geddes, J. W. (2010). Involvement of ERK2 in traumatic spinal cord injury. *J. Neurochem.* 113, 131–142. doi: 10.1111/j.1471-4159.2010.06579.x

Zada, D., Tovin, A., Lerer-Goldshtein, T., and Appelbaum, L. (2016). Pharmacological treatment and BBB-targeted genetic therapy for MCT8-dependent hypomyelination in zebrafish. *Dis. Model. Mech.* 9, 1339–1348. doi: 10.1242/dmm.027227

Zhang, S., Huang, F., Gates, M., and Holmberg, E. G. (2012). Tail nerve electrical stimulation combined with scar ablation and neural transplantation promotes locomotor recovery in rats with chronically contused spinal cord. *Brain Res.* 1456, 22–35. doi: 10.1016/j.brainres.2012.03.054

Zhao, A., Qin, H., and Fu, X. (2016). What determines the regenerative capacity in animals? *Bioscience* 66, 735–746. doi: 10.1093/biosci/biw079

Zhou, Y., Shao, A., Xu, W., Wu, H., and Deng, Y. (2019). Advance of stem cell treatment for traumatic brain injury. *Front. Cell. Neurosci.* 13:301. doi: 10.3389/fncel.2019.00301

Zibara, K., Ballout, N., Mondello, S., Karnib, N., Ramadan, N., Omais, S., et al. (2019). Combination of drug and stem cells neurotherapy: potential interventions in neurotrauma and traumatic brain injury. *Neuropharmacology* 145, 177–198. doi: 10.1016/j.neuropharm.2018.09.032

Zuchero, J. B., Fu, M., Sloan, S. A., Ibrahim, A., Olson, A., Zaremba, A., et al. (2015). CNS myelin wrapping is driven by actin disassembly. *Dev. Cell* 34, 152–167. doi: 10.1016/j.devcel.2015.06.011

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 20 January 2021
doi: 10.3389/fncel.2020.625879

A Hypomorphic *Dars1*^{D367Y} Model Recapitulates Key Aspects of the Leukodystrophy HBSL

Dominik Fröhlich[1]*, *Marisa I. Mendes*[2], *Andrew J. Kueh*[3,4], *Andre Bongers*[5], *Marco J. Herold*[3,4], *Gajja S. Salomons*[2], *Gary D. Housley*[1] and *Matthias Klugmann*[1]*

[1] Translational Neuroscience Facility & Department of Physiology, School of Medical Sciences, UNSW Sydney, Kensington, NSW, Australia, [2] Metabolic Unit/Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry, Amsterdam Neuroscience, Amsterdam Gastroenterology & Metabolism, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [3] The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia, [4] Department of Medical Biology, University of Melbourne, Parkville, VIC, Australia, [5] Biomedical Resources Imaging Laboratory, UNSW Sydney, Kensington, NSW, Australia

Hypomyelination with brain stem and spinal cord involvement and leg spasticity (HBSL) is a leukodystrophy caused by missense mutations of the aspartyl-tRNA synthetase-encoding gene *DARS1*. The clinical picture includes the regression of acquired motor milestones, spasticity, ataxia, seizures, nystagmus, and intellectual disabilities. Morphologically, HBSL is characterized by a distinct pattern of hypomyelination in the central nervous system including the anterior brainstem, the cerebellar peduncles and the supratentorial white matter as well as the dorsal columns and the lateral corticospinal tracts of the spinal cord. Adequate HBSL animal models are lacking. *Dars1* knockout mice are embryonic lethal precluding examination of the etiology. To address this, we introduced the HBSL-causing *Dars1*^{D367Y} point mutation into the mouse genome. Surprisingly, mice carrying this mutation homozygously were phenotypically normal. As hypomorphic mutations are more severe in trans to a deletion, we crossed *Dars1*$^{D367Y/D367Y}$ mice with *Dars1-null* carriers. The resulting *Dars1*$^{D367Y/-}$ offspring displayed a strong developmental delay compared to control *Dars1*$^{D367Y/+}$ littermates, starting during embryogenesis. Only a small fraction of *Dars1*$^{D367Y/-}$ mice were born, and half of these mice died with hydrocephalus during the first 3 weeks of life. Of the few *Dars1*$^{D367Y/-}$ mice that were born at term, 25% displayed microphthalmia. Throughout postnatal life, *Dars1*$^{D367Y/-}$ mice remained smaller and lighter than their *Dars1*$^{D367Y/+}$ littermates. Despite this early developmental deficit, once they made it through early adolescence *Dars1*$^{D367Y/-}$ mice were phenotypically inconspicuous for most of their adult life, until they developed late onset motor deficits as well as vacuolization and demyelination of the spinal cord white matter. Expression levels of the major myelin proteins were reduced in *Dars1*$^{D367Y/-}$ mice compared to controls. Taken together, *Dars1*$^{D367Y/-}$ mice model aspects of the clinical picture of the corresponding missense mutation in HBSL. This model will enable studies of late onset deficits, which is precluded in *Dars1* knockout mice, and can be leveraged to test potential HBSL therapeutics including *DARS1* gene replacement therapy.

Keywords: HBSL, DARS1, AspRS, aminoacyl-tRNA synthetase, aspartyl-tRNA synthetase, leukodystrophy

OPEN ACCESS

Edited by:
Rosanna Parlato,
University of Heidelberg, Germany

Reviewed by:
James N. Sleigh,
University College London, United Kingdom
Bilal Ersen Kerman,
Istanbul Medipol University, Turkey

***Correspondence:**
Dominik Fröhlich
d.frohlich@unsw.edu.au
Matthias Klugmann
m.klugmann@unsw.edu.au

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *04 November 2020*
Accepted: *21 December 2020*
Published: *20 January 2021*

Citation:
Fröhlich D, Mendes MI, Kueh AJ, Bongers A, Herold MJ, Salomons GS, Housley GD and Klugmann M (2021) A Hypomorphic Dars1^{D367Y} Model Recapitulates Key Aspects of the Leukodystrophy HBSL. Front. Cell. Neurosci. 14:625879. doi: 10.3389/fncel.2020.625879

INTRODUCTION

Leukodystrophies are inherited white matter disorders often associated with an early onset, lack of treatment options and premature death. The population incidence of all leukodystrophies taken together is relatively high with one in 7,600 live births (Bonkowsky et al., 2010) underpinning the high unmet medical need. *H*ypomyelination with *b*rain stem and *s*pinal cord involvement and *l*eg spasticity (HBSL) belongs to this group of diseases and was first described in 2013 (Taft et al., 2013). Following the initial discovery, two additional case studies, together with the original study, described a total of 16 HBSL patients (Wolf et al., 2015; Ong et al., 2020). HBSL can be seen as a spectrum disorder with a high variance in severity (mild to severe forms) and onset of the disease (4 months to 22 years) (Wolf et al., 2015). An early onset form of HBSL usually results in a more severe course of disease. The clinical symptoms typically include motor deficits, leg spasticity, regression, or delay of developmental milestones, hypertonia, hyperreflexia, positive Babinski sign, nystagmus, and gait abnormalities in patients who are able to mobilize (Taft et al., 2013; Wolf et al., 2015; Ong et al., 2020). For a comprehensive clinical review of HBSL see Muthiah et al. (2020) in this issue.

The underlying cause of HBSL are missense mutations of the aspartyl-tRNA synthetase (AspRS) gene *DARS1*. AspRS belongs to a group of enzymes termed aminoacyl-tRNA synthetases (ARSs) that catalyze an aminoacylation reaction in which transfer ribonucleic acids (tRNAs) are linked to their cognate amino acids. This process is known as tRNA charging and is an essential prerequisite for successful protein biosynthesis. Each ARS is specific for the charging of one tRNA with its corresponding amino acid, e.g., AspRS specifically links $tRNA^{Asp}$ to aspartate, and there is no redundancy amongst these enzymes. ARSs can be subdivided into two classes depending on the cell compartment in which they catalyze the aminoacylation reaction: Cytosolic ARSs and mitochondrial ARSs (mt-ARSs). AspRS functions as a homodimer (Kim et al., 2013) and is ubiquitously expressed in all cells and tissues. However, our previous studies revealed a strong prevalence in neurons of the cerebellum in the murine (Fröhlich et al., 2017) and human brain (Fröhlich et al., 2018). All *DARS1* mutations identified to date are located within the catalytic domain of AspRS and are likely having a direct impact on the aminoacylation reaction and consequently on protein synthesis. Why *DARS1* mutations specifically manifest in neurologic deficits remains unresolved.

Previous attempts at creating a mouse model for HBSL through complete knockout of the *Dars1* gene were unsuccessful and either resulted in early embryonic lethality (homozygous *Dars1*-null mice), or the lack of HBSL pathology (heterozygous *Dars1*-null mice). A more promising strategy is to introduce HBSL-causing *DARS1* point mutations into the mouse gene. The HBSL index patient was reported to be a compound heterozygous carrier of the *DARS1*^{A274V} and *DARS1*^{D367Y} point mutations. In this study, we employed CRISPR/Cas9-mediated gene editing to introduce the *Dars1*^{D367Y} mutation into the murine *Dars1* gene. Surprisingly, homozygous *Dars1*$^{D367Y/D367Y}$ mice were only mildly affected. The human *DARS1* missense mutations identified so far can be classified as hypomorphic mutations, which means that they cause a partial loss of gene function either through reduced expression or through impaired activity. Hypomorphs do not usually result in a complete loss of function and are always more severe in trans to a deletion mutation. In order to enhance the phenotype of *Dars1*$^{D367Y/D367Y}$ mice, we bred them with *Dars1-null* carriers. The *Dars1*$^{D367Y/-}$ offspring displayed a strong developmental delay, which often resulted in early embryonic or pre-weaning death. The few surviving *Dars1*$^{D367Y/-}$ mice were smaller and lighter compared to *Dars1*$^{D367Y/+}$ littermates and developed late onset motor deficits as well as vacuolization of the white matter of the spinal cord. In summary, *Dars1*$^{D367Y/-}$ mice recapitulate some HBSL aspects and will be instrumental for examinations of the disease etiology or therapeutic proof-of-concept studies.

METHODS

Ethics

All procedures were approved by the University of New South Wales Animal Care and Ethics Committee and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animals

Mice were housed in ventilated cages in groups of 2 to 5 animals and fed *ad libitum* with standard chow. The c.1099G>T (D367Y) point mutation was introduced into the *Dars1* locus using the CRISPR/Cas9 technique as previously described (Kueh et al., 2017) on a C57BL/6J background. To generate mice carrying the *Dars1* c.1099G>T (D367Y) point mutation, 20 ng/μl of Cas9 mRNA, 10 ng/μl of sgRNA (CGATGAGGAAGATCTAAG GT) and 40 ng/μl of oligo donor (gagtattgcgaggctttggcaatgct tagagaagctggagttgaaatggacgatgaggaaTatctaaggttAgtctctgatatttt ctctttcaactttaatgcttaggtttaatgtatttcacccagctga) were injected into the cytoplasm of fertilized one-cell stage embryos. Twenty-four hours later, two-cell stage embryos were transferred into the oviducts of pseudo-pregnant female mice. Viable offspring were genotyped by next-generation sequencing. Additionally, correct targeting was also confirmed by Sanger sequencing. The region containing the c.1099G>T mutation was amplified by PCR using the following primers: Forward 5′-GCTTTTCT TGGTTCAGTCGC-3′, reverse 5′-CGGGTTACAACTAGGGGA AA-3′. The resulting 449 bp PCR fragment was purified by ethanol precipitation and subsequently sequenced using the forward primer.

Dars1-null mice were created and genotyped as previously described (Fröhlich et al., 2017). *Dars1*$^{D367Y/-}$ mice were generated by breeding homozygous *Dars1*$^{D367Y/D367Y}$ mice with heterozygous *Dars1*-null mice.

Behavioral Testing

Behavioral tests were either performed in *Dars1*$^{D367Y/D367Y}$ mice together with age- and sex-matched wildtype mice or in age- and sex-matched *Dars1*$^{D367Y/-}$ and *Dars1*$^{D367Y/+}$ littermates. Locomotor behavior was assessed using the rotarod test as described (von Jonquieres et al., 2018). For habituation, mice

were placed on the rotarod apparatus (Ugo Basile, Comerio, Italy) for 1 min at a constant speed of 4 revolutions per minute (rpm) before the test trials started. During the test, the speed of the rotarod constantly increased from 4 to 40 rpm over a 4-min period. Cut-off time was 5 min with the last min on full speed. Mice were tested in 3 trials per day on two consecutive days (6 trials in total). Inter-trial interval was 30 min. The latency to fall was averaged over the six trials.

The open field-test was performed as described (Fröhlich et al., 2017). Mice were placed in the center of an open field box (40 × 40 × 40 cm^3) under bright light conditions (100 lux). Mice were video recorded during the 30 min trial and subsequently analyzed using the ANY-Maze^{TM} tracking software (Stoelting, Illinois, USA) for total distance traveled as well as the distance traveled in the inner compartment.

To determine the acoustic startle response (ASR) as well as the pre-pulse inhibition (PPI) we used the SR-LAB Startle Response System (San Diego Instruments, San Diego, USA) as described (Schneider et al., 2007; Fröhlich et al., 2017). During ASR and PPI, mice were exposed to 60 dB sound pressure level (SPL) background white noise and both the ASR and PPI, were preceded by a 5 min habituation period of 60 dB SPL white noise only. The ASR was measured as the maximum amplitude detected by the accelerometer in response to 40 ms white noise pulses with increasing intensities (60–120 dB SPL) with a 10 s interval. In order to determine the PPI, the 120 dB SPL startle pulse was preceded (100 ms) by a 20 ms pre-pulse (72, 76, or 80 dB SPL). Each trial was repeated 10 times. PPI was determined as the decrease of the ASR amplitude in response to the pre-pulse compared to the 120 dB SPL pulse alone.

Magnetic Resonance Imaging (MRI) and Body Composition Analysis

Following a lethal intraperitoneal (IP) injection of pentobarbital, mice were transcardially perfused with 10 ml phosphate buffered saline (PBS) for 5 min, followed by 10 ml 4% paraformaldehyde (PFA) for 5 min. Brains were post-fixed in 4% PFA for 2 h at room temperature (RT). Prior to imaging the fixed brains were immersed in in 9 g/l NaCl/H_2O solution for 24 h at 21°C to remove fixation residues in the brain tissue. The brains were then transferred into a 1.3 mm ID, 2 ml Cryovial (Greiner, Germany) and submersed in Perfluoro-Polyether Fomblin^{TM} 6Y for susceptibility matching. The Cryovial was then mounted on the tip of a plastic tube, which was attached to the automatic positioning system of the MRI system. MRI was performed using a 9.4T BioSpec Avance III 94/20 (Bruker, Ettlingen, Germany) magnetic resonance microimaging system equipped with BGA-12S HP gradients with maximum strength 660 mT/m and slew rate 4,570 Tm/s. A dedicated 15 mm internal diameter quadrature specimen volume coil was used for radiofrequency transmission and reception. Anatomical images were acquired using an optimized isotropic 3D multi gradient echo sequence (MGE) with 106 coronal partitions and 10 gradient echoes with the following major parameters: First TE = 2.7 ms, ΔTE = 3.45 ms, #echos = 28, TR = 100 ms, FA = 30°, FOV = 15 × 15 × 8 mm, matrix = 200 × 200× 106, image resolution = 75 μm^3 (isotropic), eff. spectral BW = 78,125 Hz, total acquisition time with 2 ADC averages: 1 h and 46 min per specimen. Segmentation and surface models of the entire brain as well as the ventricles were generated using the 3D slicer software (Kikinis et al., 2014).

Body composition analysis (EchoMRI) was performed as described (von Jonquieres et al., 2018) in accordance to the manufacturer's instructions using the EchoMRI-900^{TM} scanner equipped with A100 mouse antenna insert (Echo Medical Systems). Fat and lean mass were calculated as percentage of total mass.

Mouse Histopathology Evaluation

This study utilized the Phenomics Australia (PA; formerly known as Australian Phenomics Network) Histopathology and Organ Pathology Service (HOPS) at the University of Melbourne. This service included: Harvesting of 25 organs, fixation and embedding in paraffin blocks, sectioning, Haematoxylin and Eosin (H&E; all organs; **Figures 3H,I**, **5C**) and Luxol Fast Blue (LFB; brain and spinal cord; **Figures 5B,D**) staining, slide scanning, images of histopathology, and detailed histology and pathology reports.

Immunohistochemistry

Following cardiac perfusion as described above, brains were post-fixed in 4% PFA for 2 h at RT and cryoprotected in 30% sucrose. Brains were cut in the coronal plane into 40 µm sections using a cryostat as described (von Jonquieres et al., 2013). Sections were permeabilized with 0.2% TritonX-100 in PBS (PBS-Tx) and non-specific binding was blocked with 4% goat serum in PBS-Tx. Primary antibodies were applied in 4% goat serum in PBS-Tx over night at 4°C. The following antibodies were used: rabbit anti-NF200 (neurofilament 200; 1:1000; Sigma-Aldrich no. N4142) and rat anti-PLP clone aa3 (proteolipid protein; 1:10; gift from Prof. J. Trotter, Mainz, Germany). After washing with PBS, sections were incubated with goat anti-rabbit Alexa-488 and goat anti-rat Alexa-594 secondary antibodies (1:400; Thermo Fisher Scientific no. A11012 and A11006) in 4% goat serum in PBS-Tx for 4 h at RT. After washing in PBS-Tx, sections were mounted in Mowiol (Calbiochem, Darmstadt, Germany) and imaged using an LSM710 confocal microscope (Carl Zeiss, Berlin, Germany).

RNA Isolation and qRT-PCR

Animals were euthanized at 10 months by cervical dislocation. The brain regions cortex (CX), cerebellum (CB), brainstem (BS), and basal ganglia (BG) were dissected and snap frozen. RNA extraction was performed as described before (Fröhlich et al., 2018). Briefly, brain tissue was homogenized in liquid nitrogen using mortar and pestle followed by RNA extraction according to the manufacturer's instructions (RNeasy MiniKit, Qiagen no. 74106) including on-column DNase digest (RNase-Free DNase Set, Qiagen no. 79254). RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems no. 4368813) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed in triplicate using the StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems). The following TaqMan probes (Applied Biosystems) were used: *Dars1* (Mm00624185_m1),

Dars2 (Mm01296063_m1), *Aspa* (Mm004808667_m1), *Plp1* (Mm00456892_m1), *Cnp* (Mm01306640), *Mbp* (Mm01266402_m1), *GusB* (Mm01197698_m1). Data was normalized to the housekeeper *GusB* and the CX region using the comparative ΔΔCT method.

SDS-PAGE and Western-Blotting

Animals were euthanized at 10 months by cervical dislocation. The brain regions CX, CB, BS, and BG were dissected, snap frozen and homogenized under liquid nitrogen using mortar and pestle. 10 μl lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA pH 8.0, 250 mM NaCl, and 1% Triton-X) including a cocktail of protease inhibitors (Complete, Roche) were added per mg of brain tissue. Lysates were sonicated using a Branson 450 Digital Probe Sonifier at 10% sonication amplitude and protein concentration was determined by Bradford protein assay (Bio-Rad no. 5000006).

SDS-PAGE and Western-blotting was performed as described (Fröhlich et al., 2018). In summary, 20 μg of protein mixed with 5x Laemmli reducing sample buffer were loaded onto a 10% acrylamide gel, separated by SDS-PAGE, and transferred onto a PVDF membrane (Bio-Rad no. 162-0177). Membranes were incubated with 4% skim milk powder in PBS plus 0.1% Tween (PBS-T) to prevent unspecific binding of antibodies. Subsequently, membranes were probed with the following primary antibodies in 4% skim milk in PBS-T: mouse anti-AspRS (1:1000; SantaCruz no. sc-393275), rabbit anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase; 1:4000, Cell Signaling no. 2118S), mouse anti-CNP (2',3'-cyclic nucleotide 3' phosphodiesterase; 1:3000, Abcam no. ab6319), rat anti-MBP (myelin basic protein; 1:1000; Abcam no. ab7349), rat anti-PLP aa3 (proteolipid protein; 1:200, gift from Prof. J. Trotter, Mainz, Germany). Following 3 wash steps (15 min each) with PBS-T, membranes were probed with appropriate HRP-conjugated secondary antibodies (1:10000; Dianova, Hamburg, Germany) in 4% skim milk in PBS-T. After 3 additional wash steps with PBS-T, membranes were incubated for 1 min with 1 ml Clarity Western ECL substrate (Bio-Rad no. 170-5060) and imaged in the ChemiDoc MP system (Bio-Rad, Hercules, USA).

AspRS Enzyme Activity Assay

HEK293 cells were cultured to 70% confluency for transient expression with *DARS1*^{WT}, *DARS1*^{D367Y}, *DARS1*^{A274V}, or an equimolar mix of *DARS1*^{A274V} and *DARS1*^{D367Y}. For transfection, 12 μg of plasmid DNA were mixed with 60 μl Fugene (Promega, Madison, WI) in serum–free DMEM medium (Thermo Fisher Scientific, Waltham, USA) and incubated for 15 min at room temperature. Subsequently, the mix was applied to the cells. Cells transfected with an empty vector (mock transfected) and untransfected cells were included as controls. Fluorescent microscopy was used to estimate transfection efficiency and Western blotting was performed to confirm comparable expression levels of wildtype and mutant AspRS. Cells were harvested 48 h post transfection, pelleted, snap-frozen, and stored at −80°C until further use. All transfections were performed in triplicate.

To determine aminoacylation activity, pellets were suspended in ddH_2O and cells were lysed in three freeze-thaw cycles. Protein amount was quantified using the BCA assay and protein samples were adjusted to 1 mg/ml. Cell lysates were incubated in triplicate for 10 min at 37°C in reaction buffer [50 mM Tris buffer pH 7.5, 12 mM MgCl2, 25 mM KCl, 1 mg/ml bovine serum albumin, 0.5 mM spermine, 1 mM ATP, 0.2 mM *E. coli* total tRNA, 1 mM dithiothreitol, and 0.3 mM ($^{13}C_4$, ^{15}N)-aspartate]. The reaction was stopped using trichloroacetic acid. After washing the samples with trichloroacetic acid, ammonia was added to release the labeled amino acids from the tRNAs. [D3]-aspartate was added as internal standard and the labeled amino acids were quantified by LC-MS/MS. Aminoacylation activity measured for mock transfected cells was subtracted from *DARS1*-transfected samples. *DARS1*^{WT} transfected cells were used as a positive control and activities were expressed as percentage of *DARS1*^{WT} transfected cells.

Statistics

Statistical analysis was performed with the GraphPad Prism 8 software (La Jolla, CA, USA). Following validation of normal distribution of data, one-way or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons *post-hoc* test was performed as indicated. Values are displayed as mean ± SEM with $p < 0.05$ regarded as statistically significant.

RESULTS

Phenotype of Homozygous *Dars1*$^{D367Y/D367Y}$ Mice

In our previous study we demonstrated that the early embryonic lethality of *Dars1*-null mice precluded their use as an accurate model for HBSL (Fröhlich et al., 2017). In this study we introduced the c.1099G>T (D367Y) point mutation, which was first identified in the compound heterozygous index patient, into the mouse *Dars1* locus using the CRISPR/Cas9 gene editing technology. This single nucleotide change located on exon 11 of the *Dars1* locus results in a TAT codon coding for tyrosine instead of GAT coding for aspartic acid (**Figure 1A**). To our surprise, homozygous *Dars1*$^{D367Y/D367Y}$ mice were only mildly affected. The body weight of homozygous *Dars1*$^{D367Y/D367Y}$ mice, compared to wildtype controls at 4 months of age, was unchanged (**Figure 1B**). Body composition analysis using EchoMRI revealed only a slight shift from fat to lean mass without reaching statistical significance (**Figure 1C**). We performed behavioral tests with these mice at two different timepoints, at 4 and 10 months, to detect both early as well as late onset deficits. Motor coordination was assessed using the rotarod test but revealed no differences between homozygous *Dars1*$^{D367Y/D367Y}$ mice and wildtype controls (**Figure 1D**). Explorative behavior, locomotor activity and anxiety were tested in the open field paradigm. Total distance traveled as well as distance traveled in the inner compartment of the apparatus were unchanged between groups (**Figures 1E,F**). The acoustic startle response (ASR), a reflex resulting in muscular activity in response to an acoustic stimulus, was determined to test *Dars1*$^{D367Y/D367Y}$ mice for more subtle changes in information processing speed. Homozygous *mutants,* exposed to short sound pulses (40 ms) with increasing intensities ranging from 60 to

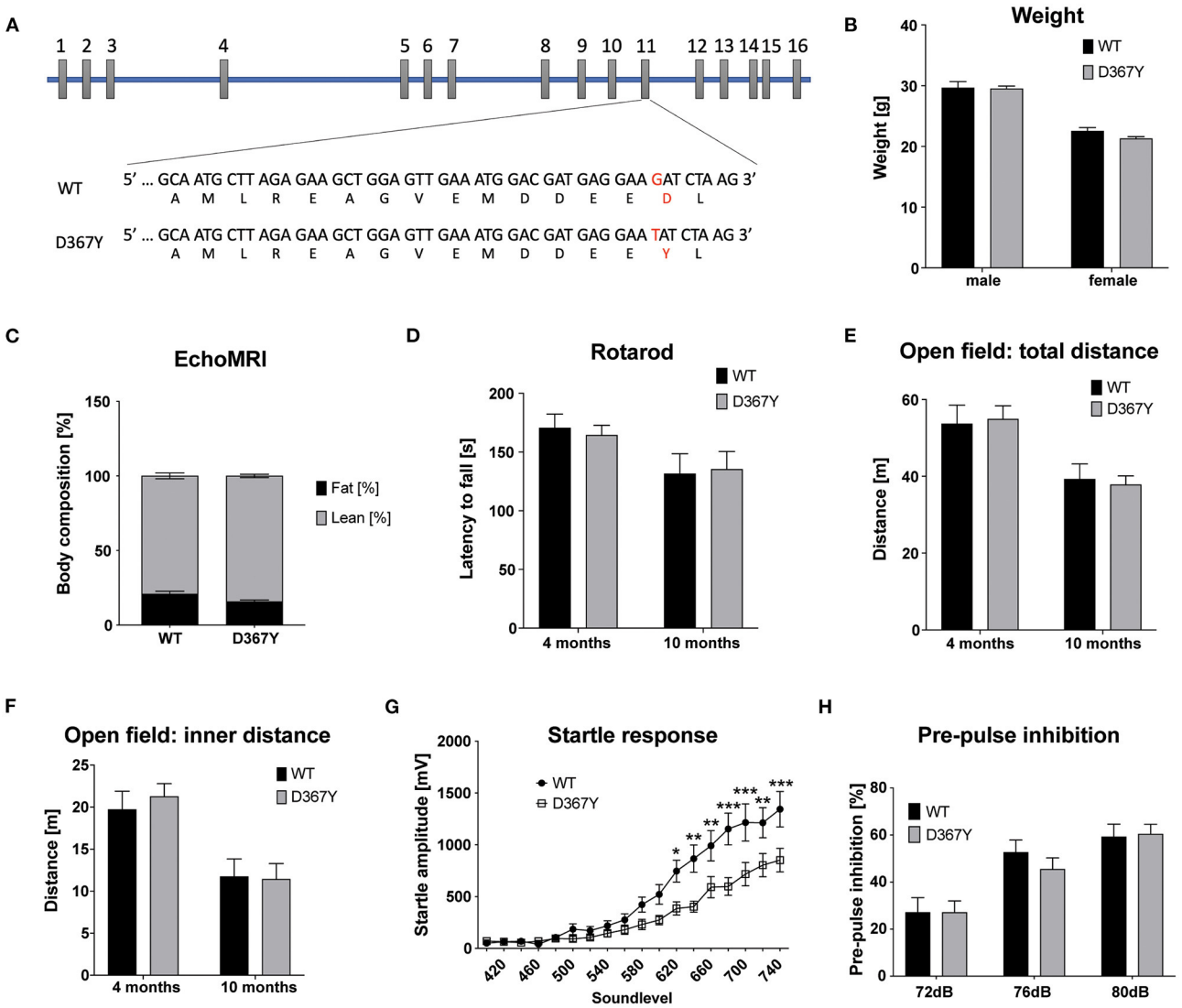


FIGURE 1 | Characterization of homozygous *Dars1*^{D367Y} mice. **(A)** Schematic depicting the genomic location of the D367Y mutation on exon 11 of the Dars1 gene. **(B)** Body weight of homozygous D367Y mice compared to wildtype controls (WT) at 4 months of age (n = 4–6). **(C)** EchoMRI body composition analysis revealed a slight shift from fat to lean mass in homozygous D367Y mice (n = 9). **(D)** Motor coordination assessed by the rotarod test was unchanged between homozygous D367Y mice and wildtype controls (n = 8–24). **(E,F)** Total distance traveled in an open field test apparatus [**(E)**; n = 7–28] and distance in the inner compartment of the apparatus [**(F)**; n = 7–28]. **(G)** Four-months-old homozygous D367Y mice exposed to sound pulses with increasing intensities (60–120 dB SPL, 40 ms) showed a lowered acoustic startle response compared to wildtype controls (n = 33–38). **(H)** Pre-pulse inhibition (72, 76, or 80 dB SPL pre-pulse 100 ms before the 120 dB SPL startle pulse) was unchanged between genotypes (n = 19–36). Data represent mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001; Two-way ANOVA).

120 dB SPL, showed a significantly lower ASR compared to wildtype controls from 105 dB SPL to 120 dB SPL (sound levels 620 to 740; **Figure 1G**). The pre-pulse inhibition (PPI) measures sensorimotor gating mechanisms, and an impairment is an indicator for attentional processing deficits (Koch, 1999). We had observed PPI deficits in heterozygous *Dars1*-null mice (Fröhlich et al., 2017), however, *Dars1*$^{D367Y/D367Y}$ mice were normal (**Figure 1H**).

Aminoacylation Activity of Mutant AspRS

The HBSL index patient was compound heterozygous for the A274V and D367Y *DARS1* mutations. Both mutations are located in the catalytic domain of the AspRS enzyme (Taft et al., 2013) indicating a direct impact on enzymatic activity. Therefore, we measured aminoacylation activity in lysates of HEK293 cells transiently expressing *DARS1*^{WT}, *DARS1*^{A274V}, *DARS1*^{D367Y}, or an equimolar mix of *DARS1*^{A274V} and *DARS1*^{D367Y}. Aminoacylation activity of mutant AspRS variants was normalized to the aminoacylation activity of wildtype AspRS (**Figure 2**). A higher value indicates more efficient charging of tRNA^{Asp} with aspartate. Strikingly, the A274V mutation completely abolished aminoacylation activity of AspRS, resembling a functional null mutation. In contrast, the D367Y mutation led to a slight increase in enzymatic

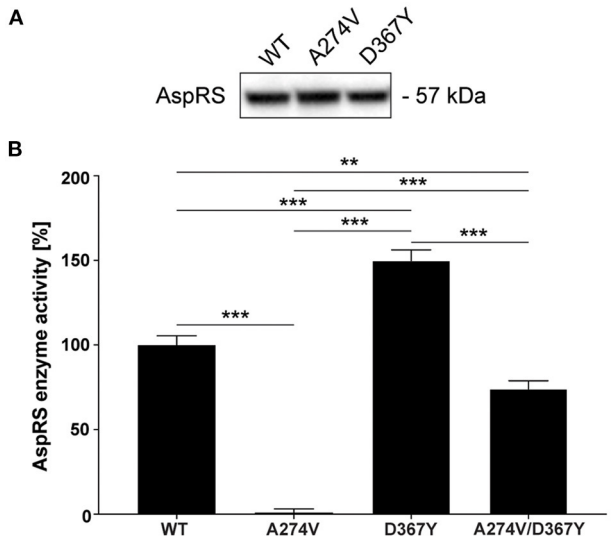


FIGURE 2 | Aminoacylation activity of wildtype AspRS compared to AspRS carrying the A274V or D367Y mutations. **(A)** Western-blot depicting comparable expression levels of DARS1^{WT}, DARS1^{A274V}, DARS1^{D367Y} in HEK293 cell lysates. **(B)** Enzyme activity was measured using lysates of HEK293 cells transiently expressing DARS1^{WT}, DARS1^{A274V}, DARS1^{D367Y}, or an equimolar mix of DARS1^{A274V} and DARS1^{D367Y}. Cell lysates were incubated with *E. coli* tRNA, [$^{13}C_4$, ^{15}N]-aspartate and ATP followed by quantification of [$^{13}C_4$,^{15}N]-aspartate using LC-MS/MS. Aminoacylation activity of AspRS variants is displayed as percentage of WT AspRS activity. Data represent mean ± SEM (**$p < 0.01$, ***$p < 0.001$; One-way ANOVA).

activity. The enzyme activity measured in lysates of HEK293 cells transiently expressing a mix of *DARS1*^{A274V} and *DARS1*^{D367Y}, which corresponds to the enzyme configuration of the index patient, ranged between the activities measured for *DARS1*^{A274V} and *DARS1*^{D367Y} alone (about 70% of wildtype AspRS activity).

Compound Heterozygous *Dars1*$^{D367Y/-}$ Mice Display Developmental Deficits

We next crossed homozygous *Dars1*$^{D367Y/D367Y}$ mice with heterozygous *Dars1*-null mice to create compound heterozygous *Dars1*$^{D367Y/-}$ mice. Since the A274V mutation completely abolished AspRS activity, *Dars1*$^{D367Y/-}$ mice can be considered a phenocopy of the compound heterozygous index patient (*DARS1*$^{A274V/D367Y}$) in regard to enzyme activity.

The expected Mendelian ratio of the *Dars1*$^{D367Y/-}$ and *Dars1*$^{D367Y/+}$ offspring in the F1 generation is 50:50. However, of 286 born mice (72 litters), only 25 mice (9%) had the genotype *Dars1*$^{D367Y/-}$. This developmental disadvantage was sustained postnatally as 48% of *Dars1*$^{D367Y/-}$ mice (4% of all mice) developed hydrocephalus during the first 3 weeks of life and either died naturally or had to be euthanized (**Figures 3A,G**). 24% of *Dars1*$^{D367Y/-}$ (2% of all littermates) suffered from microphthalmia or retinal degeneration in one or both eyes (**Figures 3A,H**). The remainder of the born *Dars1*$^{D367Y/-}$ mice (28% of *Dars1*$^{D367Y/-}$ mice or 3% of total mice) showed growth retardation and were significantly smaller and lighter compared to *Dars1*$^{D367Y/+}$ littermates (**Figures 3A,D,E**). Bodyweight of *Dars1*$^{D367Y/-}$ mice remained lower throughout life (**Figure 3D**) and EchoMRI body composition analysis revealed a significant reduction in body fat mass of *Dars1*$^{D367Y/-}$ mice (13.5% body fat) compared to *Dars1*$^{D367Y/+}$ littermates (24.3% body fat; **Figure 3F**). Lastly, H&E staining of cross sections of the inner ear showed a reduced density of spiral ganglion cells in adult *Dars1*$^{D367Y/-}$ mice (**Figure 3I**).

Intriguingly, at embryonic day 18 (E18), 57% of the embryos present *in utero* were *Dars1*$^{D367Y/-}$ ($n = 21$), which is in line with the expected Mendelian ratio when crossing homozygous *Dars1*$^{D367Y/D367Y}$ mice with heterozygous *Dars1*-null mice. However, these embryos were severely underdeveloped at this stage (**Figures 3B,C**).

Compound Heterozygous *Dars1*$^{D367Y/-}$ Mice Develop Late Onset Motor Deficits

Despite the developmental delay and high mortality of *Dars1*$^{D367Y/-}$ mice, once they passed early adolescence, *Dars1*$^{D367Y/-}$ mice were phenotypically inconspicuous for most of their adult life. A hallmark of HBSL are motor deficits including severe leg spasticity and gait abnormalities. To see whether *Dars1*$^{D367Y/-}$ mice develop similar motor deficits, we performed locomotor tests at two different timepoints (four and 10 months) to distinguish between early and late onset motor deficits. Motor coordination assessed by the rotarod test was unchanged at 4 months but was impaired at 10 months in *Dars1*$^{D367Y/-}$ mice compared to *Dars1*$^{D367Y/+}$ littermates (**Figure 4A**). Total distance traveled in an open field test apparatus and distance in the inner compartment of the apparatus were unchanged at 4 months of age (**Figures 4B,C**). At 10 months, however, the total distance and the distance in the inner compartment were significantly lower in *Dars1*$^{D367Y/-}$ mice compared to *Dars1*$^{D367Y/+}$ littermates indicating a reduction in locomotor activity and explorative behavior (**Figures 4B,C**). No significant differences in the ASR were observed between *Dars1*$^{D367Y/-}$ and *Dars1*$^{D367Y/+}$ mice (**Figure 4D**). PPI, on the other hand, was significantly lower in *Dars1*$^{D367Y/-}$ mice following the 72 dB SPL pre-pulse and showed a trend toward a reduction for the 76 dB SPL and 80 dB SPL pre-pulses (**Figure 4E**).

Spinal Cord Abnormalities in *Dars1*$^{D367Y/-}$ Mice

In order to determine whether the behavioral deficits were accompanied by neurological changes, we analyzed central nervous system (CNS) integrity and myelination. Brain MRI revealed a smaller brain size of *Dars1*$^{D367Y/-}$ mice but did not show any other overt abnormalities or demyelination (**Figure 5A**). LFB staining of coronal brain sections revealed intact brain morphology and no myelination differences between genotypes (**Figure 5B**). In the spinal cord, however, severe vacuolization of the ventral white matter was detected in 10-months-old *Dars1*$^{D367Y/-}$ mice (**Figure 5C**) but not in *Dars1*$^{D367Y/+}$ littermates. LFB staining confirmed white matter vacuolization in *Dars1*$^{D367Y/-}$ mice (**Figure 5D**). In addition,

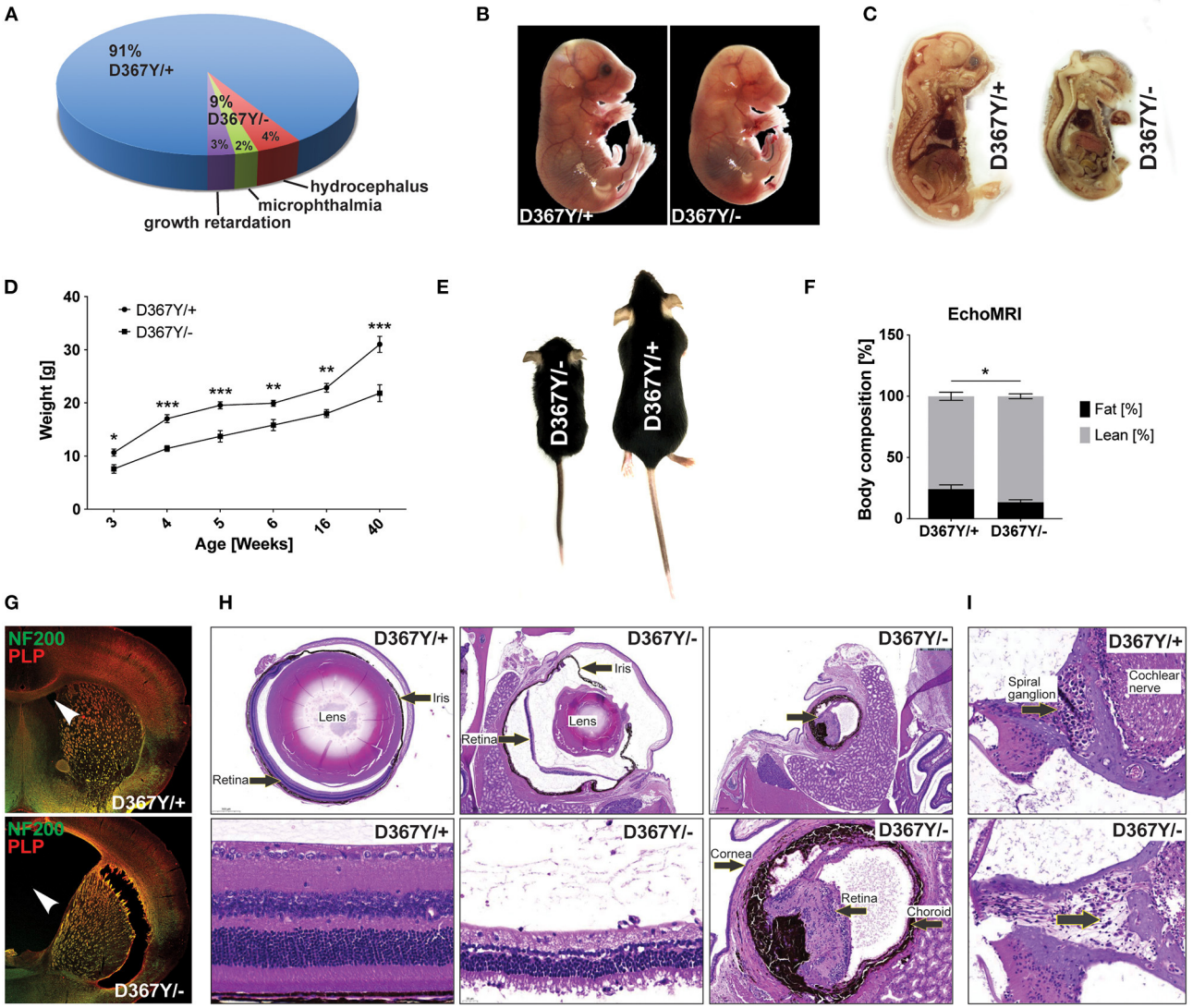


FIGURE 3 | Compound heterozygous Dars1$^{D367Y/-}$ mice display developmental deficits. **(A)** Genotype and phenotype distribution in the F1 generation resulting from breeding of homozygous D367Y and heterozygous Dars1-null mice. Of the 286 born mice (72 litters), only 25 (9%) were compound heterozygous (D367Y/–; expected: 50%). 48% of the born D367Y/– mice (4% of total mice) developed hydrocephalus during the first weeks of their life; 24% of D367Y/– mice (2% of total mice) had microphthalmia in 1 or 2 eyes. **(B)** At embryonic day 18 (E18), 57% of the embryos present *in utero* were D367Y/– ($n = 21$), however, they were significantly underdeveloped at this stage. **(C)** Sagittal sections of D367Y/+ and D367Y/– embryos at E18 showing the severe underdevelopment at this stage. **(D)** Weight of D367Y/+ and D367Y/– females ($n = 4$–11). **(E)** D367Y/– mice are smaller and lighter compared to age- and sex-matched D367Y/+ littermates. **(F)** EchoMRI body composition analysis revealed a significant reduction of body fat in D367Y/– mice ($n = 8$). **(G)** Coronal brain sections of 3-week-old D367Y/– mice with hydrocephalus and D367Y/+ control mice. Sections were labeled with NF200 (green) and PLP (red). **(H)** H&E staining of cross sections of the eyes showing various degrees of microphthalmia and retinal degeneration/atrophy in D367Y/– mice compared to D367Y/+ controls. **(I)** H&E staining of cross sections of the inner ear indicates reduced density of spiral ganglion cells (lower panel, arrow). Data represent mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Two-way ANOVA).

aged *Dars1*$^{D367Y/-}$ mice displayed severe demyelination of the lateral and dorsal white matter (**Figure 5D**).

Reduced Expression of Major Myelin Proteins in *Dars1*$^{D367Y/-}$ Mice

To determine whether myelin defects could be corroborated by reduced expression levels of the major myelin proteins in *Dars1*$^{D367Y/-}$ mice, we analyzed brain tissue of 10-month-old *Dars1*$^{D367Y/-}$ and *Dars1*$^{D367Y/+}$ mice. The brain regions analyzed included cortex, cerebellum, brainstem and basal ganglia. First, we confirmed reduction of *Dars1* mRNA and AspRS protein in *Dars1*$^{D367Y/-}$ mice. As expected, *Dars1* message and AspRS levels were reduced by 50% compared to *Dars1*$^{D367Y/+}$ mice (**Figure 6** top left and **Figures 7A,B**). mRNA levels of the mitochondrial counterpart *Dars2* were unchanged

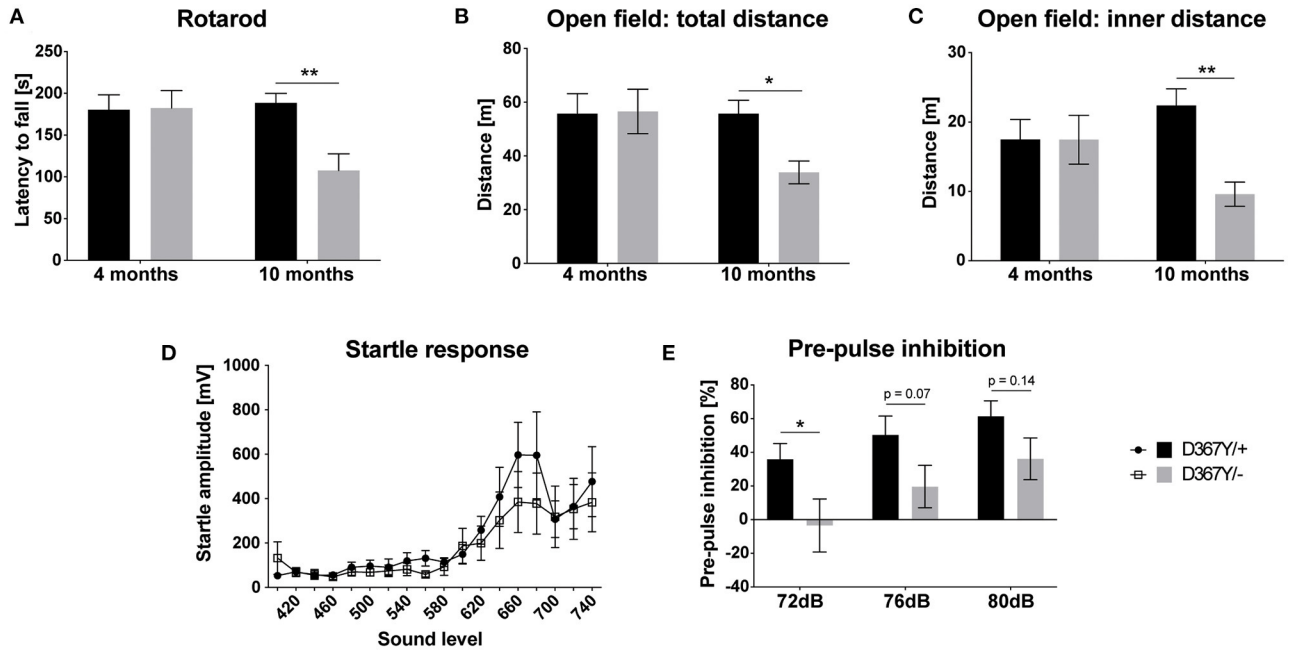


FIGURE 4 | Compound heterozygous *Dars1*$^{D367Y/-}$ mice show impaired pre-pulse inhibition and develop late onset motor deficits. **(A)** Motor coordination assessed by the rotarod test was unchanged at 4 months but was impaired at 10 months in D367Y/– mice compared to D367Y/+ controls ($n = 7$–10). **(B,C)** Total distance traveled in an open field test apparatus [**(B)**; $n = 7$–10] and distance in the inner compartment of the apparatus [**(C)**; $n = 7$–10]. No changes were observed at 4 months but at 10 months total distance and distance in the inner compartment were significantly reduced. **(D)** Four-months-old mice were exposed to sound pulses with increasing intensities (60–120 dB SPL, 40 ms) and the acoustic startle response was measured. No significant differences were observed between D367Y/– and D367Y/+ mice ($n = 8$). **(E)** Pre-pulse inhibition (72, 76, or 80 dB SPL pre-pulse 100 ms before the 120 dB SPL startle pulse) was reduced in 4-months-old D367Y/– mice ($n = 8$). Data represent mean ± SEM (*$p < 0.05$, **$p < 0.01$; Two-way ANOVA).

ruling out potential compensatory mechanisms (**Figure 6** top middle). Markers of oligodendrocytes (Aspa) or myelin (PLP, CNP and MBP) were reduced at the mRNA (**Figure 6**) and protein (**Figure 7**) level, in cerebellum and brainstem. While this trend reached statistical significance only in the brainstem, this finding suggests potential demyelination or oligodendrocyte loss in the hindbrain of *Dars1*$^{D367Y/-}$ mice.

DISCUSSION

To date, accurate HBSL models have been lacking, precluding examination of the underlying pathomechanism and testing of potential therapies. Here, we introduced the HBSL-causing *Dars1*^{D367Y} point mutation into the mouse genome. Unexpectedly, mice carrying this mutation homozygously did not show any characteristic HBSL pathology. To enhance the phenotype of *Dars1*$^{D367Y/D367Y}$ mice, we crossed them with *Dars1-null* carriers. The *Dars1*$^{D367Y/-}$ offspring displayed a severe developmental delay associated with early lethality either *in utero* or as a result of hydrocephalus during the first 3 weeks of life. Another frequently observed feature of *Dars1*$^{D367Y/-}$ mice was microphthalmia in one or both eyes. All *Dars1*$^{D367Y/-}$ mice were underdeveloped with reduced body weight and fat mass compared to *Dars1*$^{D367Y/+}$ littermates but remained otherwise phenotypically inconspicuous until 10 months of age when they developed late onset motor deficits as well as vacuolization and demyelination of the spinal cord white matter. These behavioral and morphological changes were accompanied by reduced expression levels of various myelin and oligodendrocyte markers, predominantly in the hindbrain of *Dars1*$^{D367Y/-}$ mice.

The A274V mutation resulted in a complete loss of aminoacylation activity, resembling a functional null mutation. In light of the lethal phenotype of homozygous null mice (Fröhlich et al., 2017) this suggests that a bi-allelic A274V mutation would not be viable in mouse or man. Intriguingly, the D367Y mutation did not decrease AspRS enzyme activity but instead led to a slight increase in activity. A potential explanation for the observed increase in enzymatic activity of *Dars1*^{D367Y} could be reduced specificity of AspRS for tRNA^{Asp}. As a consequence, other tRNAs would bind to AspRS and subsequently be mischarged with aspartate. These mischarged tRNAs would then incorporate the wrong amino acid into the growing polypeptide chain during translation ultimately resulting in either non-functional proteins or unfolded and misfolded proteins. An accumulation of un- and misfolded proteins in the endoplasmic reticulum (ER) can trigger the unfolded protein response (UPR), an adaptive pathway that under normal circumstances protects the cell from ER stress

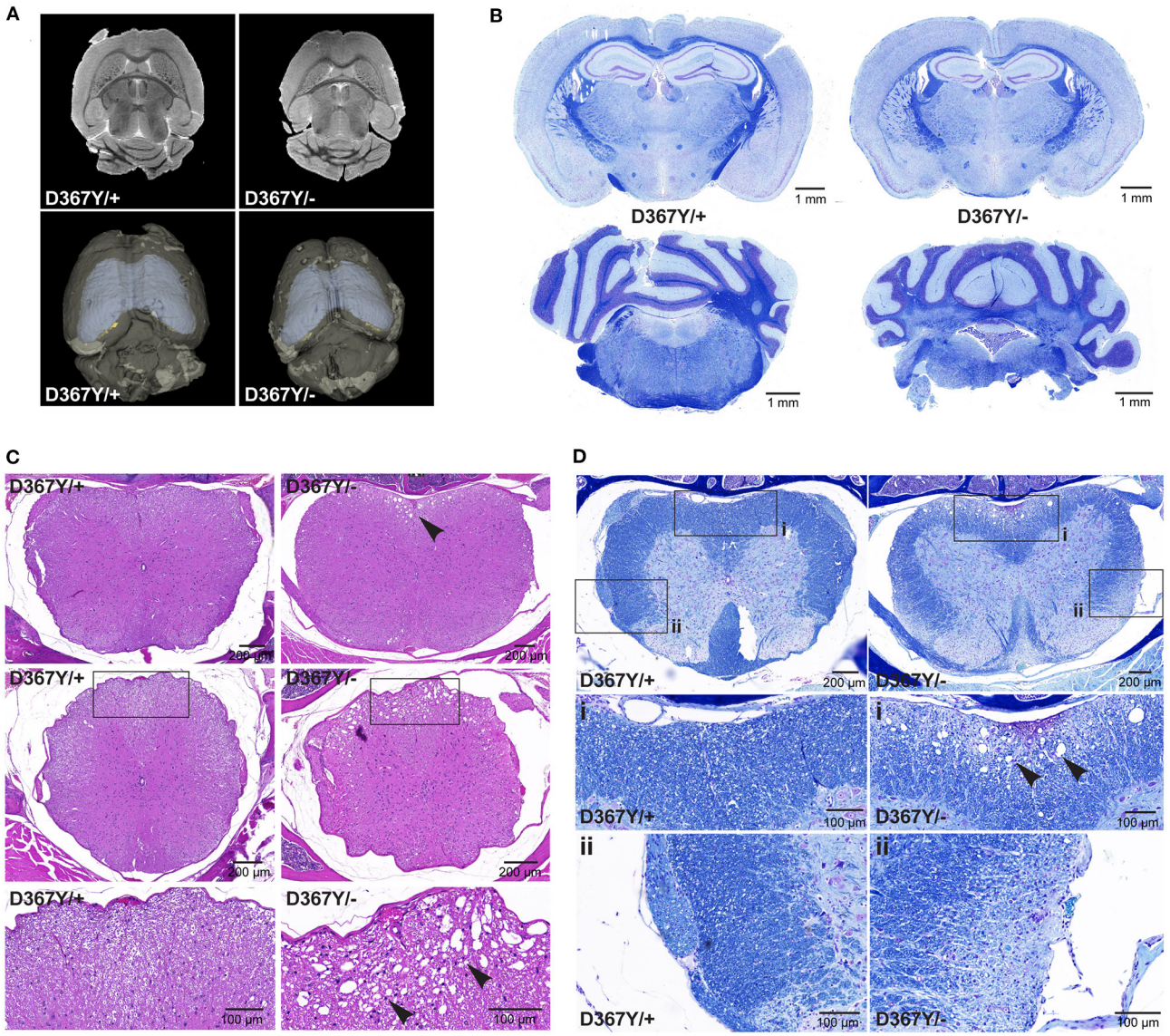


FIGURE 5 | Compound heterozygous *Dars1*$^{D367Y/-}$ mice display spinal cord white matter abnormalities. **(A)** Brain magnetic resonance imaging (MRI) revealed an overall smaller brain size of D367Y/– mice but did not show any other abnormalities or demyelination. **(B)** Luxol fast blue (LFB) staining of coronal brain sections revealed no morphological or myelination differences between genotypes. **(C)** Hematoxylin and Eosin (H&E) staining of spinal cord cross sections (top row: lumbar; middle row: thoracic) revealed severe vacuolization of the ventral white matter in D367Y/– mice (arrowheads; $n = 2$). **(D)** LFB staining of lumbar spinal cord cross sections confirmed white matter vacuolization in D367Y/– mice [close-up **(A)**, arrowheads] and revealed significant demyelination of the lateral and dorsal white matter [close-up **(B)**; $n = 2$]. Analyses were performed in 10-months-old mice.

and restores normal ER function (Lin and Popko, 2009). If the UPR fails to cope with sustained ER stress, apoptosis is triggered to eliminate malfunctioning cells (Faitova et al., 2006; Szegezdi et al., 2006). This normally protective mechanism can become pathological and has been implicated in the pathophysiology of many neurodegenerative (Szegezdi et al., 2006) and white matter diseases such as Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease, Vanishing White Matter disease, and multiple sclerosis (Lin and Popko, 2009). A similar underlying pathophysiological mechanism has been suggested for HBSL (Fröhlich et al., 2017) and is supported by data from this study. Accordingly, activation of the mitochondrial unfolded protein response (UPR^{mt}) has been demonstrated in conditional *Dars2* knockout mice (Dogan et al., 2014; Aradjanski et al., 2017). *Dars2* encodes the mitochondrial aspartyl-tRNA synthetase (mt-AspRS) and mutations in the *Dars2* gene result in *l*eukoencephalopathy with *b*rainstem and *s*pinal cord involvement and *l*actate elevation (LBSL), which was first described in 2003 (van der Knaap et al., 2003; Scheper et al., 2007).

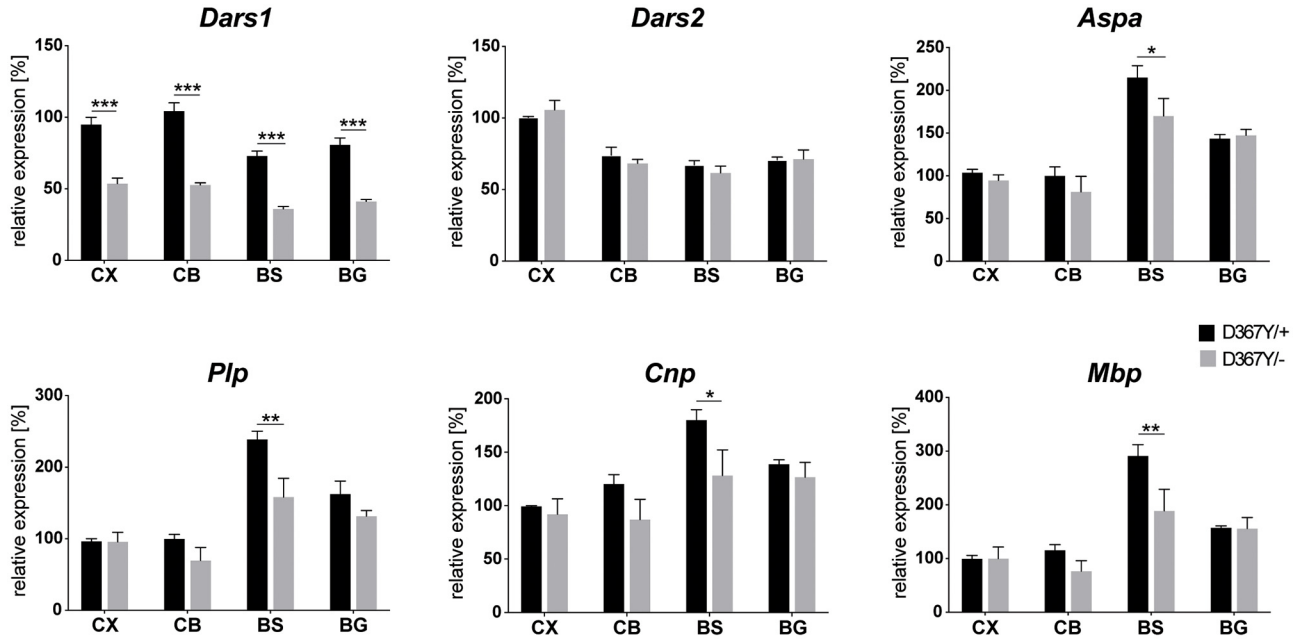


FIGURE 6 | mRNA levels of Dars1 and the major myelin proteins are reduced in compound heterozygous *Dars1*$^{D367Y/-}$ mice. mRNA was extracted from brain tissue of 10-month-old D367Y/– and D367Y/+ mice and quantified via qRT-PCR using TaqMan probes specific for Dars1, Dars2, Aspa, Plp, Cnp, and Mbp. Brain regions analyzed included cortex (CX), cerebellum (CB), brainstem (BS), and basal ganglia (BG). Expression levels were normalized to the housekeeper GusB ($n = 4$). Data represent mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Two-way ANOVA).

Patients affected by HBSL or LBSL display similar symptomology and MRI patterns involving the same CNS tracts and structures. While both diseases might share a common underlying disease mechanism, such as failure of the UPR and UPR^{mt} due to an excessive accumulation of un- and misfolded proteins, there is no functional redundancy between AspRS and mt-AspRS. This has been demonstrated in *Dars1* and *Dars2* null mice, both displaying early embryonic lethality (Dogan et al., 2014; Fröhlich et al., 2017). HBSL and LBSL mainly affect the nervous system, a phenomenon that has also been observed in other ARS-related conditions (Fuchs et al., 2019). The majority of reported LBSL patients possess a splice site mutation in intron two of the *DARS2* gene. This splice defect particularly affects neural cells providing an explanation for the CNS predilection seen in LBSL (van Berge et al., 2012). The selective vulnerability of neural cells in HBSL can be explained with the distinct AspRS expression pattern in the CNS of mice and humans (Fröhlich et al., 2017, Fröhlich et al., 2018). Accordingly, we observed CNS defects in *Dars1*$^{D367Y/-}$ mice including vacuolization and demyelination of the spinal cord white matter. White matter vacuolization is the most common myelin pathology and has been described for many leukodystrophies including Canavan's disease and Vanishing white matter disease (Duncan and Radcliff, 2016). When vacuolization occurs in conjunction with myelin degeneration it can be referred to as spongiform degeneration, however, myelin vacuolization can also occur independent of demyelination (Duncan and Radcliff, 2016). In the case of *Dars1*$^{D367Y/-}$ mice, the vacuolization in the spinal cord was accompanied by demyelination and might reflect a separation of myelin layers (Barkovich, 2000).

Protein synthesis is often regarded as a house-keeping function of all cells. Nevertheless, many aspects of protein synthesis are differently regulated across cell types and developmental stages in order to establish differences in cell identity, function and homeostasis (Buszczak et al., 2014). These differences might explain why *Dars1*$^{D367Y/-}$ mice are particularly susceptible to changes in protein synthesis during development resulting in developmental delay and in some cases premature death. Once *Dars1*$^{D367Y/-}$ mice made it through adolescence, the remaining AspRS activity seems to be sufficient to maintain normal tissue homeostasis. However, the effects of impaired protein synthesis seem to either accumulate over the lifetime or become more profound in aged mice, resulting in the observed late onset motor deficits and spinal cord abnormalities.

Many *Dars1*$^{D367Y/-}$ mice developed hydrocephalus during the first 3 weeks of life. Despite not being a frequently observed feature of HBSL, it has been described in one HBSL patient who consequently required ventriculo-peritoneal shunting to release pressure on the brain (Ong et al., 2020). Another commonly detected symptom of *Dars1*$^{D367Y/-}$ mice was severe microphthalmia or even anophthalmia, major structural malformations or even complete absence of the eye. These abnormalities were not only present in the born *Dars1*$^{D367Y/-}$ mice but also visible in 18-day-old *Dars1*$^{D367Y/-}$ embryos indicating that they occur early during embryogenesis as a result of impaired protein synthesis. The peripheral pathologies observed in *Dars1*$^{D367Y/-}$ mice, such as reduced size, body

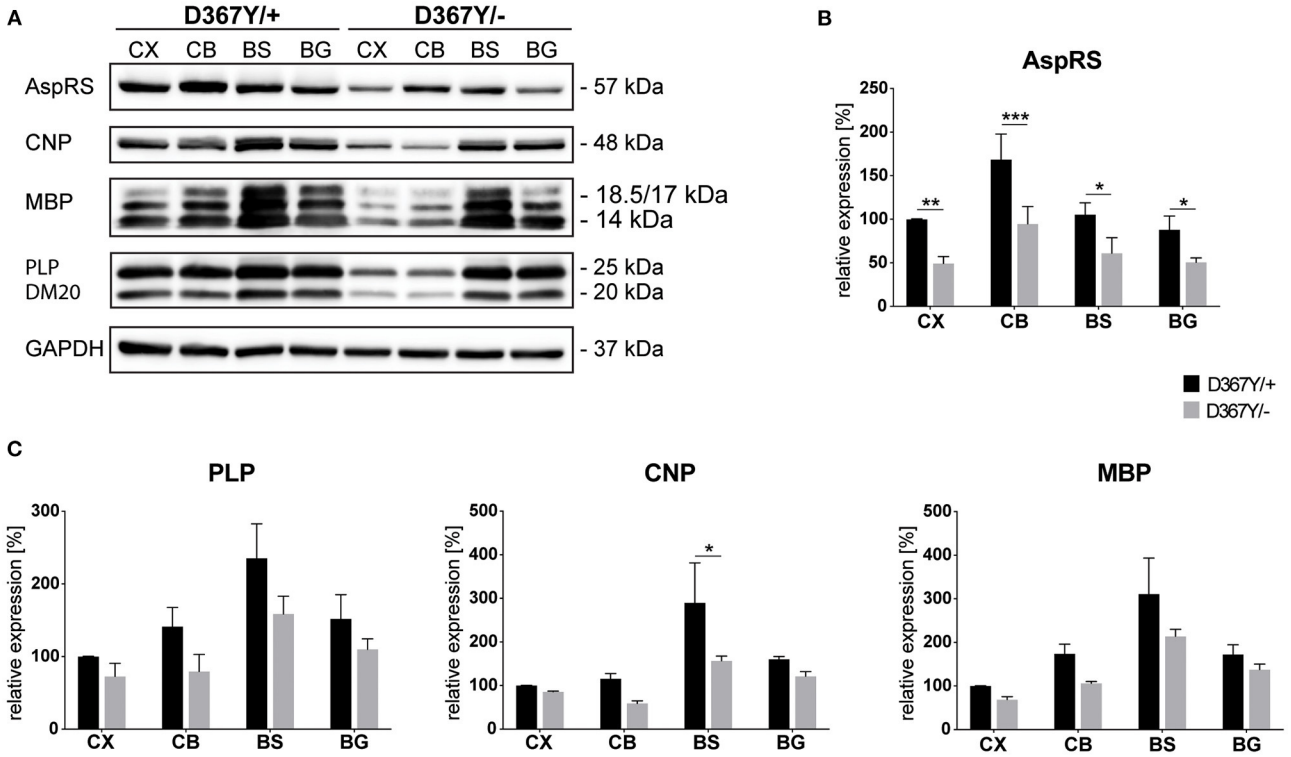


FIGURE 7 | Expression levels of AspRS and the major myelin proteins in compound heterozygous *Dars1*$^{D367Y/-}$ mice. **(A)** Western-blot depicting AspRS, the myelin proteins CNP, MBP, and PLP/DM20, as well as the housekeeping protein GAPDH in different brain regions of 10-month-old D367Y/– and D367Y/+ mice. Brain regions include cortex (CX), cerebellum (CB), brainstem (BS), and basal ganglia (BG). **(B)** Relative AspRS expression normalized to GAPDH ($n = 3$). **(C)** Relative expression of the myelin proteins PLP, CNP, and MBP normalized to GAPDH ($n = 3$). Data represent mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Two-way ANOVA).

weight and fat mass as well as microphthalmia have not been reported for HBSL patients yet. While this might be due to the low number of cases reported to date, tests for peripheral deficits should be included in the clinical context in the future.

Homozygous *Dars1*$^{D367Y/D367Y}$ mice showed a lower ASR compared to wildtype mice, whereas *Dars1*$^{D367Y/-}$ mice displayed reduced PPI of the ASR. A PPI reduction is indicative of impaired sensorimotor gating mechanisms (Koch, 1999). The genetic cause of this impairment appears to be the *Dars1*-null mutation since reduced PPI was also a feature of heterozygous *Dars1*-null mice (Fröhlich et al., 2017). The ASR is mediated by a simple neuronal circuit of the lower brainstem involving neurons of the caudal pontine reticular nucleus (PnC) (Koch, 1999). PPI, on the other hand, involves a complex interplay of many brain areas ultimately resulting in a dampening of the ASR via the PnC. PPI is reduced in a variety of neurological disorders including schizophrenia, Huntington's disease, Tourette's syndrome, and ADHD (Swerdlow and Geyer, 1998; Koch, 1999) and, based on our results, might also be a feature of HBSL. Reduced PPI was present in severely affected *Dars1*$^{D367Y/-}$ mice as well as in mildly affected heterozygous *Dars1*-null mice (Fröhlich et al., 2017), suggesting to test for ASR and PPI in the clinical context as they might support an early diagnosis of HBSL.

While *Dars1*$^{D367Y/-}$ mice do not model every aspect of the clinical HBSL picture, they enable studies of both, early and late onset deficits, which so far was precluded in *Dars1* knockout mice due to embryonic lethality. As such, mice harboring the hypomorphic *Dars1*^{D367Y} allele represent the first tool enabling therapeutic proof-of-concept studies including nutraceutical L-ornithine-L-aspartate (LOLA) supplementation to boost AspRS activity (Das et al., 2020) or *DARS1* gene replacement therapy. The introduction of other HBSL point mutations might produce a more accurate disease model with an early disease onset as seen in the majority of HBSL patients. Moreover, the use of a conditional knockout system will be instrumental in dissecting the contribution of *Dars1*-deficiency in select organs or cell lineages similar to what has been reported for *Dars2* (Aradjanski et al., 2017; Nemeth et al., 2020; Rumyantseva et al., 2020).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by University of New South Wales Animal Care and Ethics Committee.

AUTHOR CONTRIBUTIONS

DF and MK designed the study. DF, MIM, AJK, MJH, GSS, and AB conducted the research. DF, MIM, and MK analyzed the data. GDH contributed to the experimental design and manuscript preparation. DF and MK led the project and the manuscript production. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was funded by the Medical Research Future Fund (MRFF-ARLKO), the European Leukodystrophy Association (ELA 2015-016I3 and ELA 2018-014I2) and the Mission Massimo Foundation. The MAGEC and the HOPS are supported by Phenomics Australia (PA; formerly known as Australian Phenomics Network) and PA is supported by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS) program. We acknowledge the facilities, and the scientific and technical assistance of the Melbourne Advanced Genome Editing Center (MAGEC) at the Walter and Eliza Hall Institute of Medical Research (WEHI) and the Histopathology and Organ Pathology Service (HOPS), University of Melbourne. We acknowledge the National Imaging Facility (NIF), Australia, for the financial support of MR imaging instrumentation, and the scientific and technical assistance at the UNSW NIF node, Mark Wainwright Analytical Center, Biological Imaging Resources Laboratory. In this context, we also thank Mr. Brendan Lee for his help with image acquisitions.

REFERENCES

Aradjanski, M., Dogan, S. A., Lotter, S., Wang, S., Hermans, S., Wibom, R., et al. (2017). DARS2 protects against neuroinflammation and apoptotic neuronal loss, but is dispensable for myelin producing cells. *Hum. Mol. Genet*. 26, 4181–4189. doi: 10.1093/hmg/ddx307

Barkovich, A. J. (2000). Concepts of myelin and myelination in neuroradiology. *AJNR Am. J. Neuroradiol*. 21, 1099–1109. Available online at: http://www.ajnr.org/content/21/6/1099.long

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, 718–725. doi: 10.1212/WNL.0b013e3181eee46b

Buszczak, M., Signer, R. A., and Morrison, S. J. (2014). Cellular differences in protein synthesis regulate tissue homeostasis. *Cell* 159, 242–251. doi: 10.1016/j.cell.2014.09.016

Das, A., Frohlich, D., Achanta, L. B., Rowlands, B. D., Housley, G. D., Klugmann, M., et al. (2020). L-aspartate, L-ornithine and L-ornithine-L-aspartate (LOLA) and their impact on brain energy metabolism. *Neurochem. Res*. 45, 1438–1450. doi: 10.1007/s11064-020-03044-9

Dogan, S. A., Pujol, C., Maiti, P., Kukat, A., Wang, S., Hermans, S., et al. (2014). Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart. *Cell Metab*. 19, 458–469. doi: 10.1016/j.cmet.2014.02.004

Duncan, I. D., and Radcliff, A. B. (2016). Inherited and acquired disorders of myelin: the underlying myelin pathology. *Exp. Neurol*. 283 (Pt. B), 452–475. doi: 10.1016/j.expneurol.2016.04.002

Faitova, J., Krekac, D., Hrstka, R., and Vojtesek, B. (2006). Endoplasmic reticulum stress and apoptosis. *Cell. Mol. Biol. Lett*. 11, 488–505. doi: 10.2478/s11658-006-0040-4

Fröhlich, D., Suchowerska, A. K., Spencer, Z. H., von Jonquieres, G., Klugmann, C. B., Bongers, A., et al. (2017). *In vivo* characterization of the aspartyl-tRNA synthetase DARS: homing in on the leukodystrophy HBSL. *Neurobiol. Dis*. 97 (Pt. A), 24–35. doi: 10.1016/j.nbd.2016.10.008

Fröhlich, D., Suchowerska, A. K., Voss, C., He, R., Wolvetang, E., von Jonquieres, G., et al. (2018). Expression pattern of the aspartyl-tRNA synthetase DARS in the human brain. *Front. Mol. Neurosci*. 11:81. doi: 10.3389/fnmol.2018.00081

Fuchs, S. A., Schene, I. F., Kok, G., Jansen, J. M., Nikkels, P. G. J., van Gassen, K. L. I., et al. (2019). Aminoacyl-tRNA synthetase deficiencies in search of common themes. *Genet. Med*. 21, 319–330. doi: 10.1038/s41436-018-0048-y

Kikinis, R., Pieper, S. D., and Vosburgh, K. G. (2014). "3D slicer: a platform for subject-specific image analysis, visualization, and clinical support," in *Intraoperative Imaging and Image-Guided Therapy*, eds F. Jolesz (New York, NY: Springer).

Kim, K. R., Park, S. H., Kim, H. S., Rhee, K. H., Kim, B. G., Kim, D. G., et al. (2013). Crystal structure of human cytosolic aspartyl-tRNA synthetase, a component of multi-tRNA synthetase complex. *Proteins* 81, 1840–1846. doi: 10.1002/prot.24306

Koch, M. (1999). The neurobiology of startle. *Prog. Neurobiol*. 59, 107–128. doi: 10.1016/S0301-0082(98)00098-7

Kueh, A. J., Pal, M., Tai, L., Liao, Y., Smyth, G. K., Shi, W., et al. (2017). An update on using CRISPR/Cas9 in the one-cell stage mouse embryo for generating complex mutant alleles. *Cell Death Differ*. 24, 1821–1822. doi: 10.1038/cdd.2017.122

Lin, W., and Popko, B. (2009). Endoplasmic reticulum stress in disorders of myelinating cells. *Nat. Neurosci*. 12, 379–385. doi: 10.1038/nn.2273

Muthiah, A., Housley, G. D., Klugmann, M., and Fröhlich, D. (2020). The leukodystrophies HBSL and LBSL - correlates and distinction. *Front. Cell. Neurosci*. doi: 10.3389/fncel.2020.626610

Nemeth, C. L., Tomlinson, S. N., Rosen, M., O'Brien, B. M., Larraza, O., Jain, M., et al. (2020). Neuronal ablation of mt-AspRS in mice induces immune pathway activation prior to severe and progressive cortical and behavioral disruption. *Exp. Neurol*. 326:113164. doi: 10.1016/j.expneurol.2019.113164

Ong, M. T., Willoughby, J., Connolly, D. J. A., Mordekar, S., Study, D., and Johnson, D. (2020). Genotype–phenotype variability of DARS mutation—case reports of a trio of siblings. *Euro. J. Med. Case Rep*. 4, 110–115. doi: 10.24911/ejmcr/1731551044010

Rumyantseva, A., Motori, E., and Trifunovic, A. (2020). DARS2 is indispensable for purkinje cell survival and protects against cerebellar ataxia. *Hum. Mol. Genet*. 29, 2845–2854. doi: 10.1093/hmg/ddaa176

Scheper, G. C., van der Klok, T., van Andel, R. J., van Berkel, C. G., Sissler, M., Smet, J., et al. (2007). Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. *Nat. Genet*. 39, 534–539. doi: 10.1038/ng2013

Schneider, M., Spanagel, R., Zhang, S. J., Bading, H., and Klugmann, M. (2007). Adeno-associated virus. (AAV)-mediated suppression of Ca2+/calmodulin kinase IV activity in the nucleus accumbens modulates emotional behaviour in mice. *BMC Neurosci*. 8:105. doi: 10.1186/1471-2202-8-105

Swerdlow, N. R., and Geyer, M. A. (1998). Using an animal model of deficient sensorimotor gating to study the pathophysiology and new treatments of schizophrenia. *Schizophr. Bull*. 24, 285–301. doi: 10.1093/oxfordjournals.schbul.a033326

Szegezdi, E., Logue, S. E., Gorman, A. M., and Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep*. 7, 880–885. doi: 10.1038/sj.embor.7400779

Taft, R. J., Vanderver, A., Leventer, R. J., Damiani, S. A., Simons, C., Grimmond, S. M., et al. (2013). Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am. J. Hum. Genet*. 92, 774–780. doi: 10.1016/j.ajhg.2013.04.006

van Berge, L., Dooves, S., van Berkel, C. G., Polder, E., van der Knaap, M. S., and Scheper, G. C. (2012). Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation is associated with cell-type-dependent splicing of mtAspRS mRNA. *Biochem. J*. 441, 955–962. doi: 10.1042/BJ20110795

van der Knaap, M. S., van der Voorn, P., Barkhof, F., Van Coster, R., Krageloh-Mann, I., Feigenbaum, A., et al. (2003). A new leukoencephalopathy with

brainstem and spinal cord involvement and high lactate. *Ann. Neurol.* 53, 252–258. doi: 10.1002/ana.10456

von Jonquieres, G., Mersmann, N., Klugmann, C. B., Harasta, A. E., Lutz, B., Teahan, O., et al. (2013). Glial promoter selectivity following AAV-delivery to the immature brain. *PLoS ONE* 8:e65646. doi: 10.1371/journal.pone.0065646

von Jonquieres, G., Spencer, Z. H. T., Rowlands, B. D., Klugmann, C. B., Bongers, A., Harasta, A. E., et al. (2018). Uncoupling N-acetylaspartate from brain pathology: implications for Canavan disease gene therapy. *Acta Neuropathol.* 135, 95–113. doi: 10.1007/s00401-017-1784-9

Wolf, N. I., Toro, C., Kister, I., Latif, K. A., Leventer, R., Pizzino, A., et al. (2015). DARS-associated leukoencephalopathy can mimic a steroid-responsive neuroinflammatory disorder. *Neurology* 84, 226–230. doi: 10.1212/WNL.0000000000001157

Conflict of Interest: MK is an employee of Boehringer Ingelheim Pharma GmbH & Co. KG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 22 January 2021
doi: 10.3389/fncel.2020.627887

Megalencephalic Leukoencephalopathy: Insights Into Pathophysiology and Perspectives for Therapy

Assumpció Bosch[1,2,3]* *and Raúl Estévez*[4,5]*

[1]*Department of Biochemistry and Molecular Biology, Institute of Neurosciences, Univ. Autònoma de Barcelona, Barcelona, Spain,* [2]*Unitat Mixta UAB-VHIR, Vall d'Hebron Institut de Recerca (VHIR), Barcelona, Spain,* [3]*Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain,* [4]*Departament de Ciències Fisiològiques, IDIBELL—Institute of Neurosciences, Universitat de Barcelona, Barcelona, Spain,* [5]*Centro de Investigación Biomédica en Red sobre Enfermedades Raras (CIBERER), Instituto de Salud Carlos III, Madrid, Spain*

OPEN ACCESS

Edited by:
Matthias Klugmann, University of New South Wales, Australia

Reviewed by:
Arturo Ortega, Instituto Politécnico Nacional de México (CINVESTAV), Mexico
Rogier Min, Amsterdam University Medical Center, Netherlands

***Correspondence:**
Assumpció Bosch
assumpcio.bosch@uab.cat
Raúl Estévez
restevez@ub.edu

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *10 November 2020*
Accepted: *30 December 2020*
Published: *22 January 2021*

Citation:
Bosch A and Estévez R (2021) Megalencephalic Leukoencephalopathy: Insights Into Pathophysiology and Perspectives for Therapy. Front. Cell. Neurosci. 14:627887. doi: 10.3389/fncel.2020.627887

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is a rare genetic disorder belonging to the group of vacuolating leukodystrophies. It is characterized by megalencephaly, loss of motor functions, epilepsy, and mild mental decline. In brain biopsies of MLC patients, vacuoles were observed in myelin and in astrocytes surrounding blood vessels. It is mainly caused by recessive mutations in *MLC1* and *HEPACAM* (also called *GLIALCAM*) genes. These disease variants are called MLC1 and MLC2A with both types of patients sharing the same clinical phenotype. Besides, dominant mutations in *HEPACAM* were also identified in a subtype of MLC patients (MLC2B) with a remitting phenotype. MLC1 and GlialCAM proteins form a complex mainly expressed in brain astrocytes at the gliovascular interface and in Bergmann glia at the cerebellum. Both proteins regulate several ion channels and transporters involved in the control of ion and water fluxes in glial cells, either directly influencing their location and function, or indirectly regulating associated signal transduction pathways. However, the MLC1/GLIALCAM complex function and the related pathological mechanisms leading to MLC are still unknown. It has been hypothesized that, in MLC, the role of glial cells in brain ion homeostasis is altered in both physiological and inflammatory conditions. There is no therapy for MLC patients, only supportive treatment. As MLC2B patients show an MLC reversible phenotype, we speculated that the phenotype of MLC1 and MLC2A patients could also be mitigated by the re-introduction of the correct gene even at later stages. To prove this hypothesis, we injected in the cerebellar subarachnoid space of *Mlc1* knockout mice an adeno-associated virus (AAV) coding for human MLC1 under the control of the glial-fibrillary acidic protein promoter. MLC1 expression in the cerebellum extremely reduced myelin vacuolation at all ages in a dose-dependent manner. This study could be considered as the first preclinical approach for MLC. We also suggest other potential therapeutic strategies in this review.

Keywords: myelin abnormalities, ion channel, water homeostasis, chloride, cell-cell adhesion

INTRODUCTION

Leukodystrophies are human diseases affecting the central nervous system (CNS) myelin. Although individual leukodystrophies are rare, epidemiological data are indicating their high incidence. Within the leukodystrophies, Megalencephalic leukoencephalopathy with subcortical cysts (MLC; MIM 604004) is a vacuolating white matter disorder of infantile-onset (van der Knaap et al., 2012). Macrocephaly is observed in all affected individuals and develops during the first year of life. Most patients show delayed mental or motor development and seizures (Hamilton et al., 2018). Magnetic resonance imaging (MRI) is crucial to diagnose the disease in childhood, showing diffuse signal abnormality, swelling of the cerebral white matter, and the presence of subcortical cysts, which are mostly present in the anterior temporal areas and often in the frontoparietal region (van der Knaap et al., 1995).

From this initial presentation, which is common to all MLC patients, two different phenotypes have been described: a classical phenotype, which is found in the majority of patients, and an improving phenotype, which shows a bettering clinical course (van der Knaap et al., 2010). In the MLC classical phenotype, there is a gradual onset of ataxia, spasticity, and sometimes extrapyramidal findings; and late-onset of mild mental deterioration. In the improving phenotype, motor function ameliorates or normalizes; some patients have stable intellectual disability, with or without autism (Hamilton et al., 2018).

The classical MLC can be caused by two biallelic mutations in the *MLC1* or *HEPACAM* genes, as the disease is inherited in a recessive manner (Leegwater et al., 2001; López-Hernández et al., 2011a). Classical MLC patients with mutations in *MLC1* are diagnosed as MLC1 type, whereas those with mutations in *HEPACAM* are named MLC2A. The improving phenotype, on the other hand, is caused by heterozygous mutations in *HEPACAM* (López-Hernández et al., 2011a). These patients are diagnosed as MLC2B type, and the disease is inherited dominantly. Mutations in *MLC1* and *HEPACAM* are found in 76% and 22% of the patients, respectively. Pathogenic variants in *MLC1* or *HEPACAM* have not been identified in the remaining 2% of individuals showing clinical features of MLC, suggesting the existence of unknown additional genes of the disease (van der Knaap et al., 1993).

MLC1 encodes for an integral oligomeric membrane protein of unknown function expressed only in astrocytes, being one of the most specific proteins for this cellular type (Teijido et al., 2004, 2007; Boor et al., 2005; Ambrosini et al., 2008). Mutations found in MLC1 patients are distributed all over the protein and they cause protein instability, which leads to its degradation at the endoplasmic reticulum (ER) or in lysosomes (Duarri et al., 2008; Lanciotti et al., 2010; Petrini et al., 2013). *HEPACAM* encodes for a single transmembrane type I protein (Moh et al., 2005), which works as an adhesion molecule called HepaCAM or GlialCAM, the latter reflecting its higher expression in glial cells (Barrallo-Gimeno et al., 2015). GlialCAM can form homophilic interactions with other GlialCAM molecules within the same cell (*cis* interactions) and between different cells (*trans* interactions; Elorza-Vidal et al., 2020). Most missense mutations found in MLC2A or MLC2B patients are located in the extracellular domain of the protein, and affect protein localization at cell-cell junctions (López-Hernández et al., 2011a; Arnedo et al., 2014a,b; Elorza-Vidal et al., 2020). A recessive mutation in the signaling peptide of GlialCAM (p.L23H) abolishes protein expression in transfected cells *in vitro*, suggesting that the lack of GlialCAM expression can also cause the disease (Arnedo et al., 2014b). Based on recent biochemical and structural data, it seems that missense dominant MLC2B mutations specifically affect GlialCAM *cis* and *trans* interaction surfaces (Elorza-Vidal et al., 2020).

GlialCAM exerts two roles on MLC1: on one hand, it acts as an ER chaperone (Capdevila-Nortes et al., 2013) and, on the other hand, it confines MLC1 at astrocyte-astrocyte junctions (López-Hernández et al., 2011b). Thus, most MLC2A and MLC2B *HEPACAM* mutations affect GlialCAM targeting and, consequently, also MLC1 location to cell-cell junctions. As GlialCAM improves MLC1 folding, co-expression of GlialCAM with mutated MLC1 increases levels of MLC1 protein and its membrane expression (Capdevila-Nortes et al., 2013).

EFFECT OF MLC MUTATIONS INTO GLIALCAM AND MLC1 CELL BIOLOGY

Different cellular models have been used to express wild-type MLC1 and GlialCAM or containing MLC mutations, such as *Xenopus oocytes* (Teijido et al., 2004), *Spodoptera frugiperda* (Sf9) cells (Ridder et al., 2011) and other cell lines such as fibroblast HeLa (López-Hernández et al., 2011b), COS-1 (Hwang et al., 2019), HEK293 or glioblastoma U373 (Ambrosini et al., 2008) and human U251 astrocytoma (Lanciotti et al., 2020). Other studies have been performed in primary astrocyte cultures from mice, rats, and humans (Duarri et al., 2011; Ridder et al., 2011; Brignone et al., 2014). Finally, studies have also been performed in lymphoblast cell lines and human monocytes or monocyte-derived macrophages from control and MLC patients (Duarri et al., 2008; Petrini et al., 2013). Studies with human astrocytes derived from iPS cells would also be useful to gather more information on the disease.

From a pathological point of view, these studies in cell lines have defined the biochemical mechanism of how different MLC mutations affect different parameters such as protein stability, protein levels at the plasma membrane, and protein localization. Thus, it is clear that most *MLC1* missense mutations cause protein degradation. This has been verified in *Xenopus oocytes* (Duarri et al., 2008), different cell lines (Duarri et al., 2008; Brignone et al., 2014), primary astrocyte cultures (Duarri et al., 2008), monocytes from patients (Duarri et al., 2008; Petrini et al., 2013), and even in a brain biopsy of an MLC1 patient (López-Hernández et al., 2011b; **Figure 1A**). Therefore, cells or animals deficient in MLC1 constitute a model to understand the pathophysiology of MLC. Animal models containing a missense mutation in MLC1 could be used to screen for chemical chaperones that could increase MLC1 protein stability and consequently, its levels at the plasma membrane.

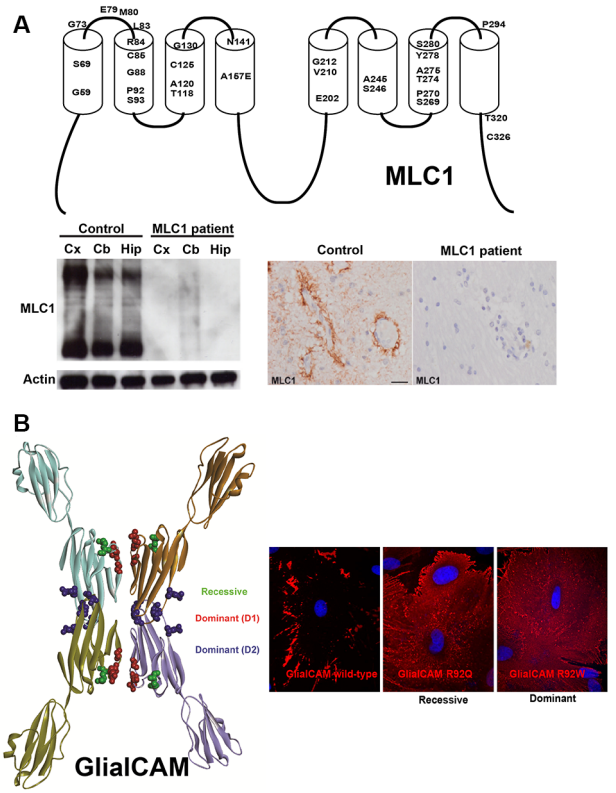


FIGURE 1 | Effect of *MLC1* **(A)** and *GLIALCAM* **(B)** mutations on the biochemistry and cell biology of their coding proteins. **(A)** Top panel: 2D-scheme of the MLC1 protein showing the residues that are mutated in Megalencephalic leukoencephalopathy with subcortical cysts (MLC) patients. Below panel: mutation S69L found in a Spanish MLC1 patient abolished MLC1 protein expression. Reprinted by permission from Oxford University Press: Human Molecular Genetics, *Molecular mechanisms of MLC1 and GLIALCAM mutations in MLC*, López-Hernández et al. (2011b). **(B)** 3D-Model of GlialCAM homo-oligomers forming cis and trans interactions. Reprinted by permission from Oxford University Press: Human Molecular Genetics, *Structural basis for the dominant or recessive character of GLIALCAM mutations found in leukodystrophies*, Elorza-Vidal et al. (2020). Expression of GlialCAM containing a recessive (R92Q) or a dominant (R92W) mutation in primary astrocytes causes mistargeting.

On the other hand, most mutations in *HEPACAM* cause protein mislocalization, as shown in cell lines and cultured astrocytes (**Figure 1B**). Biochemical studies also suggest that dominant MLC2B mutations affect the trafficking of the wild-type protein, possibly by interfering with the formation of GlialCAM complexes in the same cell (*cis*-interactions) or between different cells (*trans*-interactions; Elorza-Vidal et al., 2020). These *in vitro* studies were validated *in vivo* with the generation of a *Glialcam* knockin (KI) mouse containing a dominant mutation, which showed that GlialCAM protein also displays a trafficking defect in heterozygous mice (Hoegg-Beiler et al., 2014). With this model, it has been speculated that dominant MLC2B mutations may lead to a minor reduction of GlialCAM/MLC1 function, which might be pathogenic in humans only at the early stages of life. In agreement with the reversibility of the phenotype observed in MLC2B patients in adult stages, a decreased GlialCAM/MLC1 function may not be so pathogenic in adults as it is in infants (Hamilton et al., 2018).

Very few *HEPACAM* mutations seem to induce a gain of function, a phenotype that can only be observed in astrocytes from *Mlc1* knockout mice in depolarizing conditions (Arnedo et al., 2014b). Further work is needed to understand the molecular mechanism of the pathogenesis of these specific mutations.

INSIGHTS INTO GLIALCAM/MLC1 FUNCTION AND PATHOPHYSIOLOGY OF MLC

It has nearly been 20 years since the discovery of the first gene implicated with the disease, *MLC1* (Leegwater et al., 2001). However, we still do not have a clear picture of the function this protein exerts, and hence, the pathophysiology of the disease remains obscure (Estévez et al., 2018).

First insights into the putative role of MLC1 came from protein sequence analysis (Leegwater et al., 2001). Thus, it was suggested that MLC1 could work as an ion channel or a transporter. However, no ion or transport activity directly mediated by MLC1 has been demonstrated so far, even when co-expressed with GlialCAM (Teijido et al., 2004). Based on the clinical phenotype of the patients, it was then speculated that water/ion homeostasis should be negatively affected by the lack of MLC1 (van der Knaap et al., 2012).

To get more insights into the role of GlialCAM/MLC1, several research groups aimed to identify first the MLC1 and later GlialCAM interacting proteins. Different approaches were addressed such as yeast-to-hybrid, using MLC1 N-terminal domain (Brignone et al., 2011) or the full-length MLC1 (unpublished results from our group), immunopurification of MLC1 or GlialCAM associated complexes (López-Hernández et al., 2011a; Jeworutzki et al., 2012; Sugio et al., 2017), co-fractionation (sWGA and DEAE chromatography, ouabain chromatography; Ambrosini et al., 2008; Brignone et al., 2011, 2014), co-localization (by confocal or electronic microscopy immunogold) and by testing possible candidates (Lanciotti et al., 2012). All these studies resulted in a list of proteins that might be involved in MLC (**Table 1**).

Classically, two approaches were followed to understand the role played by MLC and MLC-interacting proteins. One method compared the effects of overexpressing wild-type MLC1 (with or without GlialCAM) with the effects of overexpressing MLC1-containing mutations. However, an important caveat has to be stated in this method. Overexpression of Mlc1 is very toxic in mice (Sugio et al., 2017), leading to a more drastic vacuolation phenotype than in the *Mlc1* knockout mice. Also, overexpression of a protein folding mutant may cause alterations in the ER and in lysosomes leading to additional effects (Duarri et al., 2008). On the other hand, other groups explored the effects of partially or totally depleting MLC proteins. Also, some studies have functionally analyzed the activity of possible interacting proteins

TABLE 1 | Proteins that have been related to the Megalencephalic leukoencephalopathy with subcortical cysts (MLC) proteins.

Protein related with MLC	Genetical, cell biology and biochemical evidence	Overexpression	Reduction of expression	Some comments
GlialCAM	1. Mutations in *GLIALCAM* cause MLC (López-Hernández et al., 2011a). 2. *GlialCAM* interact with MLC1 *in vitro*: co-IP, FRET, split-TEV (López-Hernández et al., 2011b). 3. Co-localization and co-IP *in vivo* (López-Hernández et al., 2011a). 4. GlialCAM change MLC1 localization (López-Hernández et al., 2011b).	Overexpression of GlialCAM stabilize MLC1 wild-type and mutants (Capdevila-Nortes et al., 2013).	1. KO of Glialcam affect MLC1 expression and localization (Hoegg-Beiler et al., 2014; Bugiani et al., 2017). 2. KO of *Mlc1* affect GlialCAM localization (Hoegg-Beiler et al., 2014; Bugiani et al., 2017).	There is no doubt that GlialCAM is a obligate subunit of MLC1 *in vivo*. However, MLC1 overexpressed *in vitro* arrive at the PM independently of GlialCAM.
ClC-2	1. ClC-2 interact with GlialCAM and MLC1 *in vitro*: coIP, split-TEV, stabilization at the PM (Jeworutzki et al., 2012; Gaitán-Peñas et al., 2017). 2. Co-localization and co-IP *in vivo* (Jeworutzki et al., 2012). 3. ClC-2 localization change by GlialCAM (Jeworutzki et al., 2012).	Overexpression of ClC-2 and GlialCAM change functional properties of ClC-2 (Jeworutzki et al., 2012, 2014).	1. KO of Mlc1 and Glialcam change ClC-2 localization and functional properties (Hoegg-Beiler et al., 2014; Bugiani et al., 2017). 2. In KO of ClC-2 there is a slight increase of MLC1 (Hoegg-Beiler et al., 2014).	The association between GlialCAM/MLC1 and ClC-2 is not constitutive (Sirisi et al., 2017). ClC-2 KO and/or mutations of CLCN2 have a different phenotype than MLC (Depienne et al., 2013). ClC-2 dysfunction might contribute to MLC but it is not the whole story (Hoegg-Beiler et al., 2014).
Na^+/K^+-ATPase	1. β1 subunit interact with *MLC1* N terminus by Yeast two-hybrid and pull down *in vitro* (Brignone et al., 2011). However, β1 was not co-IP *in vivo* (Sugio et al., 2017). 2. α2 and α3 subunits were co-IP *in vivo* (Sugio et al., 2017). 3. Some colocalization in astrocytes (Brignone et al., 2011). 4. Ouabain chromatography purified MLC1 (Brignone et al., 2011). 5. Other proteins copurifying with Na^+/K^+-ATPase also are found in MLC1 IP (i.e., GLAST, GLT-1; Sugio et al., 2017).	Astrocytes from mice with MLC1 OE show reduced Na^+/K^+-ATPase activity, but increased ouabain binding (Sugio et al., 2017).	1. No change in activity were observed in Mlc1 KO mice (Sugio et al., 2017). 2. No change in localization were observed in MLC KO mice (Dubey et al., 2015; Bugiani et al., 2017).	*In vitro* studies expressing these proteins are needed to prove their interaction. More evidence *in vivo* that Na^+/K^+-ATPase activity is regulated by MLC1 is necessary.
Other ATPases: PMCA1, 2, 3; SERCA1, 2; V-ATPase	1. They have been identified by coIP from brain tissue or by pulldown from astrocytes expressing His-tagged MLC1 (Brignone et al., 2014; Sugio et al., 2017). 2. Some co-localization have been demonstrated by IF in astrocytes with V-ATPase (Brignone et al., 2014).	In the case of V-ATPase: OE of MLC1 influences endosomal pH (Brignone et al., 2014).	No studies have been performed in KO animals.	*In vitro* studies expressing these proteins are needed to prove their interaction. More evidence *in vivo* that ATPase activity is regulated by MLC1 is necessary.
VRAC channel (LRRC8A)	1. No coIP *in vivo* (Elorza-Vidal et al., 2018). 2. No colocalization in tissue and in primary astrocytes (Elorza-Vidal et al., 2018).	OE MLC1 but not GlialCAM increases VRAC activity and improves RVD (regulatory volume decrease; Ridder et al., 2011; Lanciotti et al., 2012; Capdevila-Nortes et al., 2013).	1. *Mlc1* KO or *Mlc1/Glialcam* depletion reduces VRAC activity and RVD in astrocytes (Capdevila-Nortes et al., 2013; Dubey et al., 2015; Elorza-Vidal et al., 2018).	It is not clear how VRAC activity is regulated by MLC1. It may be an indirect or a compensatory mechanism. More experimental evidence is needed. For instance, VRAC current should be measured in brain slices from MLC KO models.

(Continued)

TABLE 1 | Continued

Protein related with MLC	Genetical, cell biology and biochemical evidence	Overexpression	Reduction of expression	Some comments
			2. Lymphoblast MLC cell lines show reduced VRAC activity (although MLC1 mRNA expression is nearly negligible in WT cells; Ridder et al., 2011).	
Cx43	1. coIP *in vivo* with GlialCAM (our unpublished results) and *in vitro* in U373G cells (Wu et al., 2016). 2. Partial co-localization with MLC1 and GlialCAM in primary astrocytes (Duarri et al., 2011).	1. OE MLC1 in U251 cell line reduces ERK1/2 activity which phosphorylates Cx43 increasing Cx43 in gap junctions (Lanciotti et al., 2020). 2. OE GlialCAM in U373G cell line increase Cx43 stability (Wu et al., 2016).	1. No experiments have been performed in KO animals. 2. Cx43 is misslocalized in *Glialcam* KO astrocytes (our unpublished results).	Experiments *in vivo* in KO MLC models are needed. It seems that MLC1 may influence different proteins indirectly by influencing signaling through ERK1/2 (VRAC, Cx43; Lanciotti et al., 2016).
TRPV4	1. It has been identified by pulldown from astrocytes or astrocytoma cells expressing His-tagged MLC1 (Lanciotti et al., 2012). 2. Ouabain chromatography purified also TRPV4 (Lanciotti et al., 2012).	1. Astrocytes OE MLC1 activate calcium influx in response to hyposmosis and 4αPDD more efficiently (Lanciotti et al., 2012). 2. MLC1 also favors recycling of TRPV4 (Lanciotti et al., 2012).	1. No experiments have been performed in KO animals or astrocytes. 2. In macrophages from patients Ca^{2+} responses are also affected (Petrini et al., 2013).	It is not clear how TRPV4 activity is regulated by MLC1. It may be an indirect or a compensatory mechanism. More experimental evidence is needed.
Kir4.1, AQP4	1. They have been identified by co-fractionation, ouabain chromatography or pulldown, mainly in cell culture (Brignone et al., 2011). 2. AQP4 association seem to increase in hypoosmotic conditions (Lanciotti et al., 2012). 3. *In vivo* there is no colocalization by EM with these proteins in tissue (Duarri et al., 2011).	*In vitro* experiments in *Xenopus oocytes* show that MLC1 does not influence Kir4.1 channel activity (Teijido et al., 2004).	In KO animals or in brain MLC biposies there is a redistribution of AQP4 and Kir4.1, which has been interpreted as a compensatory mechanism (Dubey et al., 2015; Wu et al., 2016; Bugiani et al., 2017).	*In vitro* studies expressing these proteins are needed to prove their interaction. More evidence *in vivo* that their activity is regulated by MLC1 is necessary.
Other proteins not related with ionic homeostasis	1. They include: syntrophin, dystrophin, caveolin-1, ZO-1. 2. ZO-1 has been identified by coIP from brain. Co-localization has been found by EM in tissue and IF in primary astrocytes (Duarri et al., 2011). 3. GlialCAM, MLC1 and other related proteins (i.e., TRPV4) are found in caveolae (Lanciotti et al., 2010). 4. Syntrophin and Dystrophin are found by co-fractionation and pulldown (Ambrosini et al., 2008). There is no colocalization with MLC1 by EM (Duarri et al., 2011). KO of *Dystrophin*, α-*Dystrobrevin* and *Utrophin* does not affect MLC1 localization (Duarri et al., 2011).	No studies have been performed in OE mice or in transfected cells.	No changes in localization have been reported in Mlc1 or *Glialcam* KO animals (Dubey et al., 2015; Bugiani et al., 2017).	1. Some of these proteins may help anchor MLC1 and GlialCAM at specific locations, so it is logical that its localization is not affected in KO animals. 2. *In vivo*, it is not clear that MLC1 forms part of the DGC complex, since there is no colocalization between proteins of the DGC complex and MLC proteins.

We indicate the biochemical evidence, the functional effects after overexpression or inhibition, and a conclusion of the effects.

in animal models of the disease (knockouts or overexpression) and/or cells obtained from patients.

In general, we can conclude that many different transporters or ion channels seem to be affected in MLC. Some of the proteins affected might be directly interacting with GlialCAM/MLC1 (i.e ClC-2, Cx43, Na^+/K^+-ATPase), whereas others might be regulated in an indirect manner (i.e., VRAC, TRPV4). An interesting new hypothesis is that GlialCAM/MLC1 may

modulate signal transduction events, and therefore, they could simultaneously influence different proteins (Lanciotti et al., 2016; Elorza-Vidal et al., 2018; Brignone et al., 2019). However, it is difficult to conclude whether the functional changes observed in these proteins are a consequence of a compensatory mechanism or a direct effect of MLC proteins. Possibly, those effects that show a different sign of change in overexpressing vs. reduced expression and that directly interact with GlialCAM/MLC proteins are probably a direct consequence of GlialCAM/MLC1 functional role. We indicate the results obtained for different proteins in **Table 1**.

An interesting issue is that some interactions might be constitutive, such as those between GlialCAM and MLC1 (Sirisi et al., 2014), whereas others might be regulated, such as the interaction between GlialCAM/MLC1 and ClC-2 (Sirisi et al., 2017). Hence, GlialCAM/MLC1 could be forming a protein scaffold, where different proteins may interact in a regulated manner with this protein network resulting in activity changes. As the interaction with ClC-2 was dependent on extracellular potassium, we proposed that GlialCAM/MLC1 might regulate brain homeostasis by influencing the activity and the interaction with different transporters/ion channels that could be involved in controlling brain potassium levels (Estévez et al., 2018). Apart from myelin vacuoles, defects in potassium clearance and increased excitability were described in MLC knockout mice and MLC patients (Dubey et al., 2018; Hamilton et al., 2018).

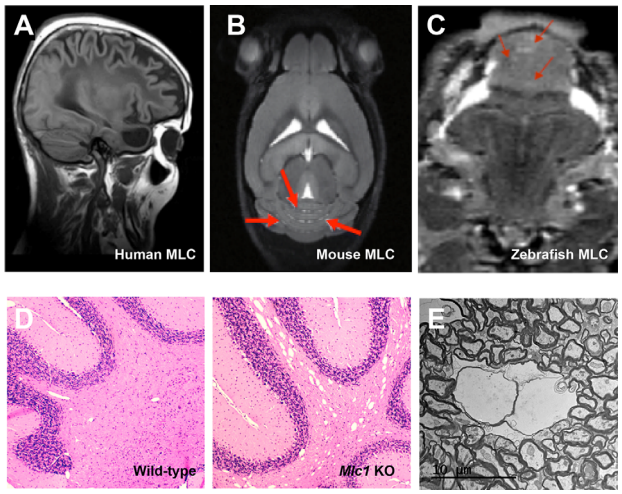


FIGURE 2 | The vacuolating phenotype of GlialCAM and MLC1 in mice and zebrafish resembles the MLC phenotype observed in humans. **(A–C)** MRI images of a human MLC1 patient **(A)**, *Mlc1* knockout mice **(B)**, and *mlc1* knockout zebrafish **(C)**. Red arrows indicate areas with increased water. Reprinted by permission from Oxford University Press: Human Molecular Genetics, *Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts*, López-Hernández et al. (2011b) and by permission from Oxford University Press: Human Molecular Genetics, *Megalencephalic leukoencephalopathy with subcortical cysts protein 1 regulates glial surface localization of GLIALCAM from fish to humans*, Sirisi et al. (2014). **(D)** Histology studies of cerebellar brain white matter comparing wild-type (left) with *Mlc1* KO mice indicate the presence of vacuoles in the KO mice. **(E)** EM images of vacuoles in *Mlc1* KO mice.

ANIMAL MODELS OF MLC: ZEBRAFISH AND MICE

To understand the pathophysiology of MLC, several animal models have been generated. In mice, three different knockout lines of *Mlc1* were produced (Hoegg-Beiler et al., 2014; Dubey et al., 2015; Sugio et al., 2017), two different knockouts for *Glialcam* (Hoegg-Beiler et al., 2014; Bugiani et al., 2017), several KI mice for *Glialcam* containing dominant or recessive mutations affecting GlialCAM targeting (Hoegg-Beiler et al., 2014; Shi et al., 2019), double KO for *Mlc1* and *Glialcam* (Pérez-Rius et al., 2019), and a transgenic mouse that overexpress *Mlc1* in astrocytes (Sugio et al., 2017). It will be interesting to use *Mlc1* floxed mice to generate conditional KO specifically in astrocytes during development, or KI mice for *Mlc1* containing missense mutations as well as to obtain tissue-specific knockouts for GlialCAM. In zebrafish, an *mlc1* KO line has been generated (Sirisi et al., 2014), with a mutation that abolishes mlc1 expression *in vitro* and *in vivo*; a *glialcama* KO and a double KO for *mlc1* and *glialcama* (Pérez-Rius et al., 2019).

All knockout animals share with human MLC patients an increase in the brain water content (**Figure 2**), which can be easily detected by MRI or histological techniques. As in biopsies of MLC patients (van der Knaap et al., 1996), vacuoles observed in animal models using electron microscopy are intramyelinic (**Figure 2**). However, some differences exist between human and animal models. MRI defects are observed in humans during the first years of life, thus there is a correlation between the period where myelination is more active and the onset of vacuolization. However, it takes much more time to be seen in mice (Hoegg-Beiler et al., 2014; Dubey et al., 2015), and even longer in zebrafish (Sirisi et al., 2014). In line with these differences, expression of MLC1 and GlialCAM is also higher in humans in the first years of life and then is reduced and maintained (Dubey et al., 2015; Bugiani et al., 2017), whereas expression of both proteins increases slowly in mice, reaching its peak at adult stage (Teijido et al., 2007; Gilbert et al., 2019).

Furthermore, there are also regional differences in vacuolated areas. It is important to mention that in humans the edema is mostly found in the subcortical white matter. While mice have a much more limited subcortical white matter, vacuoles are found in the cerebellum of KO mice, although MLC1 is broadly expressed at the endfeet of astrocytes in the whole brain (Hoegg-Beiler et al., 2014). Thus, it can be speculated that MLC1 and GlialCAM physiological roles might be more important in the cerebellum than in the rest of the brain or that they might play additional roles in cerebellar Bergmann glia. Several observations support these hypotheses: (1) in mice, expression of both proteins in the cerebellum is higher than in the rest of the brain (Teijido et al., 2007; López-Hernández et al., 2011a); (2) knockout of *Mlc1* reduces total expression of Glialcam and ClC-2 in the cerebellum, whereas no changes are observed in the cerebrum (Hoegg-Beiler et al., 2014). Similarly, knockout of *Glialcam* decreases expression of ClC-2 in the cerebellum, but not in the rest of the brain. These

results are in agreement with the formation of a ternary complex between GlialCAM, MLC1, and ClC-2 that stabilizes ClC-2 in the cerebellum (Sirisi et al., 2017); (3) in a brain biopsy of an MLC1 patient, lack of MLC1 causes GlialCAM mislocalization in Bergmann glia (Sirisi et al., 2014), but not in the rest of the brain (López-Hernández et al., 2011b); and (4) overexpression of Mlc1 causes a reduction in cerebellar size and Bergmann glia ectopia by an unknown mechanism in a development-dependent manner (Kikuchihara et al., 2018). Thus, we postulate that GlialCAM/MLC1 may have more importance or additional roles in the cerebellum, and this is perhaps more relevant in mice.

Different from MLC patients, the MLC mouse model containing a deletion of the first two exons of *Mlc1* (Hoegg-Beiler et al., 2014) does not display any cognitive or motor deficits and it is considered a model for the early stages of the disease. In contrast, the *Mlc1* KO where the full *Mlc1* gene was replaced by GFP showed some motor defects and higher susceptibility to develop epileptic seizures after kainate insults (Dubey et al., 2018). It remains to be determined whether these differences are due to the different targeting strategies and/or to genetic background.

One interesting result observed in animal models is the mutual dependence between GlialCAM and MLC1. Thus, in mice and zebrafish, lack of Mlc1 causes GlialCAM mislocalization but not the reduced expression (Hoegg-Beiler et al., 2014; Sirisi et al., 2014) and, in mice, lack of GlialCAM causes Mlc1 reduced expression and mislocalization (Hoegg-Beiler et al., 2014). These results are in agreement with similar clinical phenotypes among MLC2A and MLC1 patients (Hamilton et al., 2018).

Animal models have also been very useful in defining the biochemical relationships between different proteins related to GlialCAM/MLC1 cell biology. A clear example is a biochemical and functional interaction between GlialCAM/MLC1 and the chloride channel ClC-2. Lack of Mlc1 or Glialcam causes ClC-2 mislocalization (Hoegg-Beiler et al., 2014), which is completely abolished in Bergmann glia and strongly diminished at the astrocytic endfeet in the whole brain (Dubey et al., 2015; Bugiani et al., 2017). Furthermore, *in vivo* measurements of ClC-2 activity in cerebellar slices appeared linear in oligodendrocytes from wild-type mice, and inwardly rectifying from *Glialcam* or *Mlc1* knockout mice (Hoegg-Beiler et al., 2014). Other possible functional and biochemical interactions such as the VRAC channel (containing LRRC8A), Na^+/K^+-ATPase, Kir4.1, AQP4 or TRPV4 have not been demonstrated *in vivo*, since its localization is preserved in MLC knockout mice (Hoegg-Beiler et al., 2014; Dubey et al., 2015; Bugiani et al., 2017). Hence, these proteins might be indirectly regulated by unknown mechanisms or changes in their activity could be a consequence of a compensatory mechanism. Thus, we can conclude that many different processes might be affected by the lack of GlialCAM and MLC1. How GlialCAM and MLC1 regulate all these processes needs to be elucidated to define novel therapeutic strategies. In this respect, we explored the strategy of gene therapy as an approach to restore normal MLC1 protein levels *in vivo*, as detailed below.

GENE THERAPY FOR MLC

There are several major choices when developing a gene therapy strategy for a disease like MLC, involving the CNS, which can influence the efficiency of the treatment. This includes the type of vector, its route of administration, the diffusion of the therapeutic protein if can be secreted, or the need for a cell-specific promoter. Among the available gene delivery vectors, adeno-associated virus (AAV) vectors have several features that make them appropriate for brain gene transfer and one of the most promising for human trials: (i) the capacity for generating long-term gene expression, as long as 15 years in non-human primate CNS (Sehara et al., 2017) mostly as episomal form, thus, avoiding the risk for insertional mutagenesis reported with integrative vectors; (ii) the absence of toxicity associated with wild type virus (AAV vectors are classified as Biosafety Level 1), combined with the absence of wild type viral genes; and (iii) the ability to easily produce pure high-titer viruses in the laboratory.

Current technology allows us to efficiently target a localized brain region, for instance, the substantia nigra in Parkinson's disease, in which case direct intraparenchymal injection is needed. This brings us to another important decision: the appropriate route of administration has to be approached for each disease. Direct administration of the vector to a reduced and specific area of the brain through intracranial injection is only suitable when the therapeutic protein needs to be localized in a particular structure, limiting the diffusion of the vector. Intracranial administration is extremely invasive and there is an upper limitation of dose and volume to avoid toxicity and inflammation, although it has the advantage of using lower vector volumes, thus facilitating vector production.

When the therapeutic protein can be secreted to the extracellular space, cross-correction from a reduced number of cells producing the protein can be enough to achieve therapeutic effects in most cells, independently of the transduced cell type. This is the case of some lysosomal storage diseases like mucopolysaccharidoses, where the lysosomal enzyme is partially secreted and recaptured through the mannose-6-phosphate receptor. However, in the case of MLC disease, as MLC1 and GlialCAM proteins are located in the cellular membrane of astrocytes, we might need to transduce as many astrocytes as possible.

Indeed, many neurological disorders need a global distribution of the therapeutic protein to the whole brain or at least to large CNS structures. Moreover, some brain areas are more refractory to transduction by global routes of administration, including the cerebellum, and need a specific approach (Bosch et al., 2000). For the *Mlc1* KO mouse, where most histological abnormalities are present in the cerebellum, we explored different delivery routes involving intravenous (IV), CSF direct administration at lumbar or at the cerebellar area and directly into the cerebellar parenchyma, into the white mater or in the molecular layer (**Figure 3**). Ideally, IV administration of the vector would be the route of choice if a single injection could reach the whole brain, without using more interfering surgeries. However, most vectors are not able to cross the blood-brain

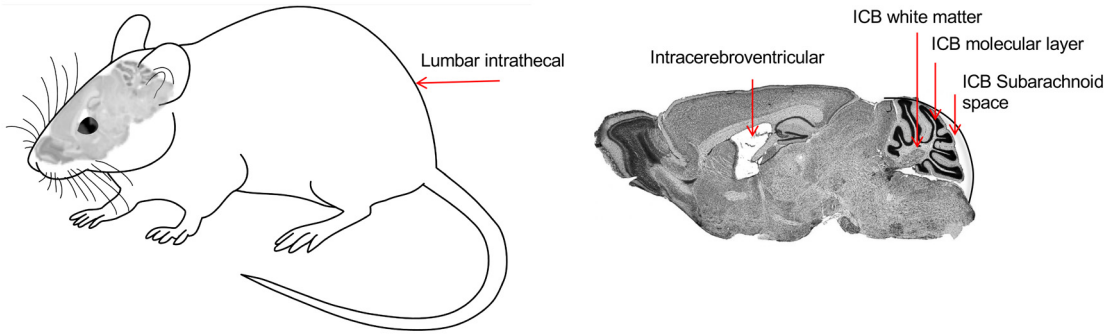


FIGURE 3 | Representation of the different routes of administration to deliver viruses into the cerebellum. Lumbar intrathecal (IT), Intracerebroventricular (ICV), Intracerebellar (ICB), white matter (ICB WM), ICB molecular layer (ICB ML), and ICB subarachnoid space (ICB SB).

barrier (BBB). Recently, the capacity of transducing the CNS through the circulation was shown for AAV9 and some derivates (like PHP.B; Foust et al., 2009; Deverman et al., 2016). However, PHP.B efficiency was demonstrated in C57/bl6 mouse strain (Deverman et al., 2016) but not in Balb/c mice (Hordeaux et al., 2018), and more importantly, the receptor used by this modified AAV capsid is not found in non-human primates (Hordeaux et al., 2019), which relegates the use of this vector to mouse preclinical studies. Intravenous AAV9 administration, however, can reach global areas in the cerebrum where it transduces astrocytes and neurons, as well as in the spinal cord, and is currently being applied to some clinical trials, particularly for very young children, in which the BBB is still not well closed. Encouraging results for spinal muscular atrophy and Mucopolysaccharidosis type IIIA have been reported so far (NCT02122952 and NCT04088734 in www.clinicaltrials.gov, respectively; Mendell et al., 2017). But, to achieve therapeutic levels of the transgene into the CNS when reaching it from the circulation, high titers of viruses need to be infused into the patients (>10^{14} vg/kg), with the risk of adverse secondary effects, some of which may be related to liver and dorsal root ganglia toxicity, as were seen in NHP and piglets (Hinderer et al., 2018). Moreover, the scale-up of vector production for human clinical trials is also challenging due to the high virus load needed for each patient.

Distribution of the vector through the cerebrospinal fluid (CSF) has many advantages, as allows for a more restricted expression, compared to the IV; but better global CNS transduction, compared to intraparenchymal administration; and it requires lower amounts of viruses, thus increasing biosafety and lowering production constraints (Pagès et al., 2019). Moreover, particularly for lumbar puncture, it can be performed in an outpatient setting, as is much less invasive than intracranial administration. In the MLC mouse model, the best results were obtained by delivering the vector into the CSF by occipital subarachnoid administration, achieving a high percentage of transduction of the cerebellum without damaging the brain parenchyma, allowing for a more localized and efficient action and avoiding non-specific effects due to systemic expression (Sánchez et al., 2020). By this route, we efficiently achieved transduction in most of the cerebellar parenchyma, with the highest efficacy in the Bergmann glia, where MLC1 is highly expressed (**Figure 4**). Given the small volume in the cerebellar intrathecal cavity, the best diffusion was achieved by loading the virus at a very slow rate (Huda et al., 2014), which in turn may decrease the amount of virus drained to the periphery (Wang et al., 2018).

It is well known that AAV vectors inherently transduce neuronal cells, so when neurons are not the target cell and to avoid undesirable secondary effects, it is important to restrict

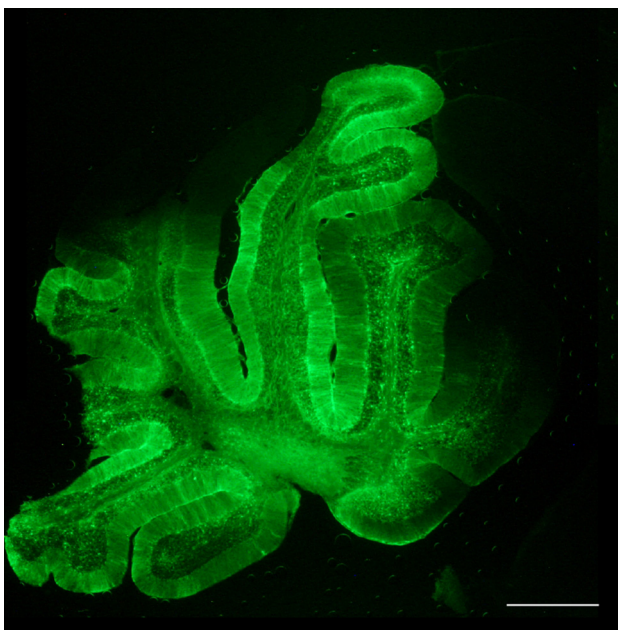


FIGURE 4 | Biodistribution of AAVrh10 coding for GFP under the regulation of Glial-derived fibrillary acidic protein recombinant promoter (GFAP). Cerebellar administration of the adeno-associated virus (AAV) vector in the subarachnoid space efficiently transduces the whole cerebellum. Scale bar, 400 μm. Reprinted by permission from Springer Nature: Springer, Neurotherapeutics, *Cerebellar Astrocyte transduction as gene therapy for megalencephalic leukoencephalopathy*, Sánchez et al. (2020).

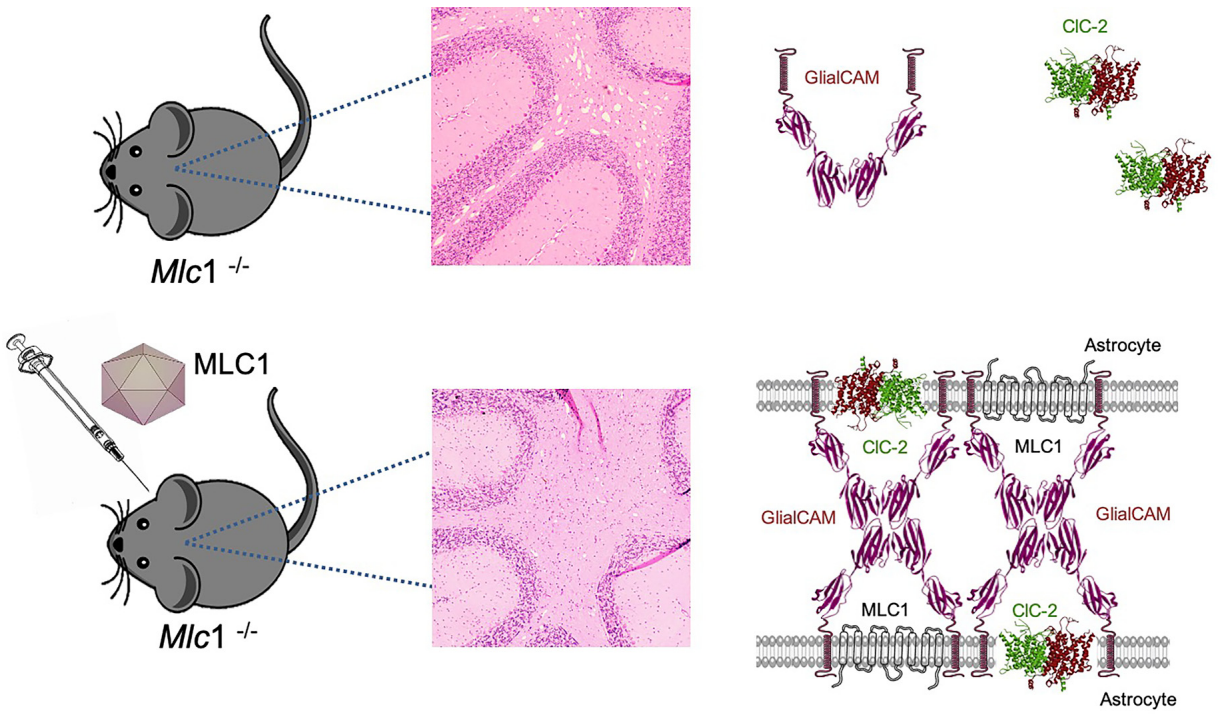


FIGURE 5 | Gene therapy for MLC corrects cerebellar leukodystrophy and MLC1, GlialCAM, and ClC-2 protein complex. *Mlc1* KO mice show myelin vacuolization in the cerebellum, as a result of lack of MLC1 and mislocalization of GlialCAM and ClC-2. AAV-mediated gene therapy restores MLC1 and allows the formation of the MLC1-GlialCAM-ClC-2 protein triad, which is enough to correct the vacuolating phenotype of this model.

expression to the target cell type. This is possible by combining a particular AAV serotype with the control of the transgene expression by recombinant promoters that recruit specific host cell-derived transcription factors (Lawlor et al., 2009). To design a gene therapy strategy for MLC, and taking into account that MLC1 is exclusively expressed by astrocytes in the brain, we choose an AAVrh10 vector (Sánchez et al., 2020), as it seems to transduce a larger number of glial cells in the adult brain, compared to AAV9 that showed a higher and specific tropism for neurons (Petrosyan et al., 2014). AAVrh10 was used in combination with a glial-derived fibrillary acidic protein recombinant promoter (GFAP), with high selectivity for astrocytes in the mature mouse brain (Brenner et al., 1994). Moreover, using a non-human primate serotype such as AAVrh10, we may increase the possibilities to avoid preexisting neutralizing antibodies in patients due to previous natural infections by human parvovirus, present in nature (Thwaite et al., 2015). Indeed, a high percentage of the human population is seropositive for AAV2 (Calcedo et al., 2009) and although there may be some cross-recognition between antibodies raised against different serotypes, this may decrease using viruses from other species (Thwaite et al., 2015). This is particularly important in adult patients, as they have been exposed to various infections throughout their life.

A dose-escalation study demonstrated a correlation between MLC1 expression and the degree of correction of the white matter vacuolation in the cerebellum of treated KO mice (Sánchez et al., 2020). However, wild-type animals injected with MLC1 did not show any deleterious effect, contrary to what was reported in the MLC1 over-expressing transgenic animal (Sugio et al., 2017), reinforcing the safety of the strategy and indicating that only supra-physiological levels of MLC1 may be toxic. Indeed, western blot analysis detected considerably lower levels of MLC1 in treated KO than in wild type animals, suggesting that a low amount of protein could still be therapeutic, as expected for a recessive disease.

We tested two different approaches, first a preventive strategy, treating the animals before the beginning of the pathology and analyzing them at 8 months of age, when the cerebellar vacuolation is evident. In the *Mlc1* KO mouse, increased MLC1 expression in Bergmann glia corrected GlialCAM and ClC-2 location in this area, confirming that the ternary complex formed by the three proteins is more stable at the plasma membrane, as previously seen *in vitro* and *in vivo* (Sirisi et al., 2017). Furthermore, myelin vacuoles were significantly reduced in number and size (**Figure 5**). More importantly, when the animals were treated at the beginning of the symptomatology, we not only prevented the onset of the cerebellar abnormalities but also significantly reduced the vacuolating phenotype in the transduced tissues. In fact, no differences were seen between the preventive and the therapeutic group at 8 months of age.

Based on the remitting edema phenotype of MLC2B patients, who carry one dominant mutation in the *HEPACAM* gene, we

hypothesized that in vacuolating leukoencephalopathies such as MLC, or as it was demonstrated for similar pathologies such as Canavan's or Pelizaeus-Merzbacher-like disease (Georgiou et al., 2017; Gessler et al., 2017), the therapeutic correction could be achieved even in advanced stages of the disease. Importantly, in this type of disease, there is no marked neuronal degeneration, and possibly the clinical phenotype could be reversible. Indeed, the results obtained from animals treated at 15 months of age, almost 1 year after the development of the vacuolating pathology, demonstrated a similar level of correction to animals treated as asymptomatic (Sánchez et al., 2020). This is relevant for therapy, as indicates that we may have a wide therapeutic window for these patients and they might be treated at any age, while in most neurodegenerative diseases, successful treatment is conditioned to very early intervention, thus, subjected to their perinatal detection, only if they have older affected siblings, or through perinatal screening, which unfortunately is still not the case for many inherited diseases.

This study is only a proof of concept of the feasibility of gene therapy for MLC. Therefore, the translatability of this approach needs to be further confirmed as human patients display pathology through the whole brain. However, some of the most severe symptoms such as mobility problems might be partially related to cerebellar pathology, so, it needs to be tested whether expression of MLC only in the cerebellum is enough to ameliorate these symptoms. Nevertheless, other phenotypes such as autism or cognitive deterioration might be more difficult to correct since these phenotypes may be the consequence of alteration of developmental processes at very early stages. Whether the mouse model may reveal higher susceptibility to develop epileptic seizures after kainate insults (Dubey et al., 2018) and if this may be corrected after gene therapy, remains to be evaluated. Anyway, extrapolating from the tiny mouse cerebellum to the same structure in young children is challenging. So, probably we will need to deliver the virus through multiple injections, although other strategies like convection-enhanced administration should be assayed in larger animals such as non-human primates, dogs, or pigs (Lonser et al., 2015).

There are still several important concerns to be studied in this disease before thinking in a human clinical trial. It would be interesting to see if gene therapy in the *Glialcam* KO mouse model can mimic the results obtained in the *Mlc1* KO mouse. Whether GlialCAM needs to be expressed in both oligodendrocytes and astrocytes to recover normal histology, or which of these cell types has a major role in the development of the disease are questions that need to be answered. Administration of AAVs coding for GlialCAM under the regulation of an astrocyte or an oligodendrocyte-specific promoter may give us the clue for this question. Other AAV serotypes or capsid modifications to increase vector tropism for glial cells (Powell et al., 2016), using tandem promoters for both cell types (Colella et al., 2019) or detargeting the neuronal tropism of AAVs by using microRNAs may also increase the efficiency of the strategy (Colin et al., 2009).

Importantly, to follow the progression of the disease in gene therapy clinical trials we need to select appropriate readouts. Alternatively, the development of larger animal models deficient for GlialCAM or MLC1 may facilitate direct invasive procedures and to follow physiological *in vivo* approaches such as structural and functional imaging of small brain structures, which is not possible in a mouse brain, and may open the possibility to develop new *in vivo* imaging biomarkers. Moreover, larger animal models may facilitate CSF collection, and will allow finding richer behavioral phenotypes which will make more complex behavioral testing possible. Besides, it will allow assessing for a larger biodistribution of the vector in the brain and possibly identifying new parameters to follow in patients during gene therapy treatment.

CONCLUSIONS AND PERSPECTIVES

The first gene for MLC (*MLC1*) was identified 20 years ago. Although many discoveries have been made in this long journey, the exact function of the MLC1 protein is not known and there are no therapeutic interventions for MLC patients. We expect that novel results will provide wider perspectives of the pathophysiology of the disease, which will help to clarify MLC protein's role in glial cell biology. Hopefully, this knowledge will be accompanied by advanced therapeutic solutions.

AUTHOR CONTRIBUTIONS

Both authors participated in the writing and revision of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by the European Leukodystrophies Association (ELA) Research Foundation (ELA 2018-0512) to AB; the Spanish Ministerio de Ciencia e Innovación (MICINN; RTI2018-093493-B-I00) to RE. RE is a recipient of an ICREA Academia prize.

REFERENCES

Ambrosini, E., Serafini, B., Lanciotti, A., Tosini, F., Scialpi, F., Psaila, R., et al. (2008). Biochemical characterization of MLC1 protein in astrocytes and its association with the dystrophin-glycoprotein complex. *Mol. Cell. Neurosci.* 37, 480–493. doi: 10.1016/j.mcn.2007.11.003

Arnedo, T., Aiello, C., Jeworutzki, E., Dentici, M. L., Uziel, G., Simonati, A., et al. (2014a). Expanding the spectrum of megalencephalic leukoencephalopathy with subcortical cysts in two patients with GLIALCAM mutations. *Neurogenetics* 15, 41–48. doi: 10.1007/s10048-013-0381-x

Arnedo, T., López-Hernández, T., Jeworutzki, E., Capdevila-Nortes, X., Sirisi, S., Pusch, M., et al. (2014b). Functional analyses of mutations in HEPACAM causing megalencephalic leukoencephalopathy. *Hum. Mutat.* 35, 1175–1178. doi: 10.1002/humu.22622

Barrallo-Gimeno, A., Gradogna, A., Zanardi, I., Pusch, M., and Estévez, R. (2015). Regulatory-auxiliary subunits of CLC chloride

channel-transport proteins. *J. Physiol.* 593, 4111–4127. doi: 10.1113/JP270057

Boor, P. K., De Groot, K., Waisfisz, Q., Kamphorst, W., Oudejans, C. B., Powers, J. M., et al. (2005). MLC1: a novel protein in distal astroglial processes. *J. Neuropathol. Exp. Neurol.* 64, 412–419. doi: 10.1093/jnen/64.5.412

Bosch, A., Perret, E., Desmaris, N., Trono, D., and Heard, J. M. (2000). Reversal of pathology in the entire brain of mucopolysaccharidosis type VII mice after lentivirus-mediated gene transfer. *Hum. Gene Ther.* 11, 1139–1150. doi: 10.1089/10430340050015194

Brenner, M., Kisseberth, W. C., Su, Y., Besnard, F., and Messing, A. (1994). GFAP promoter directs astrocyte-specific expression in transgenic mice. *J. Neurosci.* 14, 1030–1037. doi: 10.1523/JNEUROSCI.14-03-01030.1994

Brignone, M. S., Lanciotti, A., Macioce, P., Macchia, G., Gaetani, M., Aloisi, F., et al. (2011). The beta1 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. *Hum. Mol. Genet.* 20, 90–103. doi: 10.1093/hmg/ddq435

Brignone, M. S., Lanciotti, A., Serafini, B., Mallozzi, C., Sbriccoli, M., Veroni, C., et al. (2019). Megalencephalic leukoencephalopathy with subcortical cysts protein-1 (MLC1) counteracts astrocyte activation in response to inflammatory signals. *Mol. Neurobiol.* 56, 8237–8254. doi: 10.1007/s12035-019-01657-y

Brignone, M. S., Lanciotti, A., Visentin, S., De Nuccio, C., Molinari, P., Camerini, S., et al. (2014). Megalencephalic leukoencephalopathy with subcortical cysts protein-1 modulates endosomal pH and protein trafficking in astrocytes: relevance to MLC disease pathogenesis. *Neurobiol. Dis.* 66, 1–18. doi: 10.1016/j.nbd.2014.02.003

Bugiani, M., Dubey, M., Breur, M., Postma, N. L., Dekker, M. P., Ter Braak, T., et al. (2017). Megalencephalic leukoencephalopathy with cysts: the *Glialcam*-null mouse model. *Ann. Clin. Transl. Neurol.* 4, 450–465. doi: 10.1002/acn3.405

Calcedo, R., Vandenberghe, L. H., Gao, G., Lin, J., and Wilson, J. M. (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J. Infect. Dis.* 199, 381–390. doi: 10.1086/595830

Capdevila-Nortes, X., López-Hernández, T., Apaja, P. M., López de Heredia, M., Sirisi, S., Callejo, G., et al. (2013). Insights into MLC pathogenesis: GlialCAM is an MLC1 chaperone required for proper activation of volume-regulated anion currents. *Hum. Mol. Genet.* 22, 4405–4416. doi: 10.1093/hmg/ddt290

Colella, P., Sellier, P., Costa Verdera, H., Puzzo, F., Van Wittenberghe, L., Guerchet, N., et al. (2019). AAV gene transfer with tandem promoter design prevents anti-transgene immunity and provides persistent efficacy in neonate pompe mice. *Mol. Ther. Methods Clin. Dev.* 12, 85–101. doi: 10.1016/j.omtm.2018.11.002

Colin, A., Faideau, M., Dufour, N., Auregan, G., Hassig, R., Andrieu, T., et al. (2009). Engineered lentiviral vector targeting astrocytes *in vivo*. *Glia* 57, 667–679. doi: 10.1002/glia.20795

Depienne, C., Bugiani, M., Dupuits, C., Galanaud, D., Touitou, V., Postma, N., et al. (2013). Brain white matter oedema due to ClC-2 chloride channel deficiency: an observational analytical study. *Lancet Neurol.* 12, 659–668. doi: 10.1016/S1474-4422(13)70053-X

Deverman, B. E., Pravdo, P. L., Simpson, B. P., Kumar, S. R., Chan, K. Y., Banerjee, A., et al. (2016). Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat. Biotechnol.* 34, 204–209. doi: 10.1038/nbt.3440

Duarri, A., Lopez De Heredia, M., Capdevila-Nortes, X., Ridder, M. C., Montolio, M., López-Hernández, T., et al. (2011). Knockdown of MLC1 in primary astrocytes causes cell vacuolation: a MLC disease cell model. *Neurobiol. Dis.* 43, 228–238. doi: 10.1016/j.nbd.2011.03.015

Duarri, A., Teijido, O., López-Hernández, T., Scheper, G. C., Barriere, H., Boor, I., et al. (2008). Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in MLC1 cause folding defects. *Hum. Mol. Genet.* 17, 3728–3739. doi: 10.1093/hmg/ddn269

Dubey, M., Brouwers, E., Hamilton, E. M. C., Stiedl, O., Bugiani, M., Koch, H., et al. (2018). Seizures and disturbed brain potassium dynamics in the leukodystrophy megalencephalic leukoencephalopathy with subcortical cysts. *Ann. Neurol.* 83, 636–649. doi: 10.1002/ana.25190

Dubey, M., Bugiani, M., Ridder, M. C., Postma, N. L., Brouwers, E., Polder, E., et al. (2015). Mice with megalencephalic leukoencephalopathy with cysts: a developmental angle. *Ann. Neurol.* 77, 114–131. doi: 10.1002/ana.24307

Elorza-Vidal, X., Sirisi, S., Gáitan-Peñas, H., Pérez-Rius, C., Alonso-Gardón, M., Armand-Ugón, M., et al. (2018). GlialCAM/MLC1 modulates LRRC8/VRAC currents in an indirect manner: implications for megalencephalic leukoencephalopathy. *Neurobiol. Dis.* 119, 88–99. doi: 10.1016/j.nbd.2018.07.031

Elorza-Vidal, X., Xicoy-Espaulella, E., Pla-Casillanis, A., Alonso-Gardón, M., Gáitan-Peñas, H., Engel-Pizcueta, C., et al. (2020). Structural basis for the dominant or recessive character of GLIALCAM mutations found in leukodystrophies. *Hum. Mol. Genet.* 29, 1107–1120. doi: 10.1093/hmg/ddaa009

Estévez, R., Elorza-Vidal, X., Gáitan-Peñas, H., Pérez-Rius, C., Armand-Ugón, M., Alonso-Gardón, M., et al. (2018). Megalencephalic leukoencephalopathy with subcortical cysts: a personal biochemical retrospective. *Eur. J. Med. Genet.* 61, 50–60. doi: 10.1016/j.ejmg.2017.10.013

Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M., and Kaspar, B. K. (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat. Biotechnol.* 27, 59–65. doi: 10.1038/nbt.1515

Gaitán-Peñas, H., Apaja, P. M., Arnedo, T., Castellanos, A., Elorza-Vidal, X., Soto, D., et al. (2017). Leukoencephalopathy-causing CLCN2 mutations are associated with impaired Cl^- channel function and trafficking. *J. Physiol.* 595, 6993–7008. doi: 10.1113/JP275087

Georgiou, E., Sidiropoulou, K., Richter, J., Papaneophytou, C., Sargiannidou, I., Kagiava, A., et al. (2017). Gene therapy targeting oligodendrocytes provides therapeutic benefit in a leukodystrophy model. *Brain* 140, 599–616. doi: 10.1093/brain/aww351

Gessler, D. J., Li, D., Xu, H., Su, Q., Sanmiguel, J., Tuncer, S., et al. (2017). Redirecting N-acetylaspartate metabolism in the central nervous system normalizes myelination and rescues Canavan disease. *JCI Insight* 2:e90807. doi: 10.1172/jci.insight.90807

Gilbert, A., Vidal, X. E., Estévez, R., Cohen-Salmon, M., and Boulay, A.-C. (2019). Postnatal development of the astrocyte perivascular MLC1/GlialCAM complex defines a temporal window for the gliovascular unit maturation. *Brain Struct. Funct.* 224, 1267–1278. doi: 10.1007/s00429-019-01832-w

Hamilton, E. M. C., Tekturk, P., Cialdella, F., van Rappard, D. F., Wolf, N. I., Yalcinkaya, C., et al. (2018). Megalencephalic leukoencephalopathy with subcortical cysts: characterization of disease variants. *Neurology* 90, e1395–e1403. doi: 10.1212/WNL.0000000000005334

Hinderer, C., Katz, N., Buza, E. L., Dyer, C., Goode, T., Bell, P., et al. (2018). Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. *Hum. Gene Ther.* 29, 285–298. doi: 10.1089/hum.2018.015

Hoegg-Beiler, M. B., Sirisi, S., Orozco, I. J., Ferrer, I., Hohensee, S., Auberson, M., et al. (2014). Disrupting MLC1 and GlialCAM and ClC-2 interactions in leukodystrophy entails glial chloride channel dysfunction. *Nat. Commun.* 5:3475. doi: 10.1038/ncomms4475

Hordeaux, J., Wang, Q., Katz, N., Buza, E. L., Bell, P., and Wilson, J. M. (2018). The neurotropic properties of AAV-PHP.B are limited to C57BL/6J mice. *Mol. Ther.* 26, 664–668. doi: 10.1016/j.ymthe.2018.01.018

Hordeaux, J., Yuan, Y., Clark, P. M., Wang, Q., Martino, R. A., Sims, J. J., et al. (2019). The GPI-linked protein LY6A drives AAV-PHP.B transport across the blood-brain barrier. *Mol. Ther.* 27, 912–921. doi: 10.1016/j.ymthe.2019.02.013

Huda, F., Konno, A., Matsuzaki, Y., Goenawan, H., Miyake, K., Shimada, T., et al. (2014). Distinct transduction profiles in the CNS *via* three injection routes of AAV9 and the application to generation of a neurodegenerative mouse model. *Mol. Ther. Methods Clin. Dev.* 1:14032. doi: 10.1038/mtm.2014.32

Hwang, J., Vu, H. M., Kim, M.-S., and Lim, H.-H. (2019). Plasma membrane localization of MLC1 regulates cellular morphology and motility. *Mol. Brain* 12:116. doi: 10.1186/s13041-019-0540-6

Jeworutzki, E., Lagostena, L., Elorza-Vidal, X., López-Hernández, T., Estévez, R., and Pusch, M. (2014). GlialCAM, a CLC-2 Cl^- channel subunit, activates the slow gate of CLC chloride channels. *Biophys. J.* 107, 1105–1116. doi: 10.1016/j.bpj.2014.07.040

Jeworutzki, E., López-Hernández, T., Capdevila-Nortes, X., Sirisi, S., Bengtsson, L., Montolio, M., et al. (2012). GlialCAM, a protein defective in a leukodystrophy,

serves as a ClC-2 Cl^- channel auxiliary subunit. *Neuron* 73, 951–961. doi: 10.1016/j.neuron.2011.12.039

Kikuchihara, S., Sugio, S., Tanaka, K. F., Watanabe, T., Kano, M., Yamazaki, Y., et al. (2018). Ectopic positioning of Bergmann glia and impaired cerebellar wiring in Mlc1-over-expressing mice. *J. Neurochem.* 147, 344–360. doi: 10.1111/jnc.14486

Lanciotti, A., Brignone, M. S., Belfiore, M., Columba-Cabezas, S., Mallozzi, C., Vincentini, O., et al. (2020). Megalencephalic leukoencephalopathy with subcortical cysts disease-linked MLC1 protein favors gap-junction intercellular communication by regulating connexin 43 trafficking in astrocytes. *Cells* 9:1425. doi: 10.3390/cells9061425

Lanciotti, A., Brignone, M. S., Camerini, S., Serafini, B., Macchia, G., Raggi, C., et al. (2010). MLC1 trafficking and membrane expression in astrocytes: role of caveolin-1 and phosphorylation. *Neurobiol. Dis.* 37, 581–595. doi: 10.1016/j.nbd.2009.11.008

Lanciotti, A., Brignone, M. S., Molinari, P., Visentin, S., De Nuccio, C., Macchia, G., 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. *Hum. Mol. Genet.* 21, 2166–2180. doi: 10.1093/hmg/dds032

Lanciotti, A., Brignone, M. S., Visentin, S., De Nuccio, C., Catacuzzeno, L., Mallozzi, C., et al. (2016). Megalencephalic leukoencephalopathy with subcortical cysts protein-1 regulates epidermal growth factor receptor signaling in astrocytes. *Hum. Mol. Genet.* 25, 1543–1558. doi: 10.1093/hmg/ddw032

Lawlor, P. A., Bland, R. J., Mouravlev, A., Young, D., and During, M. J. (2009). Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol. Ther.* 17, 1692–1702. doi: 10.1038/mt.2009.170

Leegwater, P. A. J., Yuan, B. Q., van der Steen, J., Mulders, J., Konst, A. A. M., Boor, P. K. I., et al. (2001). Mutations of MLC1 (KIAA0027), encoding a putative membrane protein, cause megalencephalic leukoencephalopathy with subcortical cysts. *Am. J. Hum. Genet.* 68, 831–838. doi: 10.1086/319519

Lonser, R. R., Sarntinoranont, M., Morrison, P. F., and Oldfield, E. H. (2015). Convection-enhanced delivery to the central nervous system. *J. Neurosurg.* 122, 697–706. doi: 10.3171/2014.10.JNS14229

López-Hernández, T., Ridder, M. C., Montolio, M., Capdevila-Nortes, X., Polder, E., Sirisi, S., 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, 422–432. doi: 10.1016/j.ajhg.2011.02.009

López-Hernández, T., Sirisi, S., Capdevila-Nortes, X., Montolio, M., Fernández-Dueáas, V., Scheper, G. C., et al. (2011b). Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts. *Hum. Mol. Genet.* 20, 3266–3277. doi: 10.1093/hmg/ddr238

Mendell, J. R., Al-Zaidy, S., Shell, R., Arnold, W. D., Rodino-Klapac, L. R., Prior, T. W., et al. (2017). Single-dose gene-replacement therapy for spinal muscular atrophy. *N. Engl. J. Med.* 377, 1713–1722. doi: 10.1056/NEJMoa1706198

Moh, M. C., Zhang, C., Luo, C., Lee, L. H., and Shen, S. (2005). Structural and functional analyses of a novel Ig-like cell adhesion molecule, hepaCAM, in the human breast carcinoma MCF7 cells. *J. Biol. Chem.* 280, 27366–27374. doi: 10.1074/jbc.M500852200

Pagès, G., Giménez-Llort, L., García-Lareu, B., Ariza, L., Navarro, M., Casas, C., et al. (2019). Intrathecal AAVrh10 corrects biochemical and histological hallmarks of mucopolysaccharidosis VII mice and improves behavior and survival. *Hum. Mol. Genet.* 28, 3610–3624. doi: 10.1093/hmg/ddz220

Pérez-Rius, C., Folgueira, M., Elorza-Vidal, X., Alia, A., Hoegg-Beiler, M. B., Eeza, M. N.H., et al. (2019). Comparison of zebrafish and mice knockouts for megalencephalic leukoencephalopathy proteins indicates that GlialCAM/MLC1 forms a functional unit. *Orphanet J. Rare Dis.* 14:268. doi: 10.1186/s13023-019-1248-5

Petrini, S., Minnone, G., Coccetti, M., Frank, C., Aiello, C., Cutarelli, A., et al. (2013). Monocytes and macrophages as biomarkers for the diagnosis of megalencephalic leukoencephalopathy with subcortical cysts. *Mol. Cell. Neurosci.* 56, 307–321. doi: 10.1016/j.mcn.2013.07.001

Petrosyan, H. A., Alessi, V., Singh, V., Hunanyan, A. S., Levine, J. M., and Arvanian, V. L. (2014). Transduction efficiency of neurons and glial cells by AAV-1, -5, -9, -rh10, and -hu11 serotypes in rat spinal cord following contusion injury. *Gene Ther.* 21, 991–1000. doi: 10.1038/gt.2014.74

Powell, S. K., Khan, N., Parker, C. L., Samulski, R. J., Matsushima, G., Gray, S. J., et al. (2016). Characterization of a novel adeno-associated viral vector with preferential oligodendrocyte tropism. *Gene Ther.* 23, 807–814. doi: 10.1038/gt.2016.62

Ridder, M. C., Boor, I., Lodder, J. C., Postma, N. L., Capdevila-Nortes, X., Duarri, A., et al. (2011). Megalencephalic leucoencephalopathy with cysts: defect in chloride currents and cell volume regulation. *Brain* 134, 3342–3354. doi: 10.1093/brain/awr255

Sánchez, A., García-Lareu, B., Puig, M., Prat, E., Ruberte, J., Chillón, M., et al. (2020). Cerebellar astrocyte transduction as gene therapy for megalencephalic leukoencephalopathy. *Neurotherapeutics* doi: 10.1007/s13311-020-00865-y [Online ahead of print].

Sehara, Y., Fujimoto, K.-I., Ikeguchi, K., Katakai, Y., Ono, F., Takino, N., et al. (2017). Persistent expression of dopamine-synthesizing enzymes 15 years after gene transfer in a primate model of Parkinson's disease. *Hum. Gene Ther. Clin. Dev.* 28, 74–79. doi: 10.1089/humc.2017.010

Shi, Z., Yan, H.-F., Cao, B.-B., Guo, M.-M., Xie, H., Gao, K., et al. (2019). Identification in Chinese patients with GLIALCAM mutations of megalencephalic leukoencephalopathy with subcortical cysts and brain pathological study on Glialcam knock-in mouse models. *World J. Pediatr.* 15, 454–464. doi: 10.1007/s12519-019-00284-w

Sirisi, S., Elorza-Vidal, X., Arnedo, T., Armand-Ugón, M., Callejo, G., Capdevila-Nortes, X., et al. (2017). Depolarization causes the formation of a ternary complex between GlialCAM, MLC1, and ClC-2 in astrocytes: implications in megalencephalic leukoencephalopathy. *Hum. Mol. Genet.* 26, 2436–2450. doi: 10.1093/hmg/ddx134

Sirisi, S., Folgueira, M., López-Hernández, T., Minieri, L., Pérez-Rius, C., Gáitan-Peñas, H., et al. (2014). Megalencephalic leukoencephalopathy with subcortical cysts protein 1 regulates glial surface localization of GLIALCAM from fish to humans. *Hum. Mol. Genet.* 23, 5069–5086. doi: 10.1093/hmg/ddu231

Sugio, S., Tohyama, K., Oku, S., Fujiyoshi, K., Yoshimura, T., Hikishima, K., et al. (2017). Astrocyte-mediated infantile-onset leukoencephalopathy mouse model. *Glia* 65, 150–168. doi: 10.1002/glia.23084

Teijido, O., Casaroli-Marano, R., Kharkovets, T., Aguado, F., Zorzano, A., Palacin, M., et al. (2007). Expression patterns of MLC1 protein in the central and peripheral nervous systems. *Neurobiol. Dis.* 26, 532–545. doi: 10.1016/j.nbd.2007.01.016

Teijido, O., Martínez, A., Pusch, M., Zorzano, A., Soriano, E., Del Rio, J. A., et al. (2004). Localization and functional analyses of the MLC1 protein involved in megalencephalic leukoencephalopathy with subcortical cysts. *Hum. Mol. Genet.* 13, 2581–2594. doi: 10.1093/hmg/ddh291

Thwaite, R., Pagès, G., Chillón, M., and Bosch, A. (2015). AAVrh.10 immunogenicity in mice and humans. Relevance of antibody cross-reactivity in human gene therapy. *Gene Ther.* 22, 196–201. doi: 10.1038/gt.2014.103

van der Knaap, M. S., Abbink, T. E. M., and Min, R. (1993). "Megalencephalic leukoencephalopathy with subcortical cysts," in *GeneReviews((R))*, eds M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, et al. (Seattle, WA: University of Washington), 1993–2020.

van der Knaap, M. S., Barth, P. G., Stroink, H., Van Nieuwenhuizen, O., Arts, W. F., Hoogenraad, F., et al. (1995). Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. *Ann. Neurol.* 37, 324–334. doi: 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, 206–212. doi: 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. *Lancet Neurol.* 11, 973–985. doi: 10.1016/S1474-4422(12)70192-8

van der Knaap, M. S., Lai, V., Köhler, W., Salih, M. A., Fonseca, M.-J., Benke, T. A., et al. (2010). Megalencephalic leukoencephalopathy with cysts without MLC1 defect two phenotypes. *Ann. Neurol.* 67, 834–837. doi: 10.1002/ana.21980

Wang, D., Li, J., Tran, K., Burt, D. R., Zhong, L., and Gao, G. (2018). Slow infusion of recombinant adeno-associated viruses into the mouse cerebrospinal fluid space. *Hum. Gene Ther. Methods* 29, 75–85. doi: 10.1089/hgtb.2017.250

Wu, M., Moh, M. C., and Schwarz, H. (2016). HepaCAM associates with connexin 43 and enhances its localization in cellular junctions. *Sci. Rep.* 6, 36218. doi: 10.1038/srep36218

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 26 January 2021
doi: 10.3389/fncel.2020.626610

The Leukodystrophies HBSL and LBSL—Correlates and Distinctions

*Annapoorani Muthiah, Gary D. Housley, Matthias Klugmann and Dominik Fröhlich**

Translational Neuroscience Facility and Department of Physiology, School of Medical Sciences, UNSW Sydney, Kensington, NSW, Australia

Aminoacyl-tRNA synthetases (ARSs) accurately charge tRNAs with their respective amino acids. As such, they are vital for the initiation of cytosolic and mitochondrial protein translation. These enzymes have become increasingly scrutinized in recent years for their role in neurodegenerative disorders caused by the mutations of ARS-encoding genes. This review focuses on two such genes—*DARS1* and *DARS2*—which encode cytosolic and mitochondrial aspartyl-tRNA synthetases, and the clinical conditions associated with mutations of these genes. We also describe attempts made at modeling these conditions in mice, which have both yielded important mechanistic insights. *L*eukoencephalopathy with *b*rainstem and *s*pinal cord involvement and *l*actate elevation (LBSL) is a disease caused by a range of mutations in the *DARS2* gene, initially identified in 2003. Ten years later, *h*ypomyelination with *b*rainstem and *s*pinal cord involvement and *l*eg spasticity (HBSL), caused by mutations of cytosolic *DARS1*, was discovered. Multiple parallels have been drawn between the two conditions. The Magnetic Resonance Imaging (MRI) patterns are strikingly similar, but still set these two conditions apart from other leukodystrophies. Clinically, both conditions are characterized by lower limb spasticity, often associated with other pyramidal signs. However, perhaps due to earlier detection, a wider range of symptoms, including peripheral neuropathy, as well as visual and hearing changes have been described in LBSL patients. Both HBSL and LBSL are spectrum disorders lacking genotype to phenotype correlation. While the fatal phenotype of *Dars1* or *Dars2* single gene deletion mouse mutants revealed that the two enzymes lack functional redundancy, further pursuit of disease modeling are required to shed light onto the underlying disease mechanism, and enable examination of experimental treatments, including gene therapies.

Keywords: leukodystrophy, leukoencephalopathy, aminoacyl-tRNA synthetases, aspartyl-tRNA synthetase, DARS1, DARS2, hypomyelination with brainstem and spinal cord involvement and leg spasticity, leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation

OPEN ACCESS

Edited by:
Egor Dzyubenko, Essen University Hospital, Germany

Reviewed by:
Henna Tyynismaa, University of Helsinki, Finland
Isabella Zanella, University of Brescia, Italy

****Correspondence:***
Dominik Fröhlich
d.frohlich@unsw.edu.au

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *06 November 2020*
Accepted: *21 December 2020*
Published: *26 January 2021*

Citation:
Muthiah A, Housley GD, Klugmann M and Fröhlich D (2021) The Leukodystrophies HBSL and LBSL—Correlates and Distinctions. Front. Cell. Neurosci. 14:626610. doi: 10.3389/fncel.2020.626610

INTRODUCTION

Hypomyelination with Brainstem and Spinal Cord Involvement and Leg Spasticity (HBSL) and Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactate Elevation (LBSL) both belong to a group of white matter disorders termed leukodystrophies. These conditions are caused by mutations of the *DARS1* and *DARS2* genes, respectively—two genes that encode aspartyl-tRNA synthetase enzymes responsible for the accurate charging of aspartate-specific transfer ribonucleic acid (tRNA) with aspartate (Scheper et al., 2007; Taft et al., 2013). Both enzymes

are part of a larger group of enzymes known as aminoacyl-tRNA synthetases (ARSs), which are essential for protein translation. In humans, 36 ARSs exist without functional redundancy between them (Antonellis and Green, 2008).

ARSs can be divided into two subgroups based on their location of action—cytosolic and mitochondrial ARSs. In general, each ARS functions exclusively in one compartment and is responsible for the pairing of one of the 20 amino acids with its respective tRNA. Exceptions include glycyl-tRNA synthetase (GlyRS) and lysyl-tRNA synthetase (LysRS), which are capable of functioning in both compartments, and glutamyl-prolyl-tRNA synthetase (EPRS) which is able to pair both proline and glutamic acid with their respective tRNAs in the cytosolic compartment (Antonellis and Green, 2008). Interestingly, glutaminyl-tRNA synthetase (GlnRS) does not have a mitochondrial counterpart. Charging of mitochondrial $tRNA^{Gln}$ to glutamine takes place through an indirect pathway, where "mischarging" with glutamic acid by mitochondrial glutamyl-tRNA synthetase (GluRS) is the first step (Nagao et al., 2009). While *DARS1* encodes the cytosolic aspartyl-tRNA synthetase (AspRS), *DARS2* encodes its mitochondrial counterpart (mt-AspRS). AspRS catalyzes the aminoacylation reaction exclusively in the cytosol, whereas mt-AspRS is transported from the cytosol to the mitochondrial matrix where it is involved in mitochondrial protein synthesis.

CLINICAL FEATURES

LBSL was first described in 2003, based on distinctive clinical features and a characteristic Magnetic Resonance Imaging (MRI) pattern (Van Der Knaap et al., 2003). Following this initial MRI-based diagnosis, a genetic basis for the condition was established in 2007, concluding mutations harbored in the nuclear-encoded *DARS2* gene to be the cause (Scheper et al., 2007). This was the first mitochondrial aminoacyl-tRNA synthetase gene to be identified as disease-causing in its mutated state and marked the beginning of many more conditions being linked to this group of enzymes. Subsequently, in 2013, HBSL was first characterized by Taft et al., who established mutations in the cytosolic AspRS encoding gene *DARS1* as the genetic cause for the condition (Taft et al., 2013). Both diseases are inherited in an autosomal recessive manner (Scheper et al., 2007; Taft et al., 2013). To date, more than 100 LBSL patients have been reported in the literature, while only 16 HBSL patients have been described in the past 7 years. However, the actual number of diagnosed HBSL patients is recognized to be considerably higher than the number of reported cases (pers. comm.).

Of note, neither condition features extra-neurological signs, particularly cardiac and skin symptoms, even though these tissues have high energy demand and cell turnover, respectively. This selective neurological phenotype is not unique to HBSL or LBSL but has been reported for many ARS-related diseases (Fuchs et al., 2019). The reason for the neurological preponderance of both HBSL and LBSL is the subject of ongoing studies. It is worth mentioning though that few ARS-related conditions do involve non-neural tissues; for example, some *AARS2* mutations are known to cause severe cardiomyopathy (Götz et al., 2011; Taylor et al., 2014), while other *AARS2* mutations do not.

The age at which symptoms are first detected is highly variable even within each condition. The onset of HBSL ranges from 4 months (Taft et al., 2013) to 18 years (Wolf et al., 2015). The average age of onset is 3.5 years; however, most patients experience first symptoms before the age of 1 year (56% of reviewed patients). For LBSL, the onset of disease occurs on average around the age of seven, but has been detected from birth (Steenweg et al., 2012) to as old as 43 years (Ikazabo et al., 2020). It is generally understood that, for both HBSL and LBSL, the earlier the onset of symptoms the more severe and rapid the progression of disease. For instance, one study found that LBSL patients who experience symptoms before the age of 1.5 years were more likely to be wheelchair-dependent than those experiencing symptoms after this age (Van Berge et al., 2014). At present, only seven LBSL patients out of 99 patients reviewed are known to be wheelchair-dependent (Serkov et al., 2004; Távora et al., 2007; Tylki-Szymanska et al., 2014; Shimojima et al., 2017; Yelam et al., 2019). These patients had onset of symptoms in infancy or early childhood. In terms of unsupported mobility, it can be argued that patients with HBSL experience more debilitating symptoms compared to patients with LBSL. All initially described HBSL patients with infantile-onset (<2 years old) were unable to mobilize independently (Taft et al., 2013). This is due to severe spasticity, a defining feature of HBSL, with lower limbs affected more severely than upper limbs. HBSL patients with childhood-onset presented with delayed motor development but were subsequently able to mobilize independently, albeit with repeated falls (Ong et al., 2020). HBSL patients with adult-onset have been shown to experience fewer and milder symptoms compared to infantile-onset patients (Wolf et al., 2015).

The age of onset also seems to predict symptomology. While there are some common clinical features that all LBSL and HBSL patients share, clinical presentation and progression seem to differ between different age groups. Infantile-onset LBSL patients typically present with hypotonia, areflexia and developmental delay or regression of achieved developmental milestones (Miyake et al., 2011; Steenweg et al., 2012; Grechanina and Zdubskaya, 2014; Tylki-Szymanska et al., 2014; Kohler et al., 2015; Navarro Vázquez et al., 2016; Yahia et al., 2018). Progression of the disease is also more rapid and typically followed by development of upper motor neuron (UMN) signs such as spasticity, hyperreflexia, and a positive Babinski sign. To date, eight deaths associated with LBSL have been reported and all of them belonged to this group of patients. Death usually occurred within 2 years of age, or ∼1 year after the onset of symptoms with the exception of one patient who lived 7.5 years after onset of symptoms (Miyake et al., 2011; Orcesi et al., 2011; Steenweg et al., 2012; Navarro Vázquez et al., 2016; Rathore et al., 2017). The cause of death for these patients was either respiratory failure or respiratory tract infections. Children with disease onset in childhood or adolescence, on the other hand, typically present with gait difficulties, such as ataxia or unsteadiness and frequent falling. This is often accompanied by examination findings of spasticity, hyperreflexia, positive Babinski sign and distinct loss of proprioception and/or

vibration sense. Ataxic gait in LBSL is also specified to be sensory in some reports (Labauge et al., 2007; Erturk et al., 2010; Synofzik et al., 2011; Tzoulis et al., 2012), further reinforced by findings of a positive Romberg sign (Linnankivi et al., 2004; Sharma et al., 2011; Martikainen et al., 2013; Ikazabo et al., 2020) and loss of proprioceptive and vibration sense in 11% and 49% of all clinically described LBSL cases, respectively (**Table 1**). In adult-onset patients, the presenting complaints also relate to difficulties in walking, however the clinical picture often includes more atypical findings. One patient, for example, experienced episodic attacks of ataxia (Synofzik et al., 2011), while another patient experienced predominantly unilateral (rather than bilateral) symptoms of spasticity and muscle weakness (Moore et al., 2012). These two patients incidentally also belonged to the few LBSL patients who did not possess the highly prevalent *DARS2* intron 2 splice site mutation.

There are several notable exceptions to this generalized disease pattern in LBSL. The most significant is a male who experienced first symptoms at the age of 9 months (Kohler et al., 2015), where as an infant, he was irritable and had attention deficits. These worsened following a fever and respiratory infection, additionally resulting in poor eye contact and a delay in achieving motor milestones. Surprisingly, from 12.5 months of age, his condition improved to reach appropriate milestones for his age, including social smiling. His only residual symptom is mild ataxia that does not impede his ability to mobilize independently, including walking and running. It is also interesting that this patient is homozygous for *DARS2* mutations (c.172C>G; p.R58G). While the majority of LBSL patients are compound heterozygous, homozygosity has only been described in five LBSL patients from three unrelated families (Miyake et al., 2011; Synofzik et al., 2011; Kohler et al., 2015). Hence, homozygosity was initially presumed to be developmentally lethal until the first LBSL patient with homozygous *DARS2* mutations was described in 2011. Two of these patients (who are siblings) have experienced infantile-onset disease with severe disease course and subsequent death (Yamashita et al., 2013). Meanwhile, the third sibling experienced cognitive delay and severe motor deficits, and currently mobilizes by wheelchair.

Another patient with homozygous *DARS2* mutations experienced episodic bouts of ataxia that were induced by exercise, a highly unique disease presentation that has not been described in another LBSL patient (Synofzik et al., 2011). It was suggested that the finding of episodic ataxia might be incidental rather than the result of *DARS2* mutations (van der Knaap et al., 2014). Episodic ataxia, or recurring intermittent attacks of imbalance and incoordination, is a cardinal sign of a group of inherited diseases known as hereditary episodic ataxias (EA). Individuals with EA experience bouts of ataxia, of varying frequency and intensity, accompanied by other signs such as seizures and muscle fasciculation (Teive and Ashizawa, 2015). These attacks are often triggered by several environmental factors (Jen and Wan, 2018). The aforementioned patient, however, was not found to harbor any mutations for the most common EA types (Synofzik et al., 2011). Homozygous mutations, therefore, do not seem to be a reliable indicator of the prognosis in LBSL. In fact, genotype-phenotype correlations have been challenging to establish in this condition, and there are suggestions of additional genetic and non-genetic factors influencing disease presentation (Steenweg et al., 2012; Van Berge et al., 2014). This is further highlighted by the identification of a female who possessed the same two mutant *DARS2* alleles as her affected sister but experienced no clinical symptoms (Labauge et al., 2011).

TABLE 1 | Overview of clinical characteristics reported in HBSL and LBSL.

Clinical characteristics	HBSL ($n = 16$)[a], % of cases reported	LBSL ($n = 67$)[b], % of cases reported
Male	56.3	46.3
Unsupported Walking	31.3	55.2
Lower Limb Signs		
Hypertonia	87.5	65.7
Hyperreflexia	87.5	52.2
Positive Babinski Sign	81.3	52.2
Cerebellar Signs		
Ataxia	50.0	70.1
Tremor	0.0	26.9
Dysmetria and/or Dysdiadochokinesia	18.8	11.9
Nystagmus	50.0	17.9
Others		
Dysarthria	18.8	19.4
Muscle Weakness	12.5	43.3
Muscle Cramps	0.0	4.5
Peripheral Neuropathy	0.0	28.4
Dorsal Column Dysfunction	6.3	49.3
Seizures	12.5	13.4
Cognitive Impairment	37.5	34.3
Visual Changes	62.5	11.9
Hearing Changes	0.0	4.5
Urinary Changes	6.3	9.0
Foot Deformities	0.0	14.9
Anemia	0.0	1.5

Tremor encompasses intention tremor and reports of unspecified tremor. Visual changes include changes such as optic disc pallor, hypermetropia, optic atrophy, and myopia. Hearing changes include hyperacusis, hypoacusis, and sensorineural deafness. Urinary changes include increased urgency, incontinence, and changes in urine composition. Foot deformities include pes cavus, pes equinus, pes excavatus, hammertoe deformity, and contractures of the foot. Only data from full-text publications was included in this table. Characteristics were deemed to be not present if the feature was not mentioned, not investigated, or if they were stated to be normal (where applicable).

[a] *References: (Taft et al., 2013; Wolf et al., 2015; Ong et al., 2020).*

[b] *References: (Van Der Knaap et al., 2003; Linnankivi et al., 2004; Serkov et al., 2004; Petzold et al., 2006; Labauge et al., 2007, 2011; Távora et al., 2007; Uluc et al., 2008; Erturk et al., 2010; Isohanni et al., 2010; Lin et al., 2010; Galluzzi et al., 2011; Mierzewska et al., 2011; Miyake et al., 2011; Orcesi et al., 2011; Sharma et al., 2011; Synofzik et al., 2011; Moore et al., 2012; Steenweg et al., 2012; Tzoulis et al., 2012; Cheng et al., 2013; Martikainen et al., 2013; Schicks et al., 2013; Alibas et al., 2014; Kassem et al., 2014; Tylki-Szymanska et al., 2014; Kohler et al., 2015; Navarro Vázquez et al., 2016; Lan et al., 2017; Shimojima et al., 2017; Cavusoglu et al., 2018; Gonzalez-Serrano et al., 2018; Werner et al., 2018; Yahia et al., 2018; Yelam et al., 2019; Ikazabo et al., 2020).*

Ultimately, the most common sign in LBSL is cerebellar ataxia and unsteady gait. Motor impairment is mainly caused by UMN dysfunction, however lower motor neuron (LMN) signs have also been described (Miyake et al., 2011; Steenweg et al., 2012; Grechanina and Zdubskaya, 2014; Yelam et al., 2019). Some other less frequent symptoms that have been observed in LBSL patients include unspecified tremor (Isohanni et al., 2010; Galluzzi et al., 2011; Sharma et al., 2011; Yamashita et al., 2013; Werner et al., 2018) and peripheral neuropathy (Linnankivi et al., 2004; Uluc et al., 2008), among others. Cognitive deficits are usually mild, if present, and typically affect information-processing speed, concentration, and short-term memory (Martikainen et al., 2013). An overview of reported LBSL and HBSL symptoms can be found in **Table 1**.

HBSL is less diverse in its clinical presentation, and there has only been one known death associated with HBSL thus far (pers. comm.). This patient experienced infantile onset of symptoms and lived to 9 years of age. The most common presentation in infants is regression in motor development or developmental delay (Taft et al., 2013). Some additional congenital abnormalities including spinal cord tethering (Taft et al., 2013), vertebral malformations, trigonocephaly and Chiari malformations (Ong et al., 2020) have also been noted in a few patients. In older-onset HBSL patients, spasticity is one of the first symptoms (Wolf et al., 2015). Most reported patients have experienced leg spasticity—to varying degrees—that have impaired their ability to walk. Gait has been described as diplegic (Taft et al., 2013) or in-toeing (Ong et al., 2020) in those able to mobilize. In more severe cases, upper limb involvement is also noted. HBSL is predominantly a UMN disorder, with consistent hypertonia, hyperreflexia and positive Babinski sign noted in nearly all patients (**Table 1**). Nystagmus has also been a common finding. To a lesser extent, axial hypotonia (Taft et al., 2013; Wolf et al., 2015), epilepsy (Taft et al., 2013), cognitive deficits (Taft et al., 2013; Ong et al., 2020), and headaches (Ong et al., 2020) have been noted in some patients. In two adult-onset patients, symptoms of dorsal column dysfunction and urinary changes have been identified, as seen in LBSL (Wolf et al., 2015). The main differences in the clinical picture of HBSL and LBSL, are the clear lack of peripheral neuropathy and LMN signs in HBSL patients, while significant leg spasticity is the predominant feature. No hearing changes have been noted in HBSL patients. Evidence of intrafamilial variations and weak genotype-phenotype correlations, are present in HBSL as well. This is supported by a report of a family with three affected siblings carrying the same mutations but experiencing different disease courses (Ong et al., 2020).

In both conditions, a number of patients experience symptoms following clear precipitating events—for instance, fever, viral illness, or head trauma—either resulting in the first onset of symptoms (Wolf et al., 2015; Yahia et al., 2018) or further neurological deterioration (Uluc et al., 2008; Taft et al., 2013). Most patients experiencing such an exacerbation of symptoms usually recover to a new baseline with some residual deficits (Serkov et al., 2004; Isohanni et al., 2010; Wolf et al., 2015).

TABLE 2 | Overview of MRI changes reported in LBSL.

MRI Changes	LBSL ($n = 83$)[a], proportion of patients (%)
Supratentorial	
Cerebral WM with subcortical sparing*	92.8
Posterior part of the corpus callosum	77.1
Posterior limb of the internal capsule	81.9
Infratentorial	
Lateral corticospinal tracts or dorsal columns of the spinal cord	89.2
Pyramidal tract and/or medial lemniscus of the brainstem	94.0
Superior cerebellar peduncles	73.5
Inferior cerebellar peduncles	69.9
Intraparenchymal trajectories of the trigeminal nerve	63.9
Mesencephalic trigeminal tracts	47.0
Anterior spinocerebellar tracts of the medulla oblongata	32.5
Cerebellar white matter	88.0

Adapted, with some modifications, from the MRI criteria described by Steenweg et al. (2012). Only data from full-text publications was included in this table. MRI changes were deemed to be not present if the feature was not mentioned, not investigated, or if they were stated to be normal.

**Patients with cerebral white matter changes, but without subcortical u-fiber sparing, were not included in this count.*

[a] *References: (Van Der Knaap et al., 2003; Linnankivi et al., 2004; Serkov et al., 2004; Petzold et al., 2006; Labauge et al., 2007, 2011; Távora et al., 2007; Uluc et al., 2008; Erturk et al., 2010; Isohanni et al., 2010; Lin et al., 2010; Galluzzi et al., 2011; Mierzewska et al., 2011; Miyake et al., 2011; Orcesi et al., 2011; Sharma et al., 2011; Synofzik et al., 2011; Moore et al., 2012; Steenweg et al., 2012; Tzoulis et al., 2012; Cheng et al., 2013; Martikainen et al., 2013; Schicks et al., 2013; Alibas et al., 2014; Kassem et al., 2014; Tylki-Szymanska et al., 2014; Kohler et al., 2015; Navarro Vázquez et al., 2016; Lan et al., 2017; Shimojima et al., 2017; Cavusoglu et al., 2018; Gonzalez-Serrano et al., 2018; Werner et al., 2018; Yahia et al., 2018; Yelam et al., 2019; Ikazabo et al., 2020).*

NEUROIMAGING AND DIAGNOSIS

MRI is the tool of choice when investigating white matter (WM) diseases. Most leukodystrophies can be accurately diagnosed with this modality, as they present with a unique MRI signature (Schiffmann and van der Knaap, 2009). However, between HBSL and LBSL, MRI is not highly discriminatory, as both conditions affect similar brain structures and tracts (Taft et al., 2013; Wolf et al., 2015). While MRI remains the main imaging modality to initially identify HBSL and LBSL patients, genetic analysis is necessary to subsequently differentiate between the two conditions and is currently the only means to a definite diagnosis.

Nonetheless, prior to genetic analysis, a preliminary LBSL diagnosis can be made on an MRI basis. With the revised criteria proposed by Steenweg et al., three major criteria must be fulfilled—signal changes in the cerebral WM, involvement of the dorsal columns and lateral corticospinal tracts in the spinal cord and the involvement of either the pyramidal or medial lemniscal tracts in the medulla (Steenweg et al., 2012). These three major criteria are largely satisfied in all genetically confirmed LBSL patients (**Table 2**). Abnormal cerebral WM is typically observed in the periventricular region, with distinct sparing of u-fibers. A sizeable number of patients have also displayed WM changes extending into the deep cerebral WM (Kassem et al.,

2014). Changes are usually bilateral and inhomogeneous, and sometimes described as spotty. Cerebral WM has also been noted to have bilateral focal lesions in some patients (Erturk et al., 2010). Rarely, additional involvement of the u-fibers has been reported (Galluzzi et al., 2011; Orcesi et al., 2011, Yamashita et al., 2013).

In conjunction with the major criteria, at least one minor criterion should also be satisfied for diagnosis of LBSL. These minor criteria include signal changes in the cerebellar WM, posterior corpus callosum, posterior limb of the internal capsule (IC), superior and inferior cerebellar peduncles, intraparenchymal trajectories of the trigeminal nerve, mesencephalic tracts of the trigeminal nerve and anterior spinocerebellar tract in the medulla (Steenweg et al., 2012). A majority of patients display changes in the cerebellar WM, corpus callosum and posterior limb of the IC (**Table 2**). Involvement of the trigeminal nerve tracts and spinocerebellar tracts have been reported to a lesser extent (**Table 2**). Apart from these typical MRI changes, some atypical findings have been reported in genetically confirmed LBSL patients. These include the bilateral involvement of gray matter structures such as the globus pallidus (Galluzzi et al., 2011; Steenweg et al., 2012), red nucleus (Galluzzi et al., 2011), thalamus (Orcesi et al., 2011) and dentate nucleus (Galluzzi et al., 2011; Iyer and Philip, 2011; Orcesi et al., 2011; Yahia et al., 2018). Furthermore, cerebral (Tzoulis et al., 2012; Yamashita et al., 2013; Yahia et al., 2018) and cerebellar atrophy (Van Der Knaap et al., 2003; Yahia et al., 2018) has been reported, in addition to cerebellar hypoplasia (Isohanni et al., 2010). A singular case of calcifications of the cerebral WM and other regions typically affected by LBSL was identified by computed tomography (CT) scanning (Orcesi et al., 2011).

For HBSL, no such MRI criteria have been defined yet, however, some characteristic MRI features can be observed in the majority of patients (**Table 3**). These include homogenous cerebral WM changes (Taft et al., 2013; Wolf et al., 2015; Ong et al., 2020), abnormal posterior limb of the IC and hyperintensity of the corpus callosum on T2-weighted images (Taft et al., 2013; Wolf et al., 2015). Thinning of the corpus callosum has also been reported in some patients (Taft et al., 2013). In one HBSL patient, the additional involvement of the anterior corticospinal tract was observed on spinal MRI (Wolf et al., 2015). As seen in LBSL, the superior and inferior cerebellar peduncles, the pyramidal tracts in the medulla and spinal cord, and the dorsal columns of the spinal cord were affected in most patients. The cerebellar WM was also affected in several patients. Unlike in LBSL, medial lemniscal involvement was only observed in one patient (Taft et al., 2013), while no involvement of the trigeminal nerve tracts, spinocerebellar tracts and gray matter structures have been described for HBSL so far. The latter findings are also not common in LBSL and are therefore not reliable differentiators between the two conditions.

As the name suggests, lactate elevation detected by magnetic resonance spectroscopy (MRS) is often thought to be a defining feature of LBSL. However, elevated lactate levels were only detected in 68% of LBSL patients. Furthermore, even in patients where lactate was elevated, this elevation fluctuated over time (Van Der Knaap et al., 2003; Isohanni et al., 2010). Therefore, while raised lactate levels may be a good indicator for LBSL, an absence of lactate elevation does not necessarily exclude it, or automatically confirm HBSL. It has been suggested that lactate elevation might be more common in early-onset LBSL (Werner et al., 2018). Additional MRS analyses of affected WM regions of LBSL patients also revealed decreased N-acetylaspartate (NAA), elevated choline (Cho), elevated myoinositol (mIns) (Van Der Knaap et al., 2003) and occasionally elevated creatine (Cr) (Erturk et al., 2010). While no lactate elevation has been described in HBSL patients, one case report of HBSL showed mildly increased Cho in one patient and decreased NAA in another patient (Ong et al., 2020). A decrease in NAA, as observed in 52% of LBSL patients reviewed, implicates axonal damage or loss (Moffett et al., 2007). Increased Cho levels suggest demyelination (Van Der Knaap et al., 2003; Galluzzi et al., 2011) while an increase in mIns is a sign for gliosis (Chang et al., 2013). Finally, Cr when elevated can imply disturbance in cell metabolism (Rackayova et al., 2017). Taken together, a more detailed analysis of the metabolic changes in the brains of LBSL and HBSL patients could shed some light on the underlying mechanisms of these conditions.

Diffusion weighted imaging (DWI) has also been performed in LBSL patients, revealing diffusion restriction in parts of the affected WM (Mierzewska et al., 2011; Alibas et al., 2014; Kassem et al., 2014). DWI indicated that there are progressive changes that seem to correlate with different stages of the disease (Steenweg et al., 2011). Initially, the observed diffusion restriction is attributed to myelin-splitting and oedema within myelin. This is followed by shifting of water to the interstitial space, resulting in high T2 signals (Steenweg et al., 2011). Thereafter, the loss of

TABLE 3 | MRI features reported in HBSL.

MRI Changes	HBSL ($n = 15$)[a], proportion of patients (%)
Supratentorial WM	
Homogenous Cerebral WM Abnormality	80.0
Focal/Patchy Cerebral WM Changes	20.0
Hyperintense Corpus Callosum	60.0
Abnormal Posterior Limb of Internal Capsule	60.0
Brainstem	
Abnormal Signal of Pyramidal Tract	53.3
Abnormal Signal of Medial Lemniscus	6.7
Cerebellum	
Abnormal Signal of White Matter	26.7
Abnormal Signal of Superior Cerebellar Peduncle	60.0
Abnormal Signal of Inferior Cerebellar Peduncle	53.3
Spinal Cord	
Abnormal Signal of Dorsal Columns*	100.0
Abnormal Signal of Lateral Corticospinal Tracts**	90.9

*Only data from full-text publications were included in this table. MRI changes were deemed to be not present if the feature was not mentioned, or if they were stated to be normal. *n = 13, as this feature was not investigated in two patients (Taft et al., 2013). **n = 11, as this feature was not investigated in four patients (Taft et al., 2013).*
[a] *References: (Taft et al., 2013; Wolf et al., 2015; Ong et al., 2020).*

water in the interstitial space indicates the final component of the disease process, leading to intermediate T2 signals (Steenweg et al., 2011). These findings have been supported by post-mortem histopathological observations in two LBSL patients, which showed intra-lamellar splitting of myelin coupled with vacuolar, spongiform degeneration of the cerebral and cerebellar WM (Yamashita et al., 2013). Similar DWI studies in HBSL patients, in addition to more detailed MRS studies, will provide valuable insight into the progression of this leukodystrophy.

Although MRI is a reliable tool for diagnosis, clinical severity of LBSL does not always reflect the MRI results. Labauge et al. described a sibling to a proband who had characteristic MRI changes typically seen in affected LBSL patients, yet did not show any symptoms (Labauge et al., 2011). Genetic testing revealed that this person was a compound heterozygous carrier of *DARS2* mutations. Furthermore, there appears to be a delay between MRI changes and the onset of clinical symptoms in LBSL (Isohanni et al., 2010). These findings indicate that MRI could potentially be used to provide an early, pre-symptomatic diagnosis of the disease. An adult-onset LBSL patient was described to have solely right-sided spasticity, yet pyramidal tracts were equally affected bilaterally throughout their entire length (Moore et al., 2012), suggesting that MRI results do not always match clinical symptoms. MRI findings in HBSL patients are generally more consistent with the clinical presentation and MRI can be used to differentiate between infantile and adult-onset patients. Changes in adult-onset patients are more localized and less severe (Wolf et al., 2015). Moreover, in the least affected sibling of three reported HBSL patients, cerebral WM changes were noted to be patchy and less widespread compared to the more severely affected siblings (Ong et al., 2020).

Apart from HBSL and LBSL, there are other white matter disorders that affect similar regions of the CNS, which could potentially result in misdiagnosis. For example, one patient with mutations in the iron-sulfur cluster assembly 2 (ISCA2) gene was initially diagnosed with LBSL based on MRI and clinical features, prior to genetic testing (Toldo et al., 2018). Mutations in this gene cause multiple mitochondrial dysfunctions syndrome 4 (MMDS4), which has MRI features similar to LBSL and HBSL including signal changes in the periventricular WM, corpus callosum, posterior limb of the IC, cerebellar WM and cerebellar peduncles (Al-Hassnan et al., 2015; Alaimo et al., 2018; Toldo et al., 2018; Eidi and Garshasbi, 2019; Hartman et al., 2020). Additionally, a few MMDS4 patients also display lactate peaks on MRS (Alfadhel, 2019), as well as diffusion restriction in some affected WM regions on DWI (Alaimo et al., 2018; Toldo et al., 2018; Hartman et al., 2020). However, some MMDS4 patients show signal abnormality of the cerebral peduncles (Alaimo et al., 2018), as well as glycine elevation (Alfadhel, 2019), which have not been reported in LBSL or HBSL. Similarities have also been drawn between multiple sclerosis (MS) and HBSL and LBSL (Mierzewska et al., 2011; Wolf et al., 2015). This is likely because MS is seen to affect the same regions of the brain—the periventricular WM, brainstem, cerebellar peduncles, cerebellum and spinal cord (Filippi et al., 2019). Unlike MS however, abnormalities of WM in HBSL and LBSL are noted to be more symmetrical, and to affect specific tracts (Mierzewska et al., 2011; Filippi et al., 2019). Especially in the spinal cord, MS lesions are seen to be limited to a maximum of two vertebral levels and to be focal rather than affecting whole tracts (Wolf et al., 2015; Filippi et al., 2019). Adult-onset HBSL patients presenting with only focal MRI changes were originally misdiagnosed with MS, prior to genetic testing. As a result, treatment with steroids was initiated, resulting in transient recovery of symptoms (Wolf et al., 2015). The responsiveness to steroids paired with a relapsing-remitting course and focal white matter and spinal cord signal changes on MRI normally indicate inflammatory demyelinating diseases such as MS or neuromyelitis optica (Wolf et al., 2015). The similar presentation of adult-onset HBSL cases and MS patients indicates that the actual number of HBSL cases might be higher than originally anticipated and it has been suggested that HBSL be included in the differential diagnosis of CNS inflammatory diseases (Wolf et al., 2015).

As previously mentioned, the definite diagnosis of HBSL and LBSL requires genetic testing. The first LBSL cases reported were diagnosed using genome-wide linkage analysis to determine the disease-causing mutations (Scheper et al., 2007). The first diagnosis of HBSL was made using a novel technology at the time—full-genome trio sequencing (Taft et al., 2013). Using this method, the entire genome of the patient and both parents are sequenced and compared to limit the number of candidate genes. In a clinical setting, however, standard sequencing techniques such as next-generation sequencing (NGS) and Sanger sequencing are used to obtain a genetic diagnosis when HBSL and LBSL are already suspected based on clinical and MRI findings, which is faster and more cost-efficient than whole genome sequencing (WGS). Some LBSL patients with large deletion mutations have been reported (Lan et al., 2017). These mutations are not detected by standard sequencing techniques and it is recommended to follow up with copy number analysis to provide a diagnosis (Lan et al., 2017). Whole exon deletions have not been described for HBSL.

MECHANISM OF DISEASE

With the use of WGS, mutations in the nuclear genes *DARS* and *DARS2* were identified to be the cause of HBSL and LBSL, respectively. *DARS1* is located on chromosome 2 in the 2q21.3 region and encodes AspRS (Taft et al., 2013). *DARS2* is located on chromosome 1 in the 1q25.1 region and encodes the mitochondrial counterpart mt-AspRS (Scheper et al., 2007). While AspRS acts exclusively in the cytosol, mt-AspRS is transported into the mitochondrial matrix and is responsible for the translation of mitochondrial transcripts.

The majority of LBSL and HBSL patients are compound heterozygous. Only very few LBSL patients are homozygous (5.6% of reported cases). In comparison, 37.5% of reported HBSL cases are homozygous. All *DARS1* mutations identified thus far are missense mutations, affecting amino acids primarily located in and around the catalytic domain of AspRS (Taft et al., 2013; Wolf et al., 2015; Ong et al., 2020) (**Table 4**). These mutations are thought to impair aminoacylation directly through changes of the active site, or through destabilization of the side chain

TABLE 4 | Summary of known HBSL mutations and their corresponding amino acid change.

Nucleotide change	Amino acid change	Reference(s)
c.536G>A	p.R179K	Ong et al., 2020
c.599C>G	p.S200C	Wolf et al., 2015
c.766A>C	p.M256L	Taft et al., 2013
c.821C>T	p.A274V	Taft et al., 2013
c.830C>T	p.S277F	Wolf et al., 2015
c.839A>T	p.H280L	Wolf et al., 2015
c.1099G>T	p.D367Y	Taft et al., 2013
c.1099G>C	p.D367H	Wolf et al., 2015
c.1277T>C	p.L426S	Wolf et al., 2015
c.1379G>A	p.R460H	Taft et al., 2013
c.1391C>T	p.P464L	Taft et al., 2013
c.1459C>T	p.R487C	Taft et al., 2013
c.1480C>G	p.R494G	Taft et al., 2013; Ong et al., 2020
c.1480C>T	p.R494C	Taft et al., 2013

interactions with the active site (Taft et al., 2013). Many of these mutations occur in highly conserved amino acids of AspRS (Taft et al., 2013; Ong et al., 2020).

In contrast, LBSL-causing mutations are scattered throughout the length of the *DARS2* gene (**Table 5**). These mutations include missense, non-sense, deletion, and splice site mutations. Most patients carry a mutation in the intron 2 splice acceptor region, several nucleotides upstream of exon 3 (Scheper et al., 2007). A mutation in this region affects about 88% of LBSL patients reviewed, and one mutation at this site (c.228-20_21delTTinsC) is estimated to have a carrier frequency of 1:95 in the Finnish population (Isohanni et al., 2010). A mutation at this splice acceptor region causes the exclusion of exon 3 in mt-AspRS transcripts. This exclusion ultimately results in a frame shift (p.R76SfsX5) and premature stop (van Berge et al., 2012), possibly producing a truncated, non-functional protein. It is important to note that this mutation is considered "leaky," allowing low-level production of wild-type, functional mt-AspRS protein from transcripts including exon 3 (van Berge et al., 2012). Remarkably, this splicing defect is particularly profound within neural cells, and specifically in neurons, significantly lowering the amount of functional mt-AspRS produced by these cells (van Berge et al., 2012). Even in healthy individuals with two unaffected *DARS2* copies, splicing of this region is less efficient in neurons compared to other cell types. When this mutation is coupled with another *DARS2* mutation on the second allele, the amount of wild-type protein produced becomes insufficient (van Berge et al., 2012). The splicing differences between individuals potentially explain the large clinical variation among patients, including intrafamilial variations where affected family members possess the same *DARS2* mutations (Labauge et al., 2011). Despite its high prevalence, homozygous mutations at this site have only been reported in one family with three affected siblings

TABLE 5 | Summary of known LBSL mutations and their corresponding amino acid change.

Nucleotide change	Amino acid change	Reference(s)
c.133A>G	p.S45G	Scheper et al., 2007
c.172C>G	p.R58G	Kohler et al., 2015
c.228-10C>A	p.R76SfsX5	Scheper et al., 2007; Steenweg et al., 2012
c.228-11C>G	p.R76SfsX5	Scheper et al., 2007; Schicks et al., 2013
c.228-12C>A	p.R76SfsX5	Galluzzi et al., 2011; Sharma et al., 2011
c.228-12C>G	p.R76SfsX5	Orcesi et al., 2011; Steenweg et al., 2012
c.228-15C>A	p.R76SfsX5	Scheper et al., 2007; Steenweg et al., 2012; Yelam et al., 2019
c.228-15C>G	p.R76SfsX5	Scheper et al., 2007; Shimojima et al., 2017
c.228-16C>A	p.R76SfsX5	Scheper et al., 2007; Lin et al., 2010; Steenweg et al., 2012; Lan et al., 2017
c.228-16C>G	p.R76SfsX5	Labauge et al., 2011; Steenweg et al., 2012
c.228-20_21delTTinsC	p.R76SfsX5	Scheper et al., 2007; Uluc et al., 2008; Erturk et al., 2010; Isohanni et al., 2010; Mierzewska et al., 2011; Tzoulis et al., 2012; Cheng et al., 2013; Martikainen et al., 2013; Alibas et al., 2014; Tylki-Szymanska et al., 2014; Cavusoglu et al., 2018; Gonzalez-Serrano et al., 2018; Werner et al., 2018
c.228-22T>A	p.R76SfsX5	Miyake et al., 2011
c.228-24insT	p.R76SfsX5	Orcesi et al., 2011
c.259G>A	p.D87N	Rathore et al., 2017
c.295-2A>G	p.A100_P132del	Scheper et al., 2007
c.396+2T>G	p.A100_P132del	Scheper et al., 2007
c.358_359delinsTC	p.G120S	Shimojima et al., 2017
c.374G>A	p.R125H	Ikazabo et al., 2020
c.397-2A>G	p.M134_K165del	Scheper et al., 2007; Erturk et al., 2010
c.492+2T>C	p.M134_K165del	Scheper et al., 2007; Isohanni et al., 2010; Galluzzi et al., 2011; Moore et al., 2012; Steenweg et al., 2012; Martikainen et al., 2013; Alibas et al., 2014; Grechanina and Zdubskaya, 2014; Tylki-Szymanska et al., 2014
c.416T>C	p.I139T	Steenweg et al., 2012
c.455G>T	p.C152F	Scheper et al., 2007; Isohanni et al., 2010; Steenweg et al., 2012; Cavusoglu et al., 2018; Werner et al., 2018
c.473A>T	p.E158V	Moore et al., 2012
c.536G>A	p.R179H	Scheper et al., 2007
c.550C>A	p.Q184K	Scheper et al., 2007
c.563G>A	p.R188Q	Yahia et al., 2018

(Continued)

TABLE 5 | Continued

Nucleotide change	Amino acid change	Reference(s)
c.617_663del	p.F207CfsX25	Schicks et al., 2013
c.716T>C	p.L239P	Lin et al., 2010
c.742C>A	p.Q248K	Scheper et al., 2007
c.745C>A	p.L249I	Labauge et al., 2011
c.749T>C	p.L250P	Steenweg et al., 2012
c.787C>T	p.R263X	Scheper et al., 2007
c.788G>A	p.R263Q	Scheper et al., 2007; Rathore et al., 2017
c.822_825del	p.R274SfsX9	Steenweg et al., 2012
c.850G>A	p.E284K	Cheng et al., 2013
c.1013G>A	p.G338E	Gonzalez-Serrano et al., 2018; Ikazabo et al., 2020
c.1069C>T	p.Q357X	Sharma et al., 2011
c.1129_1191	p.K377_Q397del	Lan et al., 2017
c.1272_1273G>C	p.E424NfsX1	Scheper et al., 2007
c.1273G	p.E425X	Scheper et al., 2007
c.1345-17del13	p.C449_K521del	Uluc et al., 2008
c.1395_1396delAA	p.G467SfsX7	Tzoulis et al., 2012
c.1564_1674del	p.A522_K558del	Scheper et al., 2007
c.1679A>T	p.D560V	Scheper et al., 2007
c.1762C>G	p.L588V	Yahia et al., 2018
c.1825C>T	p.R609W	Synofzik et al., 2011
c.1837C>T	p.L613F	Scheper et al., 2007
c.1875C>G	p.L626V	Scheper et al., 2007
c.1876T>A	p.L626Q	Scheper et al., 2007
c.1886A>G	p.Y629C	Scheper et al., 2007

(Miyake et al., 2011). Additionally, one patient carrying two different mutations at this site has been reported (Orcesi et al., 2011). In HBSL, the most prevalent mutation site is in exon 9 of the *DARS1* gene, causing a single amino acid change from methionine to leucine. This mutation was found in four patients from three different families (Taft et al., 2013).

ARSs are primarily responsible for the accurate pairing of tRNAs to their respective amino acids, in a two-step aminoacylation reaction (Wallen and Antonellis, 2013). This is often referred to as charging. Some ARSs have additional editing capabilities to identify and correct erroneous charging (Beuning and Musier-Forsyth, 2000). However, such a capability has not been reported for AspRS or mt-AspRS. All studies investigating the mechanism of disease in HBSL and LBSL have so far been mainly targeted toward their canonical function of aminoacylation. There is a growing body of evidence, though, that the secondary ARS functions may also play a role in ARS pathologies (Guo and Schimmel, 2013). One study found missense mutations in *DARS2* regions that are solely conserved in mammals, which could suggest that the evolutionary later-acquired supplementary functions of mt-AspRS may be involved in LBSL pathology (Sauter et al., 2015). These secondary functions include inflammatory and immune regulation by ARSs (Guo and Schimmel, 2013). In addition, cytosolic AspRS is known to interact with other cytosolic ARSs and aminoacyl tRNA synthetase complex interacting multifunctional proteins (AIMPs) to form a multi-synthetase complex (MSC) (Khan et al., 2020). While much of the MSC's function and role is shrouded, it is thought to regulate the secondary functions of the involved ARSs and to promote translation by ferrying tRNAs to ribosomes (Lee et al., 2004). Thus, impaired MSC function could play a role in HBSL (**Figure 1**).

Compromised aminoacylation may occur as a result of reduced enzyme production, decreased catalytic reaction or impaired dimerization. *In vitro* analysis of seven missense mutations of *DARS2* revealed that protein levels were greatly reduced as a result of the C152F, Q184K, and D560V mutations (van Berge et al., 2013). The D560V mutation additionally caused a decrease of catalytic activity by 83.4% (van Berge et al., 2013) while another study showed a much greater reduction of up to 99.7% (Scheper et al., 2007). The R263Q and L626Q mutations also reduced catalytic activity of mt-AspRS by 99.3 and 97.7%, respectively (van Berge et al., 2013). Reduced mt-AspRS activity was also found in patient-derived lymphoblasts of a compound heterozygous (missense mutations R125H and G338E) LBSL case (Ikazabo et al., 2020), as well as in patients with the highly prevalent splicing defect and other missense mutations (Van Berge et al., 2014). It is notable that not all missense mutations studied resulted in reduced mt-AspRS expression or activity (van Berge et al., 2013). An effect of *DARS1* mutations on AspRS expression levels or activity in HBSL patients has not been reported yet. However, the positions of affected amino acids in and around the catalytic domain strongly suggest that these mutations affect AspRS enzymatic activity (Taft et al., 2013).

In LBSL, impaired transport of mt-AspRS from the cytosol into mitochondria has been suggested as a potential cause of compromised aminoacylation. This disease mechanism was described for the S45G mutation, which is situated in the mitochondrial targeting sequence (MTS) of mt-AspRS (Messmer et al., 2011). The MTS enables nuclear-encoded mitochondrial precursor proteins to be recognized by and transported into mitochondria (Omura, 1998). The S45G mutation impaired the import of precursor mt-AspRS through the mitochondrial membranes, but did not affect targeting of mt-AspRS to the surface of mitochondria (Messmer et al., 2011). A change in mt-AspRS solubility has also been suggested as a potential cause of disease. One study found that the Q184K mutation significantly reduced the solubility of mt-AspRS, while the other four investigated mutations did not substantially affect solubility (Sauter et al., 2015). When combined, two mutations causing only a mild decrease in solubility might be sufficient to trigger LBSL pathology (Sauter et al., 2015). A second study replicated these findings showing that the Q184K mutation significantly decreased the matrix localization of mt-AspRS (Gonzalez-Serrano et al., 2018).

The same study also established that mt-AspRS is localized in the mitochondrial matrix and in the mitochondrial membranes, where it is anchored through electrostatic interactions (Gonzalez-Serrano et al., 2018). This raised the possibility of disrupted ARS localization as a disease mechanism for LBSL (Gonzalez-Serrano et al., 2018). Localization errors could also

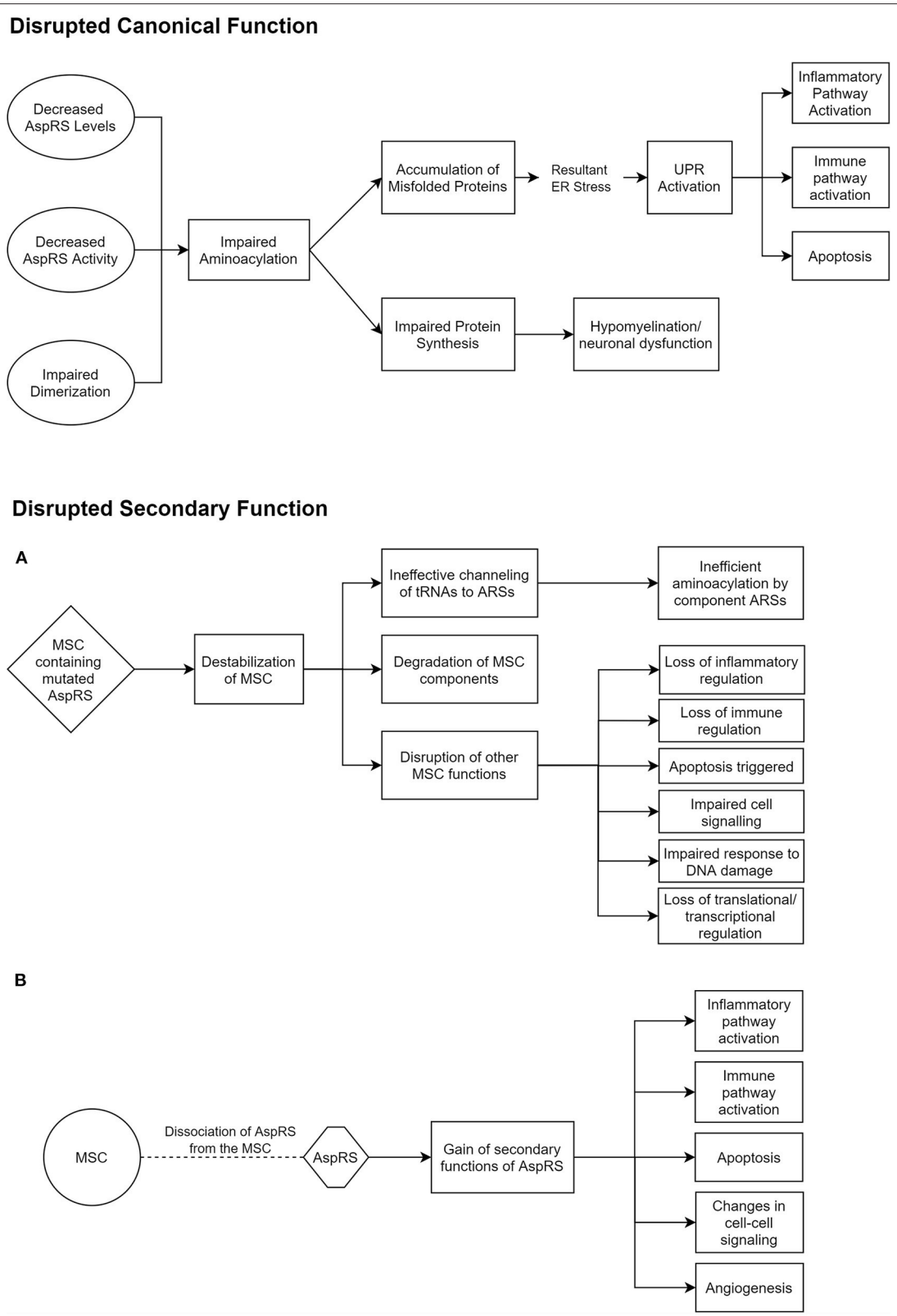


FIGURE 1 | Potential pathomechanisms underlying HBSL. These hypothesized modes of dysfunction in HBSL are based on current knowledge of DARS1, the MSC, and secondary functions of ARSs (Lee et al., 2004; Guo et al., 2010; Pang et al., 2014; Frohlich et al., 2017). **(A,B)** Offer two alternative explanations for the disruption of secondary AspRS function. AspRS, Aspartyl-tRNA Synthetase; ER, Endoplasmic Reticulum; UPR, Unfolded Protein Response; MSC, Multi-tRNA Synthetase Complex; ARS, Aminoacyl-tRNA Synthetase.

affect cytosolic ARSs that are part of the MSC. LysRS dissociates from the MSC in response to certain triggers (Ofir-Birin et al., 2013; Gonzalez-Serrano et al., 2018), which subsequently diverts LysRS away from translational sites (Ofir-Birin et al., 2013).

Finally, dimerization is an important aspect to consider in HBSL and LBSL, as both AspRS and mt-AspRS function as homodimers (Bonnefond et al., 2005; Kim et al., 2013). The R58G, T136S, and L626Q mt-AspRS mutations were found to impair dimerization with other mutant mt-AspRS monomers; while the C152F, Q184K, R263Q, and D560V mutations even impacted dimerization with wild-type mt-AspRS subunits (van Berge et al., 2013). None of the reported *DARS1* mutations is located in the dimerization domain of AspRS. However, it has previously been demonstrated in yeast that a change in the highly conserved amino acid P273 in the dimerization domain of AspRS leads to a reduction in catalytic activity of mutant homodimers (Eriani et al., 1993).

Due to the almost identical MRI pattern and similar clinical symptoms, it has been hypothesized that the two conditions could share a common, underlying disease mechanism. Both conditions particularly affect the nervous system, despite ARSs being ubiquitously expressed enzymes. As previously mentioned, in LBSL, the splicing defects seem to be more profound in neurons compared to other cell types (van Berge et al., 2012). The characteristic presentation of LBSL patients without the intron 2 splice site mutation should be further explored. Expression pattern studies of *DARS1* have revealed that neurons have a higher AspRS expression level compared to other cell types in the human brain (Frohlich et al., 2018). On a regional level, the highest AspRS expression was observed in neurons of the cerebellum, particularly in the Purkinje cell layer (Frohlich et al., 2018). To a lesser extent, the motor cortex (predominantly layers II-VI) and hippocampus (CA1, CA2, and CA3 layers) also displayed considerable *DARS1* expression levels. This specific expression pattern may explain why these areas are particularly affected in HBSL (Frohlich et al., 2018). Interestingly, the white matter of the cerebellum and brainstem showed only limited *DARS1* expression (Frohlich et al., 2018). This is in contrast to findings of *DARS2* expression pattern analysis, which was found to be comparable across different tissues (Scheper et al., 2007). However, levels of mitochondrial tRNA levels were found to be highest in the central nervous system (CNS) (Dittmar et al., 2006; Scheper et al., 2007).

MOUSE MODELS OF DISEASE

Mouse models are useful tools to explore possible disease mechanisms and are a prerequisite for trial of potential therapies. *In vitro* studies have also contributed valuable insight to our current understanding of the diseases, supplementing experiments in mice. Several attempts have been made to model HBSL and LBSL in mice which have provided important mechanistic insights.

The first attempt at an LBSL mouse model found that complete knockout of *Dars2* is lethal; these mice died around embryonic day eight, coinciding with organogenesis (Dogan et al., 2012). Subsequent efforts were directed toward conditional knockout (cKO) of *Dars2* in specific tissues. The first attempt at modeling HBSL in mice encountered the same barrier, as the complete knockout was embryologically lethal due to developmental arrest (Frohlich et al., 2017). This indicates that complete functional null mutations are not tolerated, and the resulting embryonic lethality may explain the rather low numbers of HBSL and LBSL cases diagnosed to date.

Additionally, in both HBSL and LBSL mouse models, the heterozygous null state produced largely phenotypically normal mice (Dogan et al., 2014; Frohlich et al., 2017). This is consistent with the recessive genetic trait of inheritance, that is further supported by the finding that unaffected siblings and parents of patients, who possess one wild-type copy of the *DARS1* or *DARS2* gene, are healthy. Altogether, these findings provide experimental evidence that some residual activity of both these ARSs is necessary for life, and that one unaffected copy of the gene is sufficient to avoid disease.

Two neuronal *Dars2* cKO models have been created utilizing the Cre/LoxP system (Aradjanski et al., 2017; Nemeth et al., 2020). In both models, mice were crossed to CamKIIα Cre-driver lines, expressing Cre recombinase under control of the neuronal CamKIIα promotor. Peak CamKIIα-Cre expression, and hence recombination and *Dars2* knockout, is reached at around postnatal week 4 (Aradjanski et al., 2017). Neuronal *Dars2* cKO mice displayed severe morphological and behavioral changes starting around week 24 and worsening until 28 weeks of age (Aradjanski et al., 2017). These morphological changes included severe brain atrophy concentrated around the forebrain cortex and hippocampal area (Aradjanski et al., 2017; Nemeth et al., 2020) and enlargements of the third and lateral ventricles, with a notable sparing of the cerebellum (Nemeth et al., 2020). Additionally, thinning of the corpus callosum was also noted (Aradjanski et al., 2017; Nemeth et al., 2020). Microscopically, this is accompanied by neuronal cell death, as demonstrated by the presence of pyknotic cells and vacuoles in the affected regions at 28 weeks of age (Aradjanski et al., 2017). In one study, these changes occurred on the background of limited weight gain compared to wild-type mice, and a shorter lifespan. These mice also displayed self-injuries around 28 weeks of age, coinciding with the most severe morphological changes, in addition to motor abnormalities such as tremor and ataxia (Aradjanski et al., 2017). Conversely, in the other study, neuronal *Dars2* cKO mice were of higher body mass than controls until 28 weeks of age, after which they were comparable to controls. In addition, neuronal *Dars2* cKO mice were more active compared to controls, particularly in their explorative behavior (Nemeth et al., 2020). Both models displayed an increase in mitochondria number, however, the mitochondrial ultrastructure appeared normal in one model (Nemeth et al., 2020), while there was a loss of normal cristae in the other model indicating mitochondrial dysfunction (Aradjanski et al., 2017). It remains unclear why the two studies, despite using the same neuronal *Dars2* cKO model, resulted in such different outcomes.

A third neuronal LBSL mouse model specifically targeting Purkinje cells in the cerebellum was recently reported (Rumyantseva et al., 2020). In this model, Cre recombinase

was expressed under control of the Purkinje cell protein 2 promotor. Maximal recombination of *Dars2* took place at postnatal weeks 2 to 3 (Rumyantseva et al., 2020). Significant Purkinje cell death was noted at 15 weeks, accompanied by motor dysfunction such as unsteady gait and lack of balance. Morphologically, no cerebellar atrophy was noted (Rumyantseva et al., 2020). Histological analysis revealed a loss of Purkinje cell layer organization at this time. Preceding these changes, mitochondrial respiratory chain (MRC) dysfunction was noted at 6 weeks of age (Rumyantseva et al., 2020). As the cerebellum is the CNS region with the highest AspRS expression in humans and mice, a similar approach to model HBSL in mice should be considered.

It is remarkable that in LBSL patients, mitochondrial dysfunction including altered MRC activity, are not a common feature and have only been reported in one patient to date (Orcesi et al., 2011). Increase in mitochondrial size in muscle cells has also been reported in another patient (Linnankivi et al., 2004). Interestingly, one *in vitro* study revealed a strong reduction in the number of mitochondrial DNA encoded MRC complex proteins in fibroblasts of a compound heterozygous LBSL patient, together with reduced cellular oxygen consumption and increased mitochondrial fragmentation (Lin et al., 2019).

Dars2 cKO was also performed in the myelinating cells of the central and peripheral nervous system (PNS) at the age of 4 weeks, using tamoxifen inducible Cre expression under control of the proteolipid 1 (*Plp1*) promoter. Similar to the neuronal *Dars2* cKO model produced by the same group, strong MRC deficiencies were observed in these mice, yet no other sequelae involving demyelination, apoptosis or inflammation followed (Aradjanski et al., 2017). Morphologically and behaviorally, these mice were normal too. It was suggested that myelinating cells possess a different metabolic profile compared to neurons, which enables them to produce sufficient energy through glycolysis when mitochondrial respiration is impaired. Mitochondria in mature oligodendrocytes may be more important for myelin maintenance through fatty acid oxidation and lipid synthesis rather than ATP production (Rinholm et al., 2016; Aradjanski et al., 2017). Since *Dars2* cKO only affects translation of mitochondrial DNA transcripts, and hence MRC subunit function and ATP production, myelination remained largely unaffected. Expression levels of cytosolic AspRS are much lower in oligodendrocytes compared to neurons (Frohlich et al., 2018). It has been hypothesized that the low expression levels could make these cells particularly susceptible to disturbances in protein synthesis. However, given the growing evidence of the influence of neurons on oligodendrocyte differentiation and myelination (Gibson et al., 2014; Mitew et al., 2014; Ortiz et al., 2019), it is also plausible for the primary pathology to lie in neuronal cell bodies and axonal processes. This is supported by the predominant involvement of long tracts in LBSL and HBSL (van Berge et al., 2012).

Dars2 cKO was also achieved in cardiac and skeletal muscles using the muscle creatine kinase (Ckmm) Cre-driver line. Maximal recombination of the *Dars2* gene occurred at 2 weeks of age in cardiac and skeletal muscle (SkM) cells (Dogan et al., 2014). While no cardiac defects have been reported in LBSL patients, muscle tissue has a similar energy demand and therefore findings from this study could be translationally relevant for LBSL. All mice developed hypertrophic cardiomyopathy by 6 weeks of age, quickly followed by death (Dogan et al., 2014). Microscopically, the response in each muscle tissue differed. Cardiac muscle fibers were hypertrophic and disorganized progressively, while atrophy and preserved fiber organization was observed in SkM fibers. Both the cardiac and skeletal myocytes experienced substantial MRC deficiency; however, mitochondrial biogenesis, cell stress pathway activation and suppressed autophagy was only observed in cardiomyocytes (Dogan et al., 2014). It is postulated that this absence, or rather significant delay in onset of regulatory changes is owed to the skeletal myocytes' low mitochondrial transcript turnover rate and the cells' much greater capacity to regenerate (Dogan et al., 2014).

The morphological, behavioral, and microscopic changes observed in these studies have been consistently preceded by molecular changes—mainly the activation of several stress pathways accompanied by immune and inflammatory responses (Dogan et al., 2014; Aradjanski et al., 2017; Nemeth et al., 2020). In the neuronal *Dars2* cKO model, these changes occur as early as 16 weeks of age and particularly impact immune pathways (Nemeth et al., 2020). The most notable change was the upregulation of Cystatin F (CST7). CST7 is a lysosomal protease inhibitor that is known to repress lysosomal proteases to prevent tissue damage (Kos et al., 2018). In the CNS, *Cst7* has been shown to be expressed, and then released from activated microglia (Nemeth et al., 2020). This typically occurs in areas of active demyelination and simultaneous remyelination (Ma et al., 2011). Interestingly, when the remyelinating potential is lost (such as in chronic demyelination), CST7 levels fall (Ma et al., 2011). Therefore, the reported 200-fold increase of CST7 levels in neuronal *Dars2* cKO mice (Nemeth et al., 2020) may indicate the enduring potential for remyelination. However, despite the CST7 increase, the neuronal *Dars2* cKO mice continued to deteriorate. Another role of CST7 is the regulation of cathepsin C (CTSC)—a known lysosomal cysteine protease, which was also upregulated in neuronal *Dars2* cKO mice (Nemeth et al., 2020). CTSC is involved in the activation of several immune cells, including T cells and neutrophils, and its absence has been shown to dampen the immune response (Colbert et al., 2009). One study found the knockout of *Ctsc* "rescued" the demyelinating process in multiple sclerosis (Shimizu et al., 2017). The upregulation of CTSC could potentially contribute to the progression of LBSL.

These immune changes coincided with signs of inflammation. In histological brain sections of neuronal *Dars2* cKO mice, a significant increase in the number of activated microglia expressing the inflammatory marker CD68 was observed (Nemeth et al., 2020). Although the role of CD68 in inflammation is unclear, it is known to be upregulated in macrophage-mediated inflammatory processes and is hence a reliable indicator for microglia activation (Chistiakov et al., 2017). This finding is further corroborated by the second neuronal *Dars2* cKO study, where increasing microglial activation was observed in the cortex until 20 weeks of age, when neuronal cells began to undergo apoptosis (Aradjanski et al., 2017). These inflammatory changes resulted in a near complete disruption of normal cellular

structures in the hippocampal area close to the time of death (Aradjanski et al., 2017), and even contributed to cell-death in CamKIIα negative neurons (Nemeth et al., 2020). In the Purkinje cell *Dars2* cKO mouse model, the observed microglial activation and associated neuroinflammatory changes have been attributed to degradation of Purkinje cell dendrites and their complex network (Rumyantseva et al., 2020).

In addition, molecular changes indicative of mitochondrial dysfunction and disrupted proteostasis have been observed. Following conditional knockout of *Dars2* in cardiac and SkM, the activation of the mitochondrial unfolded protein response (UPR^{mt}) in cardiomyocytes was evident by 6 weeks of age (Dogan et al., 2014). Stress pathway activators such as activation transcription factor 5 (ATF5) and C/EBP homologous protein (CHOP) were found to be upregulated at this time point (Dogan et al., 2014). These molecules stimulate the UPR^{mt}, which is responsible for the degradation of aberrant proteins in order to maintain cell homeostasis (Haynes and Ron, 2010). The activation of the UPR^{mt} was the first notable change in mutant cells and increased ATF5 and CHOP levels along with proteases involved in the UPR^{mt} were identified before any detectable MRC deficits (Dogan et al., 2014). In the neuronal *Dars2* cKO model, molecular changes suggestive of UPR^{mt} activation were also observed at 20 weeks of age coinciding with severe MRC defects (Aradjanski et al., 2017). Accordingly, the activation of the unfolded protein response (UPR) as a possible disease mechanism for HBSL was suggested (Frohlich et al., 2017).

A 250-fold increase of fibroblast growth factor 21 (FGF21) within the first 2 weeks of life in the heart of Ckmm *Dars2* cKO mice is further evidence of mitochondrial dysfunction preceding detectable MRC deficiency (Dogan et al., 2014). FGF21 has been shown to be elevated in mitochondrial diseases, particularly those affecting the muscles, and has even been suggested as a biomarker for mitochondrial disorders for its high sensitivity and specificity (Suomalainen et al., 2011). Notably, most of the FGF21 increase resulted from cardiomyocytes, where *Fgf21* expression levels are usually low (Dogan et al., 2014). FGF21 is predominantly known for its role in modulating metabolic pathways (Potthoff et al., 2009). This is also evident in Ckmm *Dars2* cKO mice through altered nutrient usage (Dogan et al., 2014). Additionally, FGF21 ensures the stability of the transcription factor Peroxisome proliferator-activated receptor-Gamma Coactivator 1-alpha (PGC-1α) (Fisher et al., 2012). PGC-1α is a key controller of mitochondrial biogenesis (Ventura-Clapier et al., 2008)—an adaptive response to increased ATP demand.

In addition to the UPR, the integrated stress response (ISR) pathway was also activated in neuronal *Dars2* cKO mice. This was evident through the upregulation of *Atf4*, a key component of the ISR, at 16 weeks of age (Nemeth et al., 2020). ISR activation also occurred prior to any observable neuronal death. It is remarkable that only increased mitochondrial biogenesis, without any mitochondrial dysfunction, was noted in this model. In the Purkinje cell *Dars2* cKO mouse model, ISR activation was noted at 8 weeks of age, as evidenced by the initiation of the one-carbon metabolic pathway. This pathway has been linked to the activation of the ISR through *Atf4* upregulation (Rumyantseva et al., 2020). The ISR is a cell stress pathway intended to maintain homeostasis and is activated upon dysregulation of proteostasis, oxidative damage, loss of nutrients, and other similar events (Costa-Mattioli and Walter, 2020). When the challenge becomes unmanageable, apoptosis is triggered. Intriguingly, prolonged activation of this usually protective pathway can in itself become cytotoxic, which has been shown to be the case in several neurodegenerative disorders (Rabouw et al., 2019).

Taken together, an imbalance in protein homeostasis, which is evident through a rise in ubiquitinated proteins, appears to be the initial trigger for the disease (Dogan et al., 2014). Subsequently, this leads to the activation of stress pathways, which through prolonged activation likely contributes to neuronal cell-death and neurodegeneration (**Figure 2**).

The neuronal *Dars2* knockout mouse model generated by Aradjanski et al. was able to capture some of the LBSL symptomatology seen in patients, such as ataxia and tremor (Aradjanski et al., 2017). Neuronal *Dars2* cKO mice also displayed worsening kyphosis with age. Signs of microglial activation and cell death corroborated the histological findings in LBSL patients (Yamashita et al., 2013). Still, gross pathological changes were mainly limited to cortical and hippocampal areas, while LBSL patients displayed WM changes in the cerebellum, brainstem and spinal cord in addition to cortical changes. This discrepancy can be explained by the predominant localization of CamKIIα positive neurons within the hippocampus and cortex, while the midbrain, medulla and cerebellum lack CamKIIα positive neurons (with the exception of Purkinje cells) (Wang et al., 2013). The use of a more appropriate neuronal Cre-driver to ablate *Dars2* in all neurons of the CNS might result in a more accurate LBSL model. Surprisingly, despite using the same CamKIIα Cre-driver, Nemeth et al. demonstrated increased activity of neuronal *Dars2* cKO mice without any overt motor deficits (Nemeth et al., 2020). It is also important to bear in mind that regulation of mRNA translation in the human brain likely varies from the mouse brain.

For HBSL, our lab is currently working on a *Dars1* cKO mouse model. In addition, we are attempting to model HBSL by introducing patient specific point mutations into the mouse *Dars1* gene. The first mutation we introduced was the $Dars1^{D367Y}$ point mutation found in the HBSL index patient (see Froehlich et al., in this issue). While homozygous $Dars1^{D367Y/D367Y}$ mice failed to model HBSL, mice compound heterozygous for the $Dars1^{D367Y}$ and the $Dars1^{null}$ mutation captured some aspects of the disease.

In summary, attempts at modeling HBSL and LBSL have contributed significantly to our understanding of both conditions. Nevertheless, these studies have their limitations. In all studies, *Dars2* was rendered functionally inactive postnatally. Since CNS development is accelerated in mice compared to humans, these knockouts occur relatively late during development. In contrast, *DARS2* mutations are evident from birth in LBSL patients. These mutations greatly reduce, but do not completely abolish mt-AspRS function, throughout the developmental period.

In mice, complete knockout of *Dars1* or *Dars2* were both embryonically lethal. This indicates a lack of functional

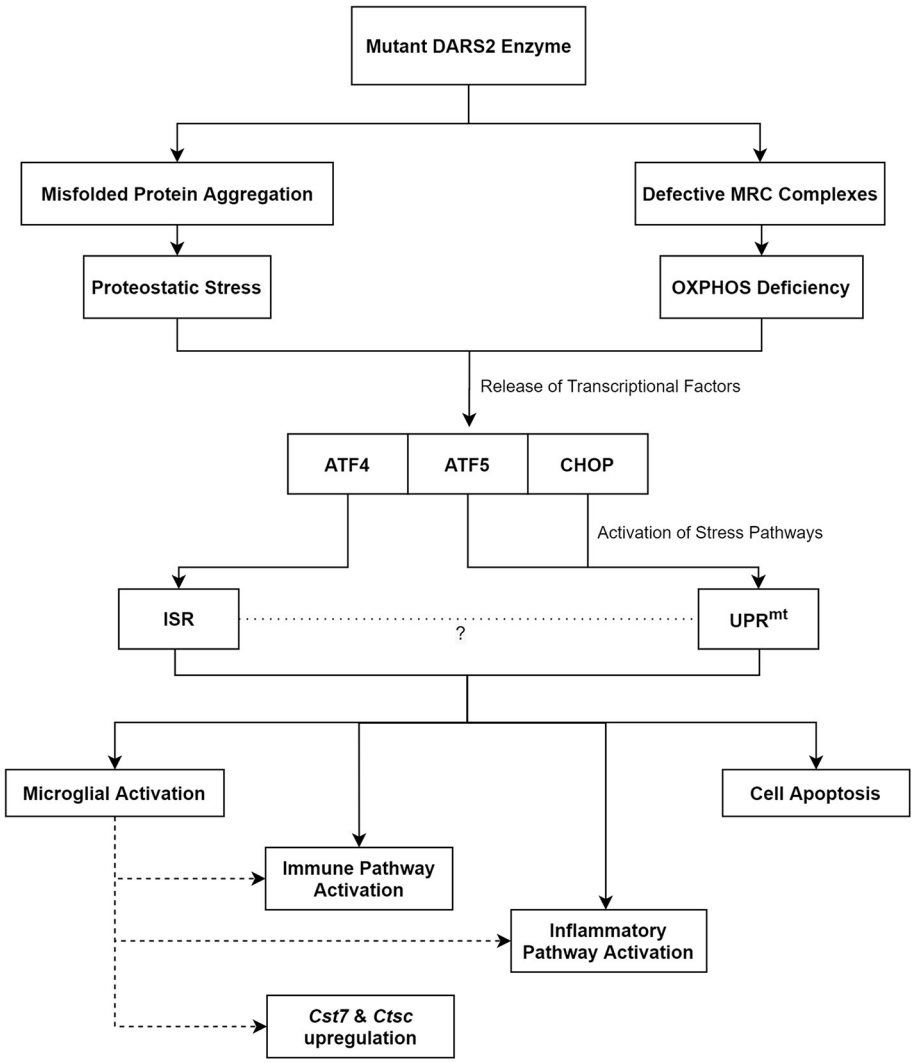


FIGURE 2 | The proposed pathomechanisms of LBSL based on findings from the neuronal *Dars2* cKO mouse models. MRC, Mitochondrial Respiratory Chain; OXPHOS, Oxidative Phosphorylation; ATF, Activation Transcription Factor; CHOP, C/EBP Homologous Protein; ISR, Integrated Stress Response; UPR^{mt}, Mitochondrial Unfolded Protein Response; Cst7, Cystatin F; Ctsc, Cathepsin C.

redundancy between the two enzymes and puts to rest questions on possible compensatory mechanisms between the two. However, other findings from these studies continue to offer support for a possible shared disease mechanism between LBSL and HBSL. Firstly, the finding of inflammatory processes and immune responses likely underlying neuronal dysfunction and cell-death in LBSL is reminiscent of the study by Wolf et al., who postulated that HBSL might possess a neuroinflammatory component based on clinical findings and the responsiveness of HBSL patients to steroids (Wolf et al., 2015). This should be addressed when studying the underlying disease mechanism in future HBSL mouse models.

Secondly, studies in LBSL models suggest the primary pathology to lie in neuronal or axonal dysfunction rather than in myelin-producing cells or the myelin itself. These findings are supported by the absence of noticeable phenotypic changes in mice following *Dars2* cKO in myelinating cells. Moreover, one study reported findings of axonal degeneration of myelinated fibers in histopathological analysis of tissues from LBSL patients. This was accompanied by detection of myelin ovoids in the peripheral sural nerve, and the absence of segmental demyelination, hence the pattern of peripheral neuropathy in these patients was suggested to be mainly axonal (Yamashita et al., 2013). Myelin ovoids are often associated with Wallerian degeneration—a pathological process where demyelination occurs secondary to axonal pathology. Myelin ovoids result from the clearance of myelin by Schwann cells (Tricaud and Park, 2017). Segmental demyelination indicates myelin degeneration with sparing of nerve fibers, yet this was not a prominent finding in the histological analysis (Yamashita et al., 2013). Accordingly, AspRS was found to be predominantly expressed by neurons, with lower expression

levels in oligodendrocytes and astrocytes. In mice, the subcellular localization of AspRS was not restricted to the cell body of neurons but also in the axons and synapses (Frohlich et al., 2017). Taken together, there is a strong rationale for a primary neuronal or axonal dysfunction underlying LBSL and HBSL pathology.

THERAPIES AND MANAGEMENT

Management of HBSL and LBSL are currently solely supportive and symptomatic. While these methods can improve the quality of life, the benefits are neither sustained nor equally effective for all patients. Hence, there is a need for the development of novel treatment strategies for patients affected by these conditions. Some medications have been shown to be effective in patients and when tested *in vitro* for LBSL (Synofzik et al., 2011; Van Berge et al., 2014). As previously stated, for the investigation and trial of new curative treatment options accurate animal models are required to conduct pre-clinical studies. In the HBSL mouse model study, phenotypically normal heterozygous null mice possessed just 20% of AspRS protein levels compared to control mice (Frohlich et al., 2017). As such, reinstating the enzyme to this "threshold" level might be sufficient to ameliorate symptoms or prevent disease.

For the symptomatic management of LBSL, the use of allied health specialties such as speech, nutrition and physiotherapy has been reported, to support patient disability (Navarro Vázquez et al., 2016; Werner et al., 2018). In addition, medications for pain and spasticity are also used for symptom management (Werner et al., 2018). Antispastic medications have been shown to improve symptoms gradually in adult-onset LBSL patients (Petzold et al., 2006). At the same time, genetic counseling is often provided to affected families. In HBSL, management is similarly symptom-based, with chemodenervation additionally suggested to manage spasticity (Ulrick and Vanderver, 2017).

In both conditions, steroid medications have been shown to reduce the severity of symptoms experienced by patients. Particularly in HBSL, 5 out of 16 patients reported in the literature showed improvements following administration of steroids, three of whom were infantile-onset patients (Taft et al., 2013; Wolf et al., 2015). One early-onset patient was described to gain muscle strength and postural tone, although these improvements were only modest (Wolf et al., 2015). In late-onset patients, the effect of steroids was inconsistent—with one patient regaining his ability to walk independently following a single course of methylprednisolone, while another patient repeatedly experienced relapse of symptoms over a few months post-steroid treatment (Wolf et al., 2015). In LBSL, to our knowledge, the use of steroids has only been described in one patient who displayed spastic bladder symptoms, which improved following the use of glucocorticoids, although other symptoms persisted (Cheng et al., 2013). Following the uncovering of activated inflammatory and immune pathways in LBSL mouse models, the use of steroids could be beneficial for symptom management in LBSL patients in the future. Concomitantly, variable suppression of symptoms through the use of steroids in HBSL hints at inflammatory and immune activation as a component of the disease process. Further investigation into the cause of the activation of inflammatory and immune pathways—such as the UPR and ISR—will help develop more effective treatment options. An ISR inhibitor (ISRIB) is of interest, as it has delivered promising results in inhibiting the harmful effects of ISR overactivation without producing excessive side effects from inhibition of healthy, basal ISR protection (Rabouw et al., 2019).

Lately, some drugs have gained special attention for the treatment of LBSL. Firstly, the carbonic anhydrase inhibitor acetazolamide was reported to improve ataxia in one patient (Synofzik et al., 2011). This medication, however, was used in a patient with a highly atypical presentation, where ataxia was episodic and triggered by exercise. In such a patient, 125 mg bi-daily acetazolamide was shown to decrease the mean number of ataxic attacks per day by about 77%. Acetazolamide is a standard medication used in episodic ataxia syndromes, for which this patient did not harbor mutations, and is thought to exert its effect in the CNS by reducing lactate and pyruvate levels (Griggs et al., 1978). Notably, this patient displayed lactate elevation on MRS. It remains to be seen if this medication will produce similar results in more typical LBSL patients. Another drug, cantharidin, has been demonstrated to improve the efficiency of splicing at the *DARS2* intron 2 splice site *in vitro* (Van Berge et al., 2014). There was a 5.9% increase in the inclusion of exon 3 in mRNA transcripts of *DARS2* following cantharidin application. While cantharidin is toxic and unsuitable for clinical use, the use of other protein phosphatase 1 and 2A inhibitors could be explored for therapeutic use. Alternatively, antisense oligonucleotides have also been shown to increase the efficiency of splicing at this site (van Berge et al., 2012), although their use is limited by their inability to cross the blood-brain barrier (Rinaldi and Wood, 2018). As the intron 2 splice site mutation is the most prevalent mutation reported in LBSL patients, this site is a valuable target for the treatment of LBSL (Van Berge et al., 2014). For HBSL, it has been suggested that boosting AspRS activity through nutraceutical L-ornithine-L-aspartate (LOLA) supplementation might have therapeutic effects (Das et al., 2020).

Finally, gene therapy is often considered for inherited conditions. This is a particularly viable option for HBSL and LBSL, as they are both monogenic disorders and therefore highly amenable to such a treatment approach. With the recent advances in creating animal models for HBSL and LBSL, attempts at establishing such a treatment modality will follow and hopefully provide a cure for these devastating conditions.

AUTHOR CONTRIBUTIONS

AM and DF led the project and the manuscript production. MK and GH contributed to the manuscript preparation. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was funded by the Medical Research Future Fund (MRFF-ARLKO), the European Leukodystrophy Association (ELA 2018-014I2), and the Mission Massimo Foundation.

REFERENCES

Alaimo, J. T., Besse, A., Alston, C. L., Pang, K., Appadurai, V., Samanta, M., et al. (2018). Loss-of-function mutations in ISCA2 disrupt 4Fe-4S cluster machinery and cause a fatal leukodystrophy with hyperglycinemia and mtDNA depletion. *Hum. Mutat*. 39, 537–549. doi: 10.1002/humu.23396

Alfadhel, M. (2019). Multiple mitochondrial dysfunctions syndrome 4 due to ISCA2 gene defects: a review. *Child Neurol. Open* 6:2329048X19847377. doi: 10.1177/2329048X19847377

Al-Hassnan, Z. N., Al-Dosary, M., Alfadhel, M., Faqeih, E. A., Alsagob, M., Kenana, R., et al. (2015). *ISCA2* mutation causes infantile neurodegenerative mitochondrial disorder. *J. Med. Genet*. 52, 186–194. doi: 10.1136/jmedgenet-2014-102592

Alibas, H., Koytak, P. K., Ekinci, G., and Uluc, K. (2014). A case with leukoencephalopathy with brainstem and spinal cord involvement and elevated lactate (LBSL) with its characteristic clinical and neuroimaging findings. *Clin. Neuroradiol*. 24, 297–300. doi: 10.1007/s00062-013-0250-x

Antonellis, A., and Green, E. D. (2008). The role of aminoacyl-tRNA synthetases in genetic diseases. *Annu. Rev. Genomics Hum. Genet*. 9, 87–107. doi: 10.1146/annurev.genom.9.081307.164204

Aradjanski, M., Dogan, S. A., Lotter, S., Wang, S., Hermans, S., Wibom, R., et al. (2017). DARS2 protects against neuroinflammation and apoptotic neuronal loss, but is dispensable for myelin producing cells. *Hum. Mol. Genet.* 26, 4181–4189. doi: 10.1093/hmg/ddx307

Beuning, P. J., and Musier-Forsyth, K. (2000). Hydrolytic editing by a class II aminoacyl-tRNA synthetase. *Proc. Natl. Acad. Sci. U.S.A*. 97, 8916–8920. doi: 10.1073/pnas.97.16.8916

Bonnefond, L., Fender, A., Rudinger-Thirion, J., Giegé, R., Florentz, C., and Sissler, M. (2005). Toward the full set of human mitochondrial aminoacyl-tRNA synthetases: characterization of AspRS and TyrRS. *Biochemistry* 44, 4805–4816. doi: 10.1021/bi047527z

Cavusoglu, D., Olgac-Dundar, N., Oztekin, O., Ozdemir, T. R., Arican, P., and Gencpinar, P. (2018). The first pediatric case of leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) from Turkey. *Turk. J. Pediatr*. 60, 216–220. doi: 10.24953/turkjped.2018.02.018

Chang, L., Munsaka, S. M., Kraft-Terry, S., and Ernst, T. (2013). Magnetic resonance spectroscopy to assess neuroinflammation and neuropathic pain. *J. Neuroimmune Pharmacol*. 8, 576–593. doi: 10.1007/s11481-013-9460-x

Cheng, F. B., Shen, P. P., Zhou, H. W., Meng, H. M., Yang, Y., and Feng, J. C. (2013). Adult-onset leukoencephalopathy with brain stem and spinal cord involvement in Chinese Han population: a case report and literature review. *Neurol. India* 61, 161–163. doi: 10.4103/0028-3886.111123

Chistiakov, D. A., Killingsworth, M. C., Myasoedova, V. A., Orekhov, A. N., and Bobryshev, Y. V. (2017). CD68/macrosialin: not just a histochemical marker. *Lab. Invest.* 97, 4–13. doi: 10.1038/labinvest.2016.116

Colbert, J. D., Matthews, S. P., Miller, G., and Watts, C. (2009). Diverse regulatory roles for lysosomal proteases in the immune response. *Eur. J. Immunol.* 39, 2955–2965. doi: 10.1002/eji.200939650

Costa-Mattioli, M., and Walter, P. (2020). The integrated stress response: from mechanism to disease. *Science* 368:eaat5314. doi: 10.1126/science.aat5314

Das, A., Fröhlich, D., Achanta, L. B., Rowlands, B. D., Housley, G. D., Klugmann, M., et al. (2020). L-Aspartate, L-Ornithine and L-Ornithine-L-Aspartate (LOLA) and their impact on brain energy metabolism. *Neurochem. Res.* 45, 1438–1450. doi: 10.1007/s11064-020-03044-9

Dittmar, K. A., Goodenbour, J. M., and Pan, T. (2006). Tissue-specific differences in human transfer RNA expression. *PLoS Genet*. 2:e221. doi: 10.1371/journal.pgen.0020221

Dogan Sukru, A., Pujol, C., Maiti, P., Kukat, A., Wang, S., Hermans, S., et al. (2014). Tissue-specific loss of DARS2 activates stress responses independently of respiratory chain deficiency in the heart. *Cell Metab*. 19, 458–469. doi: 10.1016/j.cmet.2014.02.004

Dogan, S. A., Pujol, C., and Trifunovic, A. (2012). Mitochondrial aspartyl-tRNA synthetase (DARS2) deficiency in mice. *Free Radic. Biol. Med*. 1:S183. doi: 10.1016/j.freeradbiomed.2012.08.384

Eidi, M., and Garshasbi, M. (2019). A novel ISCA2 variant responsible for an early-onset neurodegenerative mitochondrial disorder: a case report of multiple mitochondrial dysfunctions syndrome 4. *BMC Neurol*. 19:153. doi: 10.1186/s12883-019-1387-2

Eriani, G., Cavarelli, J., Martin, F., Dirheimer, G., Moras, D., and Gangloff, J. (1993). Role of dimerization in yeast aspartyl-tRNA synthetase and importance of the class II invariant proline. *Proc. Natl. Acad. Sci. U.S.A*. 90, 10816–10820. doi: 10.1073/pnas.90.22.10816

Erturk, O., Yalcinkaya, C., Siva, A., and Van Der Knaap, M. S. (2010). Adult-onset leukoencephalopathy with brain stem and spinal cord involvement and normal lactate: case report. *Turk Noroloji Dergisi*. 16, 106–109. Available online at: https://tjn.org.tr/jvi.aspx?pdir=tjn&plng=eng&un=TJN-07752

Filippi, M., Preziosa, P., Banwell, B. L., Barkhof, F., Ciccarelli, O., De Stefano, N., et al. (2019). Assessment of lesions on magnetic resonance imaging in multiple sclerosis: practical guidelines. *Brain* 142, 1858–1875. doi: 10.1093/brain/awz144

Fisher, F. M., Kleiner, S., Douris, N., Fox, E. C., Mepani, R. J., Verdeguer, F., et al. (2012). FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev*. 26, 271–281. doi: 10.1101/gad.177857.111

Frohlich, D., Suchowerska, A. K., Spencer, Z. H. T., von Jonquieres, G., Klugmann, C. B., Bongers, A., et al. (2017). *In vivo* characterization of the aspartyl-tRNA synthetase DARS: homing in on the leukodystrophy HBSL. *Neurobiol Dis. Part A* 97, 24–35. doi: 10.1016/j.nbd.2016.10.008

Frohlich, D., Suchowerska, A. K., Voss, C., He, R., Wolvetang, E., von Jonquieres, G., et al. (2018). Expression pattern of the aspartyl-tRNA Synthetase DARS in the human brain. *Front. Mol. Neurosci*. 11:81. doi: 10.3389/fnmol.2018.00081

Fuchs, S. A., Schene, I. F., Kok, G., Jansen, J. M., Nikkels, P. G. J., van Gassen, K. L. I., et al. (2019). Aminoacyl-tRNA synthetase deficiencies in search of common themes. *Genet. Med*. 21, 319–330. doi: 10.1038/s41436-018-0048-y

Galluzzi, P., Sacchini, M., Bartalini, G., Monti, L., Cerase, A., Lamantea, E., et al. (2011). LBSL (leukoencephalopathy with brain stem and spinal cord involvement and high lactate) without sparing of the u-fibers and globi pallidi: a case report. *Eur. J. Radiol Extra* 79, e73–e76. doi: 10.1016/j.ejrex.2011.06.002

Gibson, E. M., Purger, D., Mount, C. W., Goldstein, A. K., Lin, G. L., Wood, L. S., et al. (2014). Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. *Science* 344:1252304. doi: 10.1126/science.1252304

Gonzalez-Serrano, L. E., Karim, L., Pierre, F., Schwenzer, H., Rotig, A., Munnich, A., et al. (2018). Three human aminoacyl-tRNA synthetases have distinct sub-mitochondrial localizations that are unaffected by disease-associated mutations. *J. Biol. Chem*. 293, 13604–13615. doi: 10.1074/jbc.RA118.003400

Götz, A., Tyynismaa, H., Euro, L., Ellonen, P., Hyötyläinen, T., Ojala, T., et al. (2011). Exome sequencing identifies mitochondrial alanyl-trna synthetase mutations in infantile mitochondrial cardiomyopathy. *Am. J. Hum. Genet*. 88, 635–642. doi: 10.1016/j.ajhg.2011.04.006

Grechanina, E. Y., and Zdubskaya, E. P. (2014). An early manifestation of LBSL (leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation) syndrome, case description. *J. Inherit. Metab. Dis*. 37:114. doi: 10.1007/s10545-014-9740-5

Griggs, R. C., Moxley, R. T., Lafrance, R. A., and McQuillen, J. J. N. (1978). Hereditary paroxysmal ataxia: response to acetazolamide. *Neurology* 28:1259. doi: 10.1212/WNL.28.12.1259

Guo, M., and Schimmel, P. (2013). Essential nontranslational functions of tRNA synthetases. *Nat. Chem. Biol*. 9, 145–153. doi: 10.1038/nchembio.1158

Guo, M., Yang, X.-L., and Schimmel, P. (2010). New functions of aminoacyl-tRNA synthetases beyond translation. *Nat. Rev. Mol. Cell Biol.* 11, 668–674. doi: 10.1038/nrm2956

Hartman, T. G., Yosovich, K., Michaeli, H. G., Blumkin, L., Ben-Sira, L., Lev, D., et al. (2020). Expanding the genotype-phenotype spectrum of ISCA2-related multiple mitochondrial dysfunction syndrome-cavitating leukoencephalopathy and prolonged survival. *Neurogenetics* 21, 243–249. doi: 10.1007/s10048-020-00611-8

Haynes, C. M., and Ron, D. (2010). The mitochondrial UPR – protecting organelle protein homeostasis. *J. Cell Sci.* 123, 3849–3855. doi: 10.1242/jcs.075119

Ikazabo, R. N. N., Mostosi, C., Jissendi, P., Labaisse, M. A., and Vandernoot, I. (2020). A new DARS2 mutation discovered in an adult patient. *Case Rep. Neurol*. 12, 107–113. doi: 10.1159/000506190

Isohanni, P., Linnankivi, T., Buzkova, J., Lonnqvist, T., Pihko, H., Valanne, L., et al. (2010). DARS2 mutations in mitochondrial leucoencephalopathy and multiple sclerosis. *J. Med. Genet*. 47, 66–70. doi: 10.1136/jmg.2009.068221

Iyer, A., and Philip, S. (2011). Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation in a 2-year-old male child. *Dev. Med. Child Neurol*. 53:30. doi: 10.1111/j.1469-8749.2010.03866.x

Jen, J. C., and Wan, J. (2018). "Chapter 13 - episodic ataxias," in *Handbook of Clinical Neurology, Vol. 155*, eds M. Manto and T. A. G. M. Huisman (Amsterdam: Elsevier), 205–215.

Kassem, H., Wafaie, A., Abdelfattah, S., and Farid, T. (2014). Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL): assessment of the involved white matter tracts by MRI. *Eur. J. Radiol.* 83, 191–196. doi: 10.1016/j.ejrad.2013.09.023

Khan, K., Baleanu-Gogonea, C., Willard, B., Gogonea, V., and Fox, P. L. (2020). 3-Dimensional architecture of the human multi-tRNA synthetase complex. *Nucleic Acids Res.* 48, 8740–8754. doi: 10.1093/nar/gkaa569

Kim, K. R., Park, S. H., Kim, H. S., Rhee, K. H., Kim, B.-G., Kim, D. G., et al. (2013). Crystal structure of human cytosolic aspartyl-tRNA synthetase, a component of multi-tRNA synthetase complex. *Proteins* 81, 1840–1846. doi: 10.1002/prot.24306

Kohler, C., Heyer, C., Hoffjan, S., Stemmler, S., Lucke, T., Thiels, C., et al. (2015). Early-onset leukoencephalopathy due to a homozygous missense mutation in the DARS2 gene. *Mol. Cell. Probes* 29, 319–322. doi: 10.1016/j.mcp.2015.06.005

Kos, J., Nanut, M. P., Prunk, M., Sabotič, J., Dautović, E., and Jewett, A. (2018). Cystatin F as a regulator of immune cell cytotoxicity. *Cancer Immunol. Immunother.* 67, 1931–1938. doi: 10.1007/s00262-018-2165-5

Labauge, P., Dorboz, I., Eymard-Pierre, E., Dereeper, O., and Boespflug-Tanguy, O. (2011). Clinically asymptomatic adult patient with extensive LBSL MRI pattern and DARS2 mutations. *J. Neurol.* 258, 335–337. doi: 10.1007/s00415-010-5755-5

Labauge, P., Roullet, E., Boespflug-Tanguy, O., Nicoli, F., Le Fur, Y., Cozzone, P. J., et al. (2007). Familial, adult onset form of leukoencephalopathy with brain stem and spinal cord involvement: inconstant high brain lactate and very slow disease progression. *Eur. Neurol.* 58, 59–61. doi: 10.1159/000102171

Lan, M. Y., Chang, Y. Y., Yeh, T. H., Lin, T. K., and Lu, C. S. (2017). Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) with a novel DARS2 mutation and isolated progressive spastic paraparesis. *J. Neurol. Sci.* 372, 229–231. doi: 10.1016/j.jns.2016.11.058

Lee, S. W., Cho, B. H., Park, S. G., and Kim, S. (2004). Aminoacyl-tRNA synthetase complexes: beyond translation. *Cell. Mol. Life Sci.* 117, 3725–3734. doi: 10.1242/jcs.01342

Lin, J., Chiconelli Faria, E., Da Rocha, A. J., Rodrigues Masruha, M., Pereira Vilanova, L. C., Scheper, G. C., et al. (2010). Leukoencephalopathy with brainstem and spinal cord involvement and normal lactate: a new mutation in the DARS2 gene. *J. Child Neurol.* 25, 1425–1428. doi: 10.1177/0883073810370897

Lin, T. K., Chang, Y. Y., Lin, H. Y., Liou, C. W., Wang, P. W., Chuang, J. H., et al. (2019). Mitochondrial dysfunctions in leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL). *PLoS ONE* 14:e0224173. doi: 10.1371/journal.pone.0224173

Linnankivi, T., Lundbom, N., Autti, T., Häkkinen, A.-M., Koillinen, H., Kuusi, T., et al. (2004). Five new cases of a recently described leukoencephalopathy with high brain lactate. *Neurology* 63, 688–692. doi: 10.1212/01.WNL.0000134658.35601.41

Ma, J., Tanaka, K. F., Shimizu, T., Bernard, C. C. A., Kakita, A., Takahashi, H., et al. (2011). Microglial cystatin F expression is a sensitive indicator for ongoing demyelination with concurrent remyelination. *J. Neurosci. Res.* 89, 639–649. doi: 10.1002/jnr.22567

Martikainen, M. H., Ellfolk, U., and Majamaa, K. (2013). Impaired information-processing speed and working memory in leukoencephalopathy with brainstem and spinal cord involvement and elevated lactate (LBSL) and DARS2 mutations: a report of three adult patients. *J. Neurol.* 260, 2078–2083. doi: 10.1007/s00415-013-6940-0

Messmer, M., Florentz, C., Schwenzer, H., Scheper, G. C., van der Knaap, M. S., Marechal-Drouard, L., et al. (2011). A human pathology-related mutation prevents import of an aminoacyl-tRNA synthetase into mitochondria. *Biochem. J.* 433, 441–446. doi: 10.1042/BJ20101902

Mierzewska, H., van der Knaap, M. S., Scheper, G. C., Bekiesinska-Figatowska, M., Szczepanik, E., and Jurkiewicz, E. (2011). Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation in the first Polish patient. *Brain Dev.* 33, 713–717. doi: 10.1016/j.braindev.2010.12.005

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M., and Emery, B. (2014). Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. *Neuroscience* 276:29–47. doi: 10.1016/j.neuroscience.2013.11.029

Miyake, N., Yamashita, S., Kurosawa, K., Miyatake, S., Tsurusaki, Y., Doi, H., et al. (2011). A novel homozygous mutation of DARS2 may cause a severe LBSL variant. *Clin. Genet.* 80, 293–296. doi: 10.1111/j.1399-0004.2011.01644.x

Moffett, J. R., Ross, B., Arun, P., Madhavarao, C. N., and Namboodiri, A. M. A. (2007). N-Acetylaspartate in the CNS: from neurodiagnostics to neurobiology. *Prog Neurobiol.* 81, 89–131. doi: 10.1016/j.pneurobio.2006.12.003

Moore, S. A., Kumar, N., and Gavrilova, R. H. (2012). Leukoencephalopathy with brain stem and spinal cord involvement (and high lactate): raising the bar for diagnosis. *J. Neurol.* 259, 2494–2497. doi: 10.1007/s00415-012-6596-1

Nagao, A., Suzuki, T., Katoh, T., Sakaguchi, Y., and Suzuki, T. (2009). Biogenesis of glutaminyl-mt tRNAGln in human mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16209–16214. doi: 10.1073/pnas.0907602106

Navarro Vázquez, I., Maestre Martínez, L., Lozano Setién, E., and Menor Serrano, F. (2016). Leucoencephalopathy with brain stem and spinal cord involvement and lactate elevation: report of two new cases. *An Pediatr.* 84, 291–293. doi: 10.1016/j.anpede.2015.07.034

Nemeth, C. L., Tomlinson, S. N., Rosen, M., O'Brien, B. M., Larraza, O., Jain, M., et al. (2020). Neuronal ablation of mt-AspRS in mice induces immune pathway activation prior to severe and progressive cortical and behavioral disruption. *Exp. Neurol.* 326:113164. doi: 10.1016/j.expneurol.2019.113164

Ofir-Birin, Y., Fang, P., Bennett, S. P., Zhang, H.-M., Wang, J., Rachmin, I., et al. (2013). Structural switch of lysyl-tRNA synthetase between translation and transcription. *Mol. Cell.* 49, 30–42. doi: 10.1016/j.molcel.2012.10.010

Omura, T. (1998). Mitochondria-targeting sequence, a multi-role sorting sequence recognized at all steps of protein import into mitochondria. *J. Biochem.* 123, 1010–1016. doi: 10.1093/oxfordjournals.jbchem.a022036

Ong, M. T., Willoughby, J., Connolly, D. J. A., Mordekar, S., Study, D. D. D., and Johnson, D. (2020). Genotype–phenotype variability of DARS mutation-case reports of a trio of siblings. *Eur. J. Med. Case Rep.* 4, 110–115. doi: 10.24911/ejmcr/1731551044010

Orcesi, S., La Piana, R., Uggetti, C., Tonduti, D., Pichiecchio, A., Pasin, M., et al. (2011). Spinal cord calcification in an early-onset progressive leukoencephalopathy. *J. Child Neurol.* 26, 876–880. doi: 10.1177/0883073810390038

Ortiz, F. C., Habermacher, C., Graciarena, M., Houry, P.-Y., Nishiyama, A., Nait Oumesmar, B., et al. (2019). Neuronal activity *in vivo* enhances functional myelin repair. *JCI Insight* 5:e123434. doi: 10.1172/jci.insight.123434

Pang, Y. L., Poruri, K., and Martinis, S. A. (2014). tRNA synthetase: tRNA aminoacylation and beyond. *Wiley Interdiscip. Rev. RNA* 5, 461–480. doi: 10.1002/wrna.1224

Petzold, G. C., Bohner, G., Klingebiel, R., Amberger, N., van der Knaap, M. S., and Zschenderlein, R. (2006). Adult onset leucoencephalopathy with brain stem and spinal cord involvement and normal lactate. *J. Neurol. Neurosurg. Psychiatr.* 77, 889–891. doi: 10.1136/jnnp.2005.078568

Potthoff, M. J., Inagaki, T., Satapati, S., Ding, X., He, T., Goetz, R., et al. (2009). FGF21 induces PGC-1α and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10853–10858. doi: 10.1073/pnas.0904187106

Rabouw, H. H., Langereis, M. A., Anand, A. A., Visser, L. J., de Groot, R. J., Walter, P., et al. (2019). Small molecule ISRIB suppresses the integrated stress response within a defined window of activation. *Proc. Natl. Acad. Sci. U.S.A.* 116, 2097–2102. doi: 10.1073/pnas.1815767116

Rackayova, V., Cudalbu, C., Pouwels, P. J. W., and Braissant, O. (2017). Creatine in the central nervous system: from magnetic resonance spectroscopy to creatine deficiencies. *Anal. Biochem.* 529:144–157. doi: 10.1016/j.ab.2016.11.007

Rathore, G., Star, L., Larsen, P., and Rizzo, W. (2017). "Novel mutation of DARS2 gene leads to a rare and aggressive presentation of leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)," in *Neurology Conference: 69th American Academy of Neurology Annual Meeting, AAN* (Boston, MA).

Rinaldi, C., and Wood, M. J. A. (2018). Antisense oligonucleotides: the next frontier for treatment of neurological disorders. *Nat. Rev. Neurol.* 14, 9–21. doi: 10.1038/nrneurol.2017.148

Rinholm, J. E., Vervaeke, K., Tadross, M. R., Tkachuk, A. N., Kopek, B. G., Brown, T. A., et al. (2016). Movement and structure of mitochondria

in oligodendrocytes and their myelin sheaths. *Glia* 64, 810–825. doi: 10.1002/glia.22965

Rumyantseva, A., Motori, E., and Trifunovic, A. (2020). DARS2 is indispensable for Purkinje cell survival and protects against cerebellar ataxia. *Hum. Mol. Genet.* 29, 2845–2854. doi: 10.1093/hmg/ddaa176

Sauter, C., Lorber, B., Gaudry, A., Karim, L., Schwenzer, H., Wien, F., et al. (2015). Neurodegenerative disease-associated mutants of a human mitochondrial aminoacyl-tRNA synthetase present individual molecular signatures. *Sci. Rep.* 5:17332. doi: 10.1038/srep17332

Scheper, G. C., Van Der Klok, T., Van Andel, R. J., Van Berkel, C. G. M., Sissler, M., Smet, J., et al. (2007). Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. *Nat. Genet.* 39, 534–539. doi: 10.1038/ng2013

Schicks, J., Schols, L., van der Knaap, M. S., and Synofzik, M. (2013). Teaching NeuroImages: MRI guides genetics: leukoencephalopathy with brainstem and spinal cord involvement (LBSL). *Neurology* 80, e176–e177. doi: 10.1212/WNL.0b013e31828cf846

Schiffmann, R., and van der Knaap, M. S. (2009). Invited article: an MRI-based approach to the diagnosis of white matter disorders. *Neurology* 72, 750–759. doi: 10.1212/01.wnl.0000343049.00540.c8

Serkov, S. V., Pronin, I. N., Bykova, O. V., Maslova, O. I., Arutyunov, N. V., Muravina, T. I., et al. (2004). Five patients with a recently described novel leukoencephalopathy with brainstem and spinal cord involvement and elevated lactate. *Neuropediatrics* 35, 1–5. doi: 10.1055/s-2003-43548

Sharma, S., Sankhyan, N., Kumar, A., Scheper, G. C., van der Knaap, M. S., and Gulati, S. (2011). Leukoencephalopathy with brain stem and spinal cord involvement and high lactate: a genetically proven case without elevated white matter lactate. *J. Child Neurol.* 26, 773–776. doi: 10.1177/0883073810390695

Shimizu, T., Wisessmith, W., Li, J., Abe, M., Sakimura, K., Chetsawang, B., et al. (2017). The balance between cathepsin C and cystatin F controls remyelination in the brain of Plp1-overexpressing mouse, a chronic demyelinating disease model. *Glia* 65, 917–930. doi: 10.1002/glia.23134

Shimojima, K., Higashiguchi, T., Kishimoto, K., Miyatake, S., Miyake, N., Takanashi, J. I., et al. (2017). A novel DARS2 mutation in a Japanese patient with leukoencephalopathy with brainstem and spinal cord involvement but no lactate elevation. *Hum. Genome Var.* 4:17051. doi: 10.1038/hgv.2017.51

Steenweg, M. E., Pouwels, P. J. W., Wolf, N. I., van Wieringen, W. N., Barkhof, F., and van der Knaap, M. S. (2011). Leucoencephalopathy with brainstem and spinal cord involvement and high lactate: quantitative magnetic resonance imaging. *Brain* 134, 3333–3341. doi: 10.1093/brain/awr254

Steenweg, M. E., van Berge, L., van Berkel, C. G., de Coo, I. F., Temple, I. K., Brockmann, K., et al. (2012). Early-onset LBSL: how severe does it get? *Neuropediatrics* 43, 332–338. doi: 10.1055/s-0032-1329395

Suomalainen, A., Elo, J. M., Pietiläinen, K. H., Hakonen, A. H., Sevastianova, K., Korpela, M., et al. (2011). FGF-21 as a biomarker for muscle-manifesting mitochondrial respiratory chain deficiencies: a diagnostic study. *Lancet Neurol.* 10, 806–818. doi: 10.1016/S1474-4422(11)70155-7

Synofzik, M., Schicks, J., Lindig, T., Biskup, S., Schmidt, T., Hansel, J., et al. (2011). Acetazolamide-responsive exercise-induced episodic ataxia associated with a novel homozygous DARS2 mutation. *J. Med. Genet.* 48, 713–715. doi: 10.1136/jmg.2011.090282

Taft, R. J., Vanderver, A., Leventer, R. J., Damiani, S. A., Simons, C., Grimmond, S. M., et al. (2013). Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am. J. Hum. Genet.* 92, 774–780. doi: 10.1016/j.ajhg.2013.04.006

Távora, D. G. F., Nakayama, M., Gama, R. L., Alvim TCd, L., Portugal, D., and Comerlato, E. A. (2007). Leukoencephalopathy with brainstem and spinal cord involvement and high brain lactate: report of three brazilian patients. *Arq. Neuropsiquiatr.* 65, 506–511. doi: 10.1590/S0004-282X20070003000028

Taylor, R. W., Pyle, A., Griffin, H., Blakely, E. L., Duff, J., He, L., et al. (2014). Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. *JAMA* 312, 68–77. doi: 10.1001/jama.2014.7184

Teive, H. A. G., and Ashizawa, T. (2015). Primary and secondary ataxias. *Curr. Opin. Neurol.* 28, 413–422. doi: 10.1097/WCO.0000000000000227

Toldo, I., Nosadini, M., Boscardin, C., Talenti, G., Manara, R., Lamantea, E., et al. (2018). Neonatal mitochondrial leukoencephalopathy with brain and spinal involvement and high lactate: expanding the phenotype of ISCA2 gene mutations. *Metab. Brain Dis.* 33, 805–812. doi: 10.1007/s11011-017-0181-3

Tricaud, N., and Park, H. T. (2017). Wallerian demyelination: chronicle of a cellular cataclysm. *Cell. Mol. Life Sci.* 74, 4049–4057. doi: 10.1007/s00018-017-2565-2

Tylki-Szymanska, A., Jurkiewicz, E., Zakharova, E. Y., and Bobek-Billewicz, B. (2014). Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation: high outcome variation between two siblings. *Neuropediatrics* 45, 188–191. doi: 10.1055/s-0033-1364105

Tzoulis, C., Tran, G. T., Gjerde, I. O., Aasly, J., Neckelmann, G., Rydland, J., et al. (2012). Leukoencephalopathy with brainstem and spinal cord involvement caused by a novel mutation in the DARS2 gene. *J. Neurol.* 259, 292–296. doi: 10.1007/s00415-011-6176-9

Ulrick, N., and Vanderver, A. (2017). "Hypomyelination with brainstem and spinal cord abnormalities and leg spasticity (HBSL)," in *Pediatric Demyelinating Diseases of the Central Nervous System and Their Mimics: A Case-Based Clinical Guide*, eds E. Waubant and T. E. Lotze (Cham: Springer International Publishing), 285–290.

Uluc, K., Baskan, O., Yildirim, K. A., Ozsahin, S., Koseoglu, M., Isak, B., et al. (2008). Leukoencephalopathy with brain stem and spinal cord involvement and high lactate: a genetically proven case with distinct MRI findings. *J. Neurol. Sci.* 273, 118–122. doi: 10.1016/j.jns.2008.06.002

van Berge, L., Dooves, S., van Berkel, C. G., Polder, E., van der Knaap, M. S., and Scheper, G. C. (2012). Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation is associated with cell-type-dependent splicing of mtAspRS mRNA. *Biochem. J.* 441, 955–962. doi: 10.1042/BJ20110795

Van Berge, L., Hamilton, E. M., Linnankivi, T., Uziel, G., Steenweg, M. E., Isohanni, P., et al. (2014). Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy. *Brain* 137, 1019–1029. doi: 10.1093/brain/awu026

van Berge, L., Kevenaar, J., Polder, E., Gaudry, A., Florentz, C., Sissler, M., et al. (2013). Pathogenic mutations causing LBSL affect mitochondrial aspartyl-tRNA synthetase in diverse ways. *Biochem. J.* 450, 345–350. doi: 10.1042/BJ20121564

van der Knaap, M. S., Hamilton, E. M., and van Berge, L. (2014). Reply: DARS2 gene clinical spectrum: new ideas regarding an underdiagnosed leukoencephalopathy. *Brain* 137(Pt. 7):e290. doi: 10.1093/brain/awu135

Van Der Knaap, M. S., Van Der Voorn, P., Barkhof, F., Van Coster, R., Krägeloh-Mann, I., Feigenbaum, A., et al. (2003). A new leukoencephalopathy with brainstem and spinal cord involvement and high lactate. *Ann. Neurol.* 53, 252–258. doi: 10.1002/ana.10456

Ventura-Clapier, R., Garnier, A., and Veksler, V. (2008). Transcriptional control of mitochondrial biogenesis: the central role of PGC-1α. *Cardiovasc. Res.* 79, 208–217. doi: 10.1093/cvr/cvn098

Wallen, R. C., and Antonellis, A. (2013). To charge or not to charge: mechanistic insights into neuropathy-associated tRNA synthetase mutations. *Curr. Opin. Genet. Dev.* 23, 302–309. doi: 10.1016/j.gde.2013.02.002

Wang, X., Zhang, C., Szábo, G., and Sun, Q.-Q. (2013). Distribution of CaMKIIα expression in the brain *in vivo*, studied by CaMKIIα-GFP mice. *Brain Res.* 1518, 9–25. doi: 10.1016/j.brainres.2013.04.042

Werner, R., Daum, E., Felber, S., and Wohrle, J. C. (2018). Leukoencephalopathy with brain stem and spinal cord involvement and not always lactate elevation. *Clin. Neuroradiol.* 28, 451–453. doi: 10.1007/s00062-017-0647-z

Wolf, N. I., Toro, C., Kister, I., Latif, K. A., Leventer, R., Pizzino, A., et al. (2015). DARS-associated leukoencephalopathy can mimic a steroid-responsive neuroinflammatory disorder. *Neurology* 84, 226–230. doi: 10.1212/WNL.0000000000001157

Yahia, A., Elsayed, L., Babai, A., Salih, M. A., El-Sadig, S. M., Amin, M., et al. (2018). Intra-familial phenotypic heterogeneity in a Sudanese family with DARS2-related leukoencephalopathy, brainstem and spinal cord involvement and lactate elevation: a case report. *BMC Neurol.* 18:175. doi: 10.1186/s12883-018-1180-7

Yamashita, S., Miyake, N., Matsumoto, N., Osaka, H., Iai, M., Aida, N., et al. (2013). Neuropathology of leukoencephalopathy with brainstem and spinal cord involvement and high lactate caused by a homozygous mutation of DARS2. *Brain Dev*. 35, 312–316. doi: 10.1016/j.braindev.2012.05.007

Yelam, A., Nagarajan, E., Chuquilin, M., and Govindarajan, R. (2019). Leucoencephalopathy with brain stem and spinal cord involvement and lactate elevation: a novel mutation in the DARS2 gene. *BMJ Case Rep*. 12:bcr-2018-227755. doi: 10.1136/bcr-2018-227755

Conflict of Interest: MK is an employee of Boehringer Ingelheim Pharma GmbH & Co. KG.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 28 January 2021
doi: 10.3389/fncel.2020.631802

POLR3-Related Leukodystrophy: Exploring Potential Therapeutic Approaches

Stefanie Perrier[1,2†], *Mackenzie A. Michell-Robinson*[1,2†] *and Geneviève Bernard*[1,2,3,4,5*]

[1]Department of Neurology and Neurosurgery, McGill University, Montréal, QC, Canada, [2]Child Health and Human Development Program, Research Institute of the McGill University Health Centre, Montréal, QC, Canada, [3]Department of Pediatrics, McGill University, Montréal, QC, Canada, [4]Department of Human Genetics, McGill University, Montréal, QC, Canada, [5]Department of Specialized Medicine, Division of Medical Genetics, Montréal Children's Hospital and McGill University Health Centre, Montréal, QC, Canada

Leukodystrophies are a class of rare inherited central nervous system (CNS) disorders that affect the white matter of the brain, typically leading to progressive neurodegeneration and early death. Hypomyelinating leukodystrophies are characterized by the abnormal formation of the myelin sheath during development. POLR3-related or 4H (hypomyelination, hypodontia, and hypogonadotropic hypogonadism) leukodystrophy is one of the most common types of hypomyelinating leukodystrophy for which no curative treatment or disease-modifying therapy is available. This review aims to describe potential therapies that could be further studied for effectiveness in pre-clinical studies, for an eventual translation to the clinic to treat the neurological manifestations associated with POLR3-related leukodystrophy. Here, we discuss the therapeutic approaches that have shown promise in other leukodystrophies, as well as other genetic diseases, and consider their use in treating POLR3-related leukodystrophy. More specifically, we explore the approaches of using stem cell transplantation, gene replacement therapy, and gene editing as potential treatment options, and discuss their possible benefits and limitations as future therapeutic directions.

Keywords: POLR3-related leukodystrophy, 4H leukodystrophy, hypomyelination, gene therapy, gene editing, cell therapy

OPEN ACCESS

Edited by:
Dominik Fröhlich, University of New South Wales, Australia

Reviewed by:
Bilal Ersen Kerman, Istanbul Medipol University, Turkey
Robert Weissert, University of Regensburg, Germany

***Correspondence:**
Geneviève Bernard
genevieve.bernard@mcgill.ca

†These authors have contributed equally to this work

***Specialty section**:*
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *21 November 2020*
Accepted: *28 December 2020*
Published: *28 January 2021*

Citation:
Perrier S, Michell-Robinson MA and Bernard G (2021) POLR3-Related Leukodystrophy: Exploring Potential Therapeutic Approaches. Front. Cell. Neurosci. 14:631802. doi: 10.3389/fncel.2020.631802

INTRODUCTION

Leukodystrophies are a class of heterogeneous inherited neurological diseases characterized by the predominant impairment of the central nervous system (CNS) white matter, with specific involvement of glial cells (Vanderver et al., 2015; Van Der Knaap and Bugiani, 2017). Affected patients typically present in childhood or adolescent years with psychomotor regression and/or

Abbreviations: 4H, hypomyelination, hypodontia, hypogonadotropic hypogonadism; AAV, adeno-associated virus; CNS, central nervous system; CRISPR, clustered short regularly interspaced palindromic repeats; DTI, diffusion tensor imaging; GPC, glial progenitor cell; HLD, hypomyelinating leukodystrophy; LD, leukodystrophy; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NAA, N-acetylaspartate; NSC, neural stem cell; nc-RNAs, non-coding RNAs; OPC, oligodendrocyte progenitor cell; POLR3, RNA polymerase III; POLR3-HLD, POLR3-related hypomyelinating leukodystrophy; RNA, ribonucleic acid; tRNAs, transfer RNAs.

neuropsychiatric manifestations. Magnetic resonance imaging (MRI) patterns, followed by genetic investigations, are used to confirm diagnoses (Parikh et al., 2015). Most leukodystrophies run a progressive disease course, with slow to rapid deterioration after onset, ultimately leading to an early death. Collectively, leukodystrophies affect approximately one in 7,500 individuals, however, there are many different subtypes with varying individual incidence rates (Bonkowsky et al., 2010; Parikh et al., 2015; Adang et al., 2017). Next-generation sequencing has proven to be a valuable first-line diagnostic tool for determining the genetic basis of the disease, and has facilitated the discovery of a variety of causal genes encoding proteins with diverse biological functions (Boycott et al., 2014; Srivastava et al., 2014; Vanderver et al., 2016). Although some leukodystrophies have successful restorative treatments if started early following diagnosis [i.e., pre-or early symptomatic stages (Krivit et al., 1999; Krivit, 2004; Van Den Broek et al., 2018)], most treatments address specific clinical features, providing supportive care (Adang et al., 2017).

Hypomyelinating leukodystrophies (HLDs) are a defined subcategory of leukodystrophies, characterized by defects in initial myelin production and formation during development (Costello et al., 2009; Pouwels et al., 2014; Wolf et al., 2020). HLDs are diagnosed using MRI patterns, notably involving hyperintensity of the white matter compared to gray matter on T2 weighted imaging, and variable signal (i.e., hyperintensity, hypointensity, or isointensity) of white matter on T1 weighted imaging compared to gray matter structures (Schiffmann and Van Der Knaap, 2009; Steenweg et al., 2010; Barkovich and Deon, 2016). Hypomyelination can be diagnosed in a single MRI in children older than 2 years of age, but not in younger children. Indeed, in children below 2 years, the diagnosis of hypomyelination (vs. myelination delay) requires that myelination does not progress between two MRIs taken 6 months apart, with the second performed after 2 years of age (Schiffmann and Van Der Knaap, 2009; Steenweg et al., 2010; Pouwels et al., 2014). As myelination of most key brain areas is virtually complete by 2 years of age, a lack of progression in myelin development seen at this age will likely result in permanent hypomyelination (Steenweg et al., 2010).

Classically, HLDs were primarily known to be caused by pathogenic variants in genes encoding for proteins directly associated with the development, structure, or integrity of the myelin sheath. For example, the prototypical HLD Pelizaeus-Merzbacher disease results from pathogenic variants in *PLP1*, a gene encoding a structural myelin protein (Garbern, 2007). However, a recently growing class of white matter disorders encompasses those caused by pathogenic variants in proteins that play key roles in transcription and translation. For example, pathogenic variants in several genes encoding for aminoacyl tRNA synthetases (e.g., *DARS1, RARS1, EPRS1*) are known to cause HLDs (Park et al., 2008; Taft et al., 2013; Wolf et al., 2014a; Ognjenović and Simonović, 2018; Mendes et al., 2018).

Within the category of white matter disorders caused by defects in transcription/translation-related genes is POLR3-related hypomyelinating leukodystrophy (POLR3-HLD), which is now considered one of the most common HLDs (Schmidt et al., 2020). POLR3-HLD is caused by biallelic pathogenic variants in genes encoding subunits of the transcription complex RNA polymerase III (POLR3), namely *POLR3A, POLR3B, POLR1C*, and *POLR3K* (Bernard et al., 2011; Tétreault et al., 2011; Daoud et al., 2013; Thiffault et al., 2015; Dorboz et al., 2018). POLR3 is responsible for the transcription of several non-coding RNAs (nc-RNAs) which have significant roles in translation and gene expression programs, including transfer RNAs (tRNAs), 5S ribosomal RNA, 7SL and 7SK RNAs, some microRNAs, vault RNAs, and a variety of small nucleolar RNAs, including U6 snRNA (Dieci et al., 2007, 2013; White, 2011; Wu et al., 2012; Lesniewska and Boguta, 2017). As the genes associated with POLR3-HLD have been discovered relatively recently and attempts at generating an animal model were predominantly unsuccessful (Choquet et al., 2017, 2019b), the cellular and molecular mechanisms underlying the white matter pathology of this disease are largely unknown. Research is ongoing regarding the investigation of the pathophysiology of POLR3-HLD; recent modeling of the disease has been accomplished in yeast (Moir et al., 2020), as well as in a conditional mouse model (pre-print data, not yet peer-reviewed; Merheb et al., 2020). Moreover, a variety of different types of pathogenic variants are known to cause POLR3-HLD, including nonsense, missense, intronic, synonymous, and splice site variants, as well as large exonic deletions, and small insertions or deletions (Bernard et al., 2011; Tétreault et al., 2011; Potic et al., 2012; Terao et al., 2012; Daoud et al., 2013; Takanashi et al., 2014; Wolf et al., 2014b; Gutierrez et al., 2015; Thiffault et al., 2015; La Piana et al., 2016; Jurkiewicz et al., 2017; Richards et al., 2017; Al Yazidi et al., 2019; Gauquelin et al., 2019; Harting et al., 2020; Hiraide et al., 2020b; Perrier et al., 2020). It is hypothesized that loss of POLR3 function disrupts the transcription of tRNAs, thereby resulting in dysregulation of global translation during peak periods of myelin development which require synthesis of large amounts of proteins (Pfeiffer et al., 1993; Elbaz and Popko, 2019). An alternative hypothesis involves hypofunction of POLR3 causing impairments in the production of specific nc-RNAs required for the formation of myelin (Choquet et al., 2019a).

Due to the classic phenotypic presentation of patients involving hypomyelination, hypodontia, and hypogonadotropic hypogonadism, POLR3-HLD is also referred to as 4H leukodystrophy. Before the discovery of the causal genes for POLR3-HLD, four other disorders with a similar set of clinical and MRI features were previously described: ataxia, delayed dentition, and hypomyelination (ADDH; Wolf et al., 2007; Wolff et al., 2010); tremor-ataxia with central hypomyelination (TACH; Bernard et al., 2010, 2011; Tétreault et al., 2011; Tetreault et al., 2012); leukodystrophy with oligodontia (LO; Atrouni et al., 2003; Chouery et al., 2011); and hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum (HCAHC; Sasaki et al., 2009; Saitsu et al., 2011; Bernard and Vanderver, 2017). Moreover, the clinical phenotype of patients with POLR3-HLD has been extensively characterized *via* large cohort studies, with the most notable features stemming

from neurological dysfunction due to hypomyelination (Wolf et al., 2014b; Gauquelin et al., 2019). The typical MRI pattern associated with POLR3-HLD involves diffuse hypomyelination with relative preservation (T2-weighted hypointensity) of the dentate nuclei, anterolateral nuclei of the thalami, globi pallidi, pyramidal tracts at the level of the posterior limb of the internal capsules, and the optic radiations (La Piana et al., 2014; Vrij-Van Den Bos et al., 2017). Thinning of the corpus callosum and cerebellar atrophy have also been noted in a proportion of cases (La Piana et al., 2014; Vrij-Van Den Bos et al., 2017). Neurological features resulting from hypomyelination typically manifest as developmental delay and motor impairment from progressive cerebellar features, such as gait ataxia, tremor, dysmetria, and dysarthria (Bernard and Vanderver, 2017; **Figure 1**). Extrapyramidal features, most commonly dystonia, have also been reported (Osterman et al., 2012; Al Yazidi et al., 2019), along with pyramidal features such as spasticity, and cognitive features such as learning difficulties and intellectual disability (Wolf et al., 2014b; Bernard and Vanderver, 2017; Gauquelin et al., 2019). Non-neurological features typically involve myopia, abnormal dentition, and endocrine abnormalities including hypogonadotropic hypogonadism, associated with arrested, delayed, or absent puberty, and short stature (Wolf et al., 2014b; Pelletier et al., 2020; **Figure 1**).

Recently, the spectrum of severity and the associated clinical and MRI features of POLR3-HLD expanded significantly, from very mild to extremely severe. Very mild presentations include asymptomatic young adults, or patients with intellectual disability and milder hypomyelination on MRI compared to the typical phenotype, discovered incidentally during unrelated investigations (Wolf et al., 2014b; Degasperis et al., 2020; Perrier et al., 2020). Isolated hypogonadotropic hypogonadism without evidence of hypomyelination has also been described on the mild end of the POLR3-HLD spectrum (Richards et al., 2017). Patients with the severe form of POLR3-HLD present much earlier compared to the typical phenotype, exhibiting developmental regression, failure to thrive, and severe dysphagia in the first few months of life, with some passing in early childhood due to respiratory complications (Wu et al., 2019; Harting et al., 2020; Perrier et al., 2020). These patients also present with a unique MRI phenotype, in fact not meeting the criteria for hypomyelination, but primarily showing neuronal involvement in specific brain regions (predominantly the putamen and thalamus) with some evidence of insufficient myelin deposition (Perrier et al., 2020). It is hypothesized that the neuronal presentation is likely linked to a specific splicing variant in *POLR3A*, given the common neuronal phenotype shared with other patients harboring the same, or an adjacent, splicing variant in a homozygous or compound heterozygous state (Azmanov et al., 2016; Minnerop et al., 2017; Wu et al., 2019; Harting et al., 2020; Hiraide et al., 2020a). In sum, it is clear that the pathophysiology underlying POLR3-HLD is complex as a broad range of phenotypes are associated with hypomorphic POLR3. In this review article, we will focus on the potential therapeutic options for the classic and most common phenotype, specifically concentrating on hypomyelination.

POLR3-RELATED LEUKODYSTROPHY: APPROACHING TREATMENT OPTIONS

With the advent of MRI pattern recognition and improvements in genetic technologies in the last decade, diagnostic rates for leukodystrophies, including POLR3-HLD, have risen in parallel. An important goal for POLR3-HLD research now lies in the determination of quantifiable markers of disease progression. Indeed, before therapeutic options can be considered, clinical outcome measures and surrogate markers of disease progression must be established and deemed accurately quantifiable. These markers are critical for assessing the effectiveness of treatment efficacy in future clinical trials. Advanced neuroimaging techniques, such as diffusion tensor imaging (DTI), pose an interesting route for measurement of improvements in myelination (Aung et al., 2013; Pouwels et al., 2014; Koob et al., 2016; Poretti et al., 2016; Sarret et al., 2018; Van Rappard et al., 2018). The heterogeneity of POLR3-HLD presents an additional limitation for assessing the effectiveness of different therapies as difficulties could arise when comparing the progression rate of phenotypes between patients. Thus far, the clinical experience of most patients with POLR3-HLD presents a relatively similar disease course according to the gene which is mutated. Indeed, those with pathogenic variants in *POLR1C* present with the most severe disease course, followed by *POLR3A*, and then *POLR3B* (Wolf et al., 2014b; Gauquelin et al., 2019). The comparative severity of patients with pathogenic variants in *POLR3K* cannot yet be determined as clinical information has only been published on two patients (Dorboz et al., 2018). In recent years, it has become clear that natural history studies concerning the delineation of disease progression and identification of surrogate markers are of the utmost importance (Pouwels et al., 2014). Hence, it is essential to complete these studies in parallel to pathophysiological investigations for clinical trials of potential therapies to progress.

Limited knowledge of the exact pathophysiological mechanisms underlying POLR3-HLD also poses a challenge for the evaluation of the most effective treatment options. When specific mechanisms are implicated in genetic diseases, it is possible to focus on targeting alternative pathways in treatment approaches, in order to overpass the mechanism containing the defective protein (Greene and Voight, 2016). Although the cellular pathophysiological mechanisms associated with POLR3-HLD have yet to be uncovered, studies have shown that mutations in POLR3 subunits can cause disruptions on several molecular levels. For example, mutational mapping onto specific protein domains suggests association with specific mechanisms of dysfunction, including modification of the catalytic cleft structure, impaired POLR3 complex assembly, perturbed interactions between subunits, and interference within POLR3 complex binding to DNA (Bernard et al., 2011; Tétreault et al., 2011; Girbig et al., 2020; Ramsay et al., 2020). Additionally, protein localization studies have shown that disease-causing *POLR1C* variants can alter assembly and nuclear import exclusively of POLR3, resulting in a lack in binding to POLR3 target genes (Thiffault et al., 2015). Protein expression studies on patient fibroblasts and brain

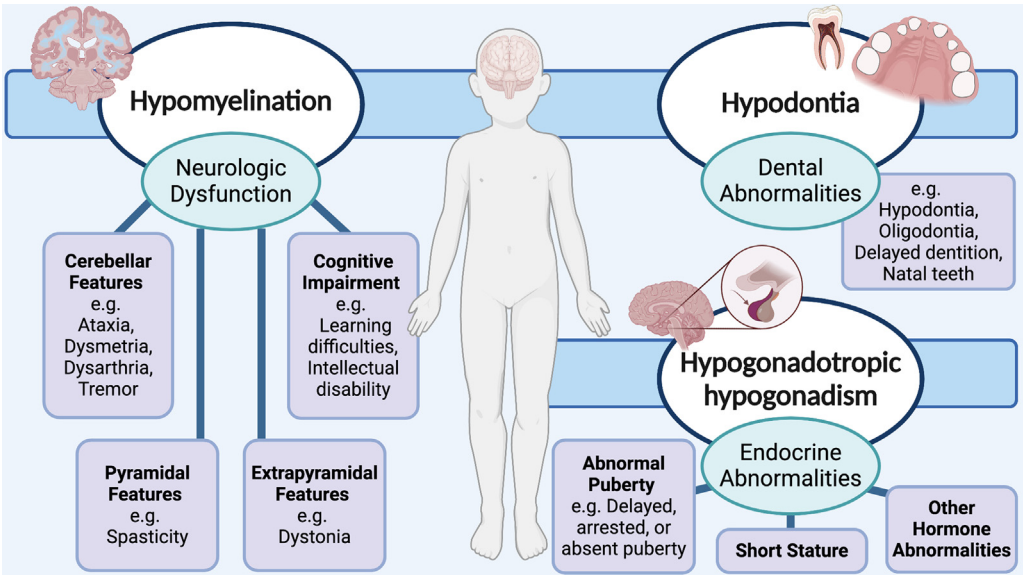


FIGURE 1 | Schematic showing the neurological and non-neurological clinical features that are associated with RNA polymerase III (POLR3)-related, or 4H (hypomyelination, hypodontia, hypogonadotropic hypogonadism) leukodystrophy. Neurological abnormalities typically include cerebellar, pyramidal, extrapyramidal, and cognitive features. Teeth and endocrine abnormalities are also common.

tissue also demonstrate a decrease in POLR3A abundance (Bernard et al., 2011). Finally, functional studies of *POLR3A* mutations associated with POLR3-HLD have demonstrated transcriptional defects when introduced in both yeast and human cells (Choquet et al., 2019a; Moir et al., 2020). Further research on molecular pathways and other POLR3 interactors will be valuable in determining whether suppression of upstream POLR3 inhibitors, such as MAF1 (Reina et al., 2006; Johnson et al., 2007; Bonhoure et al., 2020; Vorländer et al., 2020), are appropriate for future treatments. It is also possible that a small molecule screening approach could identify drugs for the treatment of specific molecular mechanisms, such as upregulation of complex assembly cofactors or signaling molecules for nuclear import of POLR3 (Cloutier and Coulombe, 2010; Lesniewska and Boguta, 2017; Willis and Moir, 2018). However, further research is required in this avenue before a molecular target approach can be considered for the repair of myelin in POLR3-HLD. Currently, as the pathophysiological processes underlying POLR3-HLD are not well known, potential therapeutic approaches can be considered in a general manner, by focusing on the replacement of defective cells (i.e., oligodendrocytes), or directly restoring POLR3 function. Specifically, this could be accomplished by: directly transplanting stem cells containing functional protein to migrate and replicate in damaged areas, using gene therapy techniques to deliver gene products and restore the functional protein in damaged cells, or repairing genetic variants in damaged cells *via* delivery of gene editing constructs (Helman et al., 2015; Gordon-Lipkin and Fatemi, 2018; **Figure 2**). This review discusses these strategies as potential avenues for treatment of POLR3-HLD, specifically considering its cellular neuropathology and discussing the benefits and limitations of treatments that have been successful in other leukodystrophies, as well as those currently being developed for clinical trials in other genetic diseases.

Myelination and POLR3-Related Leukodystrophy Cellular Pathology

Myelination is a dynamic process, involving many signaling cues, proteins, and enzymes, that begins *in utero*. The formation of myelin begins in the CNS with the development and migration of oligodendrocyte progenitor cells (OPCs), which extend their processes to contact neuronal axons and begin ensheathment (Michalski and Kothary, 2015). Upon initial axon-glial contact, key myelin membrane components are synthesized and transported to begin the extension of the processes around the axon (Emery, 2010; Mitew et al., 2014). As the processes wrap the axons and begin to compact into several thin layers, the OPCs develop into mature oligodendrocytes, thereby forming the myelin sheath (Baron and Hoekstra, 2010). Compacted myelin allows for rapid propagation of action potentials between neurons, while also providing structural protection to axons. Additionally, complex networks of microtubules in the myelin membrane support the high metabolic demand of the axon by facilitating the transport of proteins, metabolites, and other molecules (Roth et al., 2006; Lee et al., 2012). Typically, myelin deposition begins during the 4th month of gestation *in utero*, and myelination of most major tracts is essentially complete by 2 years of age (Dietrich et al., 1988; Van Der Knaap and Valk, 2005). Myelination continues on a smaller scale into the first and second decades of life, with an increase of approximately 12%

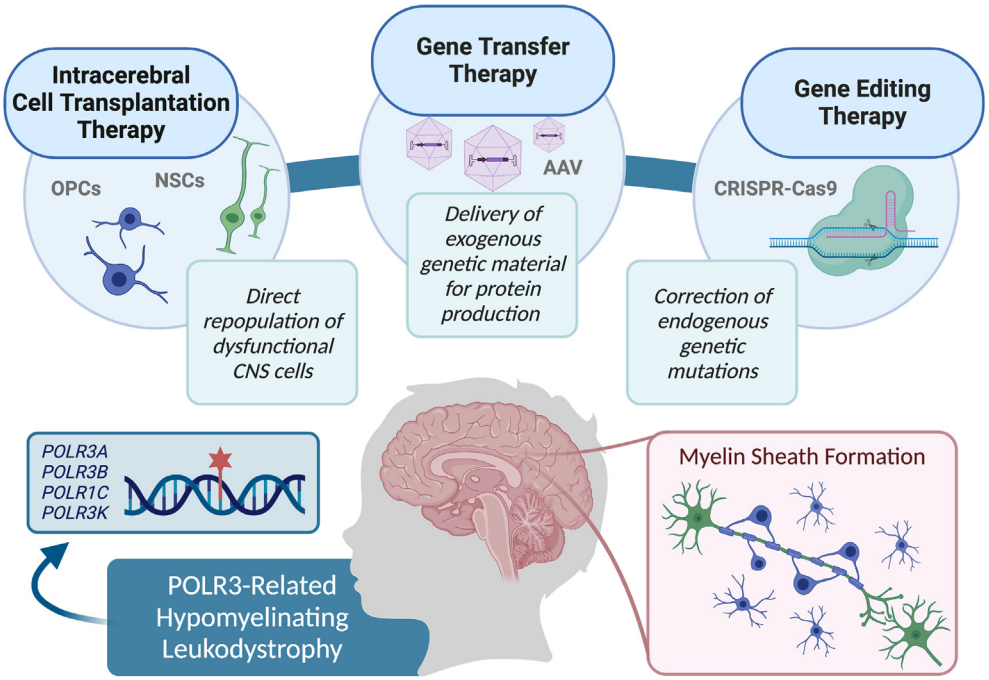


FIGURE 2 | Summary of the therapy approaches that could be explored for use in pre-clinical studies, and eventually translated in clinical trials to treat RNA polymerase III (POLR3)-related leukodystrophy, including cell transplantation therapy, gene transfer therapy, and gene editing techniques.

in total white matter volume to age 22 (Giedd et al., 1999). Additional changes in white matter volume into adulthood are both regionally and temporally associated with cognitive development and synaptic plasticity, and are also likely associated with axonal factors including pruning, branching, and packing (Sampaio-Baptista and Johansen-Berg, 2017).

Neuropathological investigations of the typical phenotype of POLR3-HLD suggest a complex pathologic process, however, the most prominent feature remains insufficient myelin deposition. The two published cases of typical POLR3-HLD pathology revealed a marked loss of oligodendrocytes, with severity varying in different brain regions (Vanderver et al., 2013; Wolf et al., 2014b). Moderate axonal loss was evident, thought to be secondary to white matter abnormalities due to its apparent proportionality to lack of myelin. Despite the uniform hypomyelinating pattern seen on MRI, it has been hypothesized that POLR3-HLD is a complex heterogenous leukodystrophy with prominent neuroaxonal and glial involvement (Vanderver et al., 2013).

As the neurological manifestations of POLR3-HLD are likely a direct result of the lack of myelin, strategies focusing on restoration of myelin are ideal therapeutic considerations. Moreover, this could be accomplished by replacement of myelinating oligodendrocytes *via* direct delivery of progenitor cells, or by gene therapy aiming at restoring the missing protein in endogenous oligodendrocytes and their progenitors. However, as the neuropathological mechanisms underlying hypomyelination in POLR3-HLD are still undetermined, it is difficult to conclude which option would be most successful in phenotypic remediation. To expand, it is currently unknown how and why cells of the oligodendrocyte lineage in POLR3-HLD are dysfunctional, and whether a defect in proliferation, migration, differentiation, signaling, or the production of myelin *per se* causes hypomyelination. Additionally, it is possible that a combination of cell types may be involved in the disease pathogenesis of POLR3-HLD, and therapies involving the direct target of glial lineages will only be partially effective. Given that POLR3 is ubiquitously expressed, it is conceivable that hypofunction of the protein in cell types other than those that directly produce myelin could play a role in disease pathogenesis. As the white matter of the brain has a complex composition involving the lipid-rich neuron-wrapping myelin sheath composed of oligodendrocytes, as well as astrocytes and microglia that provide structural and trophic support, it can be difficult to focus on specific strategies for its repair without concrete knowledge of the cellular pathogenesis of POLR3-HLD. Gathering more insight into the developmental role of POLR3 in myelin-producing cells and other neural cells, in addition to pathophysiological mechanisms of mutant POLR3 subunits, will allow the field to advance therapeutic strategies based on a deeper understanding of the underlying biology. It is important to note that each therapeutic approach has unique potential benefits and limitations, with the stage of disease progression and patient age remaining strong factors in considering the potential for therapeutic efficacy (Helman et al., 2015; Gordon-Lipkin and Fatemi, 2018).

CELL-BASED THERAPIES: TRANSPLANTATION AS TREATMENT FOR LEUKODYSTROPHIES

Cellular therapies, which involve the transplantation of stem cells into an affected individual, offer an attractive approach for treating HLDs. Stem cells can self-renew and differentiate into different lineages, including OPCs, and therefore could directly repopulate lost host cells for the regeneration of myelin in leukodystrophies. Generally, the therapeutic mechanisms of cellular therapy can be two-fold, including direct replacement of lost host cells *via* migration of transplanted cells to repopulate defective tissues, and transplanted cells acting as a source of functional exogenous enzymes (De Feo et al., 2012). While delivery of stem cells for the treatment of neurological diseases has been achieved *via* both intravenous and intracerebral administration techniques, only the latter could be applicable for the treatment of POLR3-HLD. Intravenous stem cell therapy, including bone marrow transplantation or hematopoietic stem cell transplantation, has been used in treating other monogenic neurological diseases based on the notion that monocytes could migrate through the blood-brain barrier to the CNS tissue and secrete active enzyme for cellular uptake by dysfunctional host cells, as well as differentiate into microglia and/or astrocytes that could inherently provide trophic support for diseased cells or regulate inflammation (Krivit et al., 1995; Priller et al., 2001; Asheuer et al., 2004; Sun and Kurtzberg, 2018). While this approach has been used in leukodystrophies that are associated with enzyme deficiencies [e.g., globoid cell leukodystrophy or Krabbe disease (Escolar et al., 2005; Wright et al., 2017; Laule et al., 2018), adrenoleukodystrophy (Peters et al., 2004; Mahmood et al., 2007; Matsukawa et al., 2020), and metachromatic leukodystrophy (Martin et al., 2013; Musolino et al., 2014; Boucher et al., 2015; Groeschel et al., 2016)], it is not applicable for treatment of the hypomyelinating phenotype associated with POLR3-HLD as neither the POLR3 enzyme complex nor its subunits are secreted extracellularly for reuptake, and myelination would be dependent on the delivery of functional OPCs or earlier lineages. Therefore, intracerebral administration of stem cells of neural lineage poses the most likely route for exploration in the treatment of POLR3-HLD.

Neural Stem Cell Transplantation and Remyelination

Neural stem cells (NSCs) are multipotent neural cells that give rise to radial glial progenitor cells, which can, in turn, give rise to neuron and glial cell populations, making them an attractive cell type for transplantation in the leukodystrophy setting (Brüstle et al., 1997; Temple, 2001; Zhao and Moore, 2018). During neural development, gradients of specific signaling molecules guide the fate of NSCs and provide positional information to form different regions of the brain (Wolpert, 1994; Temple, 2001). In the CNS, NSCs also have a temporal differentiation component, where the response to growth factors is altered over time, as the cells undergo repeated asymmetric divisions to first produce neurons, followed by glia (Qian et al., 2000; Okano and Temple, 2009). In the postnatal brain, NSC production and neurogenesis are restricted to certain brain areas but primarily occur in the subventricular zone (Gonzalez-Perez, 2012).

Several mouse models of dysmyelination and hypomyelination have shown that intracerebral-transplanted NSCs are effective in remyelinating the myelin-deficient brain (Duncan et al., 2011). Explored extensively is the *shiverer* mouse model, which exhibits dysmyelination and a motor phenotype due to a deletion in the *Mbp* gene, encoding for myelin basic protein, which is required for the formation of major dense lines in compact myelin (Privat et al., 1979; Roach et al., 1985). When transplanted into *shiverer* mice, NSCs can differentiate and remyelinate the brain, promoting recovery of their ataxic phenotype and prolonging survival (Yandava et al., 1999; Low et al., 2009; Uchida et al., 2012). Additionally, when transplanted into the *shiverer* spinal cord, exogenous transplanted NSCs can ensheath axons, form compact myelin, and improve nerve conduction (Eftekharpour et al., 2007; Mothe and Tator, 2008; Buchet et al., 2011). Studies on rodent models of Pelizaeus-Merzbacher disease have shown similar results; following NSC transplantation, *Plp1*-transgenic mutant mice undergo remyelination of the brain with the production of compact myelin (Marteyn et al., 2016; Gruenenfelder et al., 2020). Additionally, engraftment of NSCs into the white matter tracts of hypomyelinated mutant myelin-associated glycoprotein and nonreceptor-type tyrosine kinase Fyn (MAG/Fyn) mice produced mature oligodendrocytes and improvements in myelination (Ader et al., 2001, 2004). These studies provide evidence that mammalian NSCs can undergo functional integration into the CNS white matter, promoting remyelination and offering potential as a therapeutic approach in hypomyelinating disorders. Besides direct remyelination, it is also thought that NSC transplantation can offer an additional advantage through a neuroprotective effect *via* the release of trophic factors, which promote tissue repair and protect endogenous cells from further damage (De Feo et al., 2012).

Recently, the safety of allogeneic NSC intracerebral transplantation in humans was investigated in a phase I clinical trial including four young patients with Pelizaeus-Merzbacher disease, who were monitored over the course of 5 years (Gupta et al., 2012, 2019). The primary goal of this study was to assess the safety profile of the transplantation of allogeneic NSCs derived from human fetal brain tissue using intracerebral injections. Using MRI guidance, cells were delivered *via* four bilateral frontal burr holes to the deep white matter of the centrum semiovale or corona radiata, and patients underwent an immunosuppression regime. A 1-year evaluation determined that the procedure was well-tolerated without clinical or radiological adverse effects, and after 5-years, no tumor formation was evident and no other long-term adverse effects were noted. However, two patients had an immune response and developed donor-specific leukocyte antigen alloantibodies, pointing to the importance of monitoring immune response in future studies. Serial MRI and magnetic resonance spectroscopy (MRS), including DTI, were performed

for evaluation of remyelination, where signal changes were observed at the injection sites and some distant regions in each patient through the second year following transplantation. In the three patients who were studied up to year 5, persistent increased signal changes were noted, however, they were described as patchy and subtle, and could not be guaranteed conclusive evidence of remyelination. Although further studies are required to optimize transplantation efficacy, this study provides support for the safety of intracerebral transplantation of progenitor cells for repopulation of myelin in HLDs.

Should the transplanted NSCs be successful in migrating, signaling, and differentiating to form functional myelin in humans, this therapeutic approach would be optimal to treat the diffuse hypomyelination seen in POLR3-HLD. However, in considering the described rodent studies of remyelination following NSC transplantation, results should be interpreted with caution for their translation to the clinical setting as rodents have a much lower proportion of subcortical white matter in relation to cortical volume compared to humans (Schoenemann et al., 2005; Hofman, 2014). Therefore, transplantation in humans would more heavily depend on the severity of hypomyelination and the extent to which exogenous cells must migrate and reproduce. Moreover, to effectively correct CNS functioning *via* remyelination in humans, experimental studies on higher-order mammals, such as primates, would allow for a more comparable result in terms of determining the optimal dosage and regions of transplantation.

Glial Progenitor Cell Transplantation: A Targeted Lineage

Glial progenitor cells (GPCs), which are further patterned from NSCs towards a glial fate, have also been explored as a candidate for cerebral transplantation in leukodystrophies (Osorio and Goldman, 2016; Goldman, 2017; Chanoumidou et al., 2020). Similar to NSCs, GPCs can be generated from pluripotent stem cells or harvested and purified from fetal brain tissue for transplantation (Nunes et al., 2003; Monaco et al., 2012). Many studies have successfully performed intracerebral transplantation of glial cells in animal models and shown their effectiveness in remyelination (Duncan, 2005; Franklin and Ffrench-Constant, 2008; Goldman, 2011). Notably, several studies involving the transplantation of human glial lineage-specific cells into *shiverer* mice show consistent results, with evidence of robust remyelination, prolonged survival, and phenotypic rescue (Windrem et al., 2004, 2008, 2014, 2020; Izrael et al., 2007; Mariani et al., 2019). These results provide support for clinical exploration of this treatment, revealing that GPCs have a migratory potential and can effectively differentiate *in vivo* when transplanted into another host. Comparative efficacy between specific lineages in transplantation therapy remains to be confirmed; in one study, both NSCs and OPCs were able to remyelinate and produce compact myelin in both Pelizaeus-Merzbacher disease *Plp1*-overexpressing and *shiverer* immunodeficient mouse models, however, in the transgenic *Plp1*-overexpressing mice, NSCs more notably promoted survival and prolonged lifespan, whereas in *shiverer* mice, OPC transplantation promoted a slightly longer lifespan compared to NSCs (Marteyn et al., 2016). Nonetheless, it is important to note that the microenvironment within the CNS tissue likely had a significant impact on survival, with neuroinflammation being downregulated in NSC-grafted mice, which is an important consideration in therapy for Pelizaeus-Merzbacher disease due to the known inflammatory component of disease pathogenesis (Marteyn et al., 2016). Likewise, when OPCs were co-transplanted with mesenchymal stem cells (MSCs) into *shiverer* mice, the immune response was minimized and increased oligodendrocyte engraftment, myelination, and maturation was evident (Cristofanilli et al., 2011). Therefore, immune response could prove to be an additional important consideration when evaluating the effectiveness of stem cell therapy, and would be noteworthy to explore in POLR3-HLD pathogenesis before the development of therapeutic strategies.

Induced Pluripotent Stem Cells: Patient-Derived Cell Therapy Approaches

GPCs generated from induced pluripotent stem cells (iPSCs) have also been investigated as a prospect for cell therapy and transplantation in white matter diseases (Fox et al., 2014; Chanoumidou et al., 2020). iPSC-derived cells provide an additional advantage as they harbor the genetic background of the individual from whom they originate, thereby adding a patient-specific approach to cell-based therapies. iPSCs can be generated *via* direct reprogramming of somatic cells using a series of pluripotency factors, reverting them into a stem-like fate with the ability to renew indefinitely or differentiate into the desired lineage (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Patient-specific cells with a renewable potential are especially appealing for the treatment of genetic disorders as they can be expanded to a large number before transplantation and downstream differentiation, and importantly they can evade the possible immunologic rejection that accompanies allogeneic stem cell transplantation. However, before iPSC-derived GPCs can be considered in a clinical setting, there are several limitations to consider and study, including the possibility of tumor formation, as well as potential safety concerns of gene editing required for correction of disease-causing mutations in patient cells, including off-target effects, immunotoxicity, and DNA damage toxicity (Neofytou et al., 2015; Uddin et al., 2020).

Studies on the development of iPSC-derived oligodendrocytes have progressed in the past decade, leading to increased discussion of their utility in treating neurological diseases (Chanoumidou et al., 2020). One of the first studies of human iPSC-derived OPCs aimed to investigate their myelinating potential in the lysolecithin-induced demyelinated rat optic chiasm, in which remyelination was evident following transplantation, reinforcing the potential for iPSC-derived cell transplantation (Pouya et al., 2011). Following this direction, further studies were completed transplanting human iPSC-derived cells into the *shiverer* mouse, revealing that iPSC-derived OPCs can migrate and robustly myelinate brain tissue (Sim et al., 2011; Wang et al., 2013; Ehrlich et al., 2017). iPSC-derived cell transplantation has also

proven effective in other neurodegenerative disease models; transplantation studies using a mouse model for Huntington's disease recently demonstrated that iPSC-derived NSCs were capable of ameliorating their motor phenotype and differentiating into region-specific neurons without tumor formation, thereby providing the foundation for use of iPSC-derived cells in future studies of neurological diseases (Al-Gharaibeh et al., 2017).

Using a direct approach to replace myelin in the brain *via* NSCs, GPCs, or OPCs is an option to consider further studying in POLR3-HLD, however, studies are needed to first determine the pathophysiological mechanisms underlying hypomyelination. The use of autologous patient-specific iPSCs is also an attractive approach due to the decreased risk of transplant rejection. In these circumstances, the concern for donor cell rejection would be limited given that the patient-derived cells are nonimmunogenic, and therefore suppression of the immune system could be avoided. Moreover, iPSCs offer an accessible and renewable source of patient-derived cells, making them an optimal option for transplantation, provided that the potential for tumor formation is deemed very low risk. Further research into the potential for genetic correction of iPSCs from POLR3-HLD patients would be required to determine whether restoration of myelin would be possible with iPSC-derived OPC transplantation.

GENE TRANSFER THERAPY: CONSIDERATIONS IN LEUKODYSTROPHIES

Historically, the concept of gene therapy evolved from gene transfer experiments which suggested that supplying functional transgenes to cells with corresponding dysfunctional counterparts might provide therapeutic benefit (Rogers, 1959, 1966, 1971; Rogers and Pfuderer, 1968; Terheggen et al., 1975; Friedmann, 2001; Wirth et al., 2013). Gene therapy as a field has grown beyond gene transfer therapy, to encompass techniques such as oligonucleotide and mRNA therapy (Bennett, 2019; Kowalski et al., 2019; Setten et al., 2019) as well as gene editing (discussed below). Typically, gene transfer therapy is divided into *ex vivo* and *in vivo* approaches that make use of different viral vectors for delivery of genetic material to cells. *Ex vivo* gene therapy usually involves removing hematopoietic stem cells from the body and administering a gene therapy vector (often lentiviral) *in vitro* before re-infusing treated cells into the patient. It is methodologically similar to bone marrow transplantation therapy and has overlapping applications, with the advantage of obviating the need for long-term immunosuppression required after bone marrow transplantation. *Ex vivo* gene therapy of this type has been used successfully to treat X-linked adrenoleukodystrophy (Cartier et al., 2009, 2012; Eichler et al., 2017) and metachromatic leukodystrophy (Biffi et al., 2013; Sessa et al., 2016) if administered early. *Ex vivo* gene therapy using hematopoietic stem cells is not a viable option for POLR3-HLD due to the primary defect in POLR3 activity in brain tissues, especially because the complex is not secreted and has a primarily non-metabolic function. However, *ex vivo* gene therapy may have an application using iPSCs, if treated cells are subsequently differentiated to a glial lineage and delivered into the brain. Limitations would involve similar factors to those described above (i.e., safety, route of transplantation, migration, and differentiation capacity). Primarily, *in vivo* gene transfer has been applied successfully to treat specific leukodystrophies, and may be a candidate modality for the treatment of POLR3-HLD. Here, we will focus on *in vivo* gene transfer data, which represents a majority of the literature and clinical experience with gene therapy directly targeting the brain in leukodystrophies, such as Canavan's disease and metachromatic leukodystrophy. As our knowledge of POLR3-HLD pathology continues to evolve, so too will opportunities to advance tractable strategies for developing a disease-modifying treatment.

In vivo Approaches to Gene Therapy

In considering *in vivo* gene therapy, several viral vectors have been proposed to achieve transgene delivery, but thus far, the most clinically successful has been adeno-associated virus (AAV). *In vivo* gene therapy for leukodystrophies began with an AAV trial for Canavan disease, an autosomal recessive leukodystrophy caused by mutations in the *ASPA* gene, encoding the enzyme aspartoacyclase which functions to degrade N-acetylaspartate (NAA) in the brain (Janson et al., 2002). AAV2-*ASPA* treatment was supported by concurrent pre-clinical rodent studies suggesting human *ASPA* gene transfer to Canavan mice and rats resulted in decreased NAA concentrations in brain tissue, along with decreased seizure frequency and histopathological improvements (Matalon et al., 2003; Mcphee et al., 2005). These findings were translated into a clinical trial. Long term follow-up in a cohort of 28 patients, 13 of which were treated by intraparenchymal delivery of AAV2-*ASPA* to six sites in the brain, demonstrated a good safety profile with the most common adverse events (i.e., small subdural hemorrhage, postoperative fever) most likely associated with the neurosurgical aspect of the treatment, and no adverse events occurring after 90 days of follow-up (Leone et al., 2012). AAV2-*ASPA* was shown to decrease NAA in the brain, as measured by MRS, as well as the slow progression of brain atrophy, and was considered to have been associated with adequate safety and moderate overall clinical efficacy that warranted further clinical trials (Leone et al., 2012). This early AAV trial was instrumental in demonstrating the enhanced safety profile of AAV for *in vivo* gene therapy in leukodystrophies.

The discovery of novel AAV serotypes in nonhuman primates and human tissues elucidated numerous aspects of AAV biology, including their differences in tissue tropism, leading to an explosion of studies exploring the use of naturally occurring AAV serotypes and recombinant AAV (Gao et al., 2002, 2003, 2004). Importantly, the AAV serotypes identified in the course of Dr. Gao and colleagues' work especially AAV9, have been studied for their utility in transducing brain tissues. An important aspect surrounding the use of AAV9 for CNS diseases involves its enhanced ability to target the CNS, which

allows for intrathecal or intravenous administration (Foust et al., 2009; Mendell et al., 2017; Gessler et al., 2019). Most notably, AAV9 was successfully used in a clinical trial for spinal muscular atrophy (Mendell et al., 2017), resulting in FDA approval of Zolgensma®, an intravenously delivered gene therapy treatment. AAV9 has also recently demonstrated effectiveness in a mouse model of Canavan disease (Gessler et al., 2017), which played a role in promoting the rAAV9-*ASPA* vector transitioning to a recent open-label clinical trial for Canavan disease (*CANaspire*, ASPA Therapeutics). Finally, an exciting recent AAV9 finding is the success of AAV9-*GALC* in treating a canine model of globoid cell leukodystrophy or Krabbe disease, improving myelination and extending lifespan more than seven times beyond the typical life expectancy for model animals (Bradbury et al., 2020). However, AAV9 is not known to efficiently mediate significant transduction of oligodendrocyte lineage cells.

A Clade E AAV serotype identified in 2004 (Gao et al., 2004) called AAVrh.10, has been tested in the context of metachromatic leukodystrophy on a small number of patients (NCT01801709); however, the results of this trial have not been released. The initial preclinical data for this study suggested that intracerebral delivery of AAVrh.10-*ARSA* was superior to AAV5 both in terms of the overall impact on the model disease and its ability to transduce oligodendrocytes (Sevin et al., 2006, 2007; Piguet et al., 2012), which led to further safety and feasibility assessments in non-human primates leading up to the clinical trial (Zerah et al., 2015). Importantly, in the preclinical assessment, Sevin and colleagues evaluated the direct impact of AAVrh.10 on oligodendrocyte transduction using a GFP-containing vector and estimated that 9% of oligodendrocytes in the striatum were transduced directly, whereas 21% were found to contain ARSA enzyme after administration of AAVrh.10-*ARSA* (Piguet et al., 2012). These findings indicate that cross-correction of oligodendrocyte ARSA enzyme levels *via* transduction of non-oligodendrocyte targets plays a role in the observed improvement in oligodendrocyte sulfatide levels and brain pathology (Piguet et al., 2012). The AAVrh.10 trial excepted, in each of the mentioned leukodystrophies in which *in vivo* gene therapy has been tested, the putative improvement in oligodendrocyte function is thought to occur through cross-correction. Indeed, most AAV capsids are not known to efficiently transduce oligodendrocytes (Burger et al., 2004; Cearley and Wolfe, 2006; Cearley et al., 2008; San Sebastian et al., 2013). This fact has prompted studies evaluating the use of oligodendrocyte-specific promoters to drive expression in oligodendrocytes (Chen et al., 1998; Lawlor et al., 2009) as well as the pursuit of novel recombinant capsids with significant oligodendrocyte tropism as demonstrated in rodents (Powell et al., 2016), and the characterization of oligodendrocyte tropism in a novel naturally occurring AAV capsid (Hsu et al., 2020). Taken together, these study results indicate that AAV vector research continues to yield important advances toward achieving both safety and efficacy for *in vivo* gene therapy approaches to leukodystrophies. The increasing focus on understanding how AAV technology can be used to target oligodendrocyte lineage cells will be important for the development of an *in vivo* gene therapy approach to POLR3-HLD.

Currently, the POLR3-HLD disease population is divided with the majority (≥90%) of patients having either biallelic mutations in *POLR3A* or *POLR3B* (Bernard et al., 2011; Tétreault et al., 2011; Daoud et al., 2013; Wolf et al., 2014b) and a minority (<10%) having mutations in *POLR1C* (Thiffault et al., 2015; Gauquelin et al., 2019) or *POLR3K* (Dorboz et al., 2018). In the future, it may be possible to treat patients by grouping according to the affected subunit and administering a vector carrying the appropriate sequence *in vivo*. However, there are three key challenges for developing an *in vivo* gene therapy approach for POLR3-HLD that have not been addressed by prior *in vivo* leukodystrophy gene therapy studies. The first is the fact that in each of the previously mentioned diseases, cross-correction is possible and beneficial due to the nature of the defective enzymes and metabolites responsible for the disease. In POLR3-HLD, cross-correction is improbable because POLR3 subunits are unlikely to be secreted or transferred between cells and also because pathogenesis likely does not involve the accumulation of the enzymatic reactants (RNA nucleotides), as they are used by other RNA polymerases, and do not directly cause toxicity. Therefore, directly correcting the oligodendrocyte lineage is an important aspect of a putative gene therapy strategy for POLR3-HLD. Second, the pathophysiological axis of POLR3-HLD is hypomyelination, relating to a specific and yet poorly characterized deficit in the oligodendrocyte lineage that may occur well before the cells mature and myelinate the affected CNS regions. If the deficit occurs primarily in the cellular precursors of oligodendrocytes (i.e., a dividing cell population), the exponential dilution of non-integrating vector genomes such as those transduced using AAV is an important consideration. Third, attempts to produce a representative animal model of POLR3-HLD in which to perform pre-clinical testing have proven difficult (Choquet et al., 2017, 2019b), and this barrier will need to be overcome to properly test any novel therapeutic candidate. Recently, progress in generating an animal model has been made using an *Olig2*-Cre conditional double *Polr3a* mutant knock-in strategy, in a recent pre-print which has yet to be peer-reviewed at the time of writing (Merheb et al., 2020). Overcoming these challenges would elucidate the potential for POLR3-HLD gene transfer, and will also inform the future development of more advanced and/or personalized (e.g., gene editing) therapeutic strategies for POLR3-HLD.

GENE EDITING TECHNIQUES: A MODERN APPROACH

Recently, gene-editing research has gained traction for its utilization in the development of patient-specific therapies for genetic diseases. While these techniques are not yet employed in a large-scale clinical setting, they hold promise for treating rare genetic diseases that are without curative therapies. Moreover, the design of personalized therapies is a possibility through the use of gene editing, a technique that can create alterations in precise genomic locations to correct pathogenic

variants. Yet, to establish translational gene editing strategies, additional aspects must be investigated such as vehicles and delivery methods of editing systems, optimization of editing constructs, and elimination of off-target effects. Furthermore, with correct optimization, genome engineering can lead to the establishment of personalized therapies for diseases that are otherwise challenging to treat.

CRISPR-Cas9 Editing System

Since the discovery of its potential for human genome editing in 2013, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas gene-editing technology has been heavily investigated for its use in studying and treating genetic diseases (Cong et al., 2013; Jinek et al., 2013). The CRISPR-Cas system harnesses the cellular machinery involved in the adaptive immune response of bacterial cells against viral particles (Horvath and Barrangou, 2010). This highly specific system can target precise genomic regions and has revolutionized modern genetic research for its capability to easily manipulate the human genome. While different systems have been engineered using a series of CRISPR/Cas components and types of Cas nucleases, the Cas9 nuclease has been most commonly used in genetic editing of mammalian cells (Makarova and Koonin, 2015). In combination with a single guide-RNA (sgRNA), Cas9 can be programmed to target and cleave complementary DNA sequences, which can be subsequently repaired using a donor DNA template strand and the intrinsic homology-directed repair mechanism (Mali et al., 2013; Ran et al., 2013; Yang et al., 2014). Thus, given that the proper cell type is targeted, it is possible to use CRISPR-Cas9 technology to genetically correct mutations causing monogenic diseases, bringing to light its ability to facilitate phenotypic repair.

CRISPR-Cas9 editing has been successfully used in many *in vitro* and *in vivo* studies to both explore disease pathophysiology through the creation of transgenic or knock-out models, and investigate treatment methods *via* targeted gene editing and correction of genetic mutations (Rodríguez-Rodríguez et al., 2019). For example, in an initial study using a murine model of hereditary tyrosinemia, injection of CRISPR-Cas9 components into the liver led to phenotypic rescue, demonstrating the potential for genetic correction *in vivo* (Yin et al., 2014). Additionally, studies using the *mdx* murine model for Duchenne muscular dystrophy have shown that delivery of CRISPR-Cas9 constructs, both at the germline level with injection into zygotes and postnatally with delivery *via* AAV9, can lead to phenotypic improvements (Long et al., 2014). *In vitro* and *in vivo* studies have also been completed using CRISPR-Cas9 to correct mutations associated with Huntington's disease, with promising results demonstrating that suppression of mutant alleles can alleviate motor phenotypes in mice (Shin et al., 2016; Kolli et al., 2017; Monteys et al., 2017; Yang et al., 2017). In the field of HLDs, a recent study has demonstrated that CRISPR-Cas9 mediated germline suppression of *Plp1* in the severe *jimpy* mouse model of Pelizaeus-Merzbacher disease leads to increased myelination and restored lifespan (Elitt et al., 2020). Most recently, the first clinical trials of CRISPR-Cas9 therapies have launched, with *ex vivo* approaches centering around cancer immunotherapy, as well as gene disruption of hematological disorders including sickle-cell anemia and β-thalassemia (Li et al., 2020; Rosenblum et al., 2020; Uddin et al., 2020). Additionally, an *in vivo* approach has been employed in Leber congenital amaurosis, a monogenic disease associated with childhood blindness, involving the delivery of AAV5-packaged CRISPR-Cas9 constructs directly to the retina (Maeder et al., 2019). As these trials progress and with the assessment of long-term outcomes and safety, this gene-editing technology could show powerful potential for use in treating many classes of diseases. Moreover, research involving gene editing with CRISPR-Cas9 techniques in the CNS is constantly evolving; innovations and improvements to the editing system focus on optimizing editing efficiencies and reducing off-target effects, as well as exploring delivery methods *via* biological vesicles, nanoparticles, or viruses (Cota-Coronado et al., 2019; Sandoval et al., 2020).

While rapidly advancing, gene editing techniques would have to be studied *in vitro* and *in vivo* for their use in correcting the POLR3-HLD phenotype before they can be considered as a potential therapeutic approach. It is noteworthy that the use of CRISPR-Cas9 technology is not effective for the correction of all mutation types associated with POLR3-HLD (i.e., large exonic deletions, synonymous variants, some splice site variants), and this therapeutic approach would have to be considered on a patient-specific level. Moreover, this technique is still in the early experimental stage of study and before it can be considered in a clinical setting, its benefits and downfalls as a therapeutic tool must be explored along with the most optimal delivery methods and its potential in correcting cells of the CNS. In speculating on the use of gene editing therapy to treat the cellular pathogenesis associated with POLR3-HLD, this therapy may or may not be applicable depending on the stage of oligodendrocyte lineage that is defective. Moreover, if future studies find that early OPC proliferation or migration ability is not severely affected, and pathogenesis predominantly concerns the formation of myelin itself (due to transcriptional defects causing lack of protein availability for myelin membrane formation), the delivery of CRISPR constructs for genetic correction of myelinating cells could show high potential for phenotypic remediation. However, there are many other potential scenarios in which different cell types or mechanisms could be affected (e.g., differentiation of NSCs to a glial fate, impairments in migration of OPCs, maturation of OPCs into oligodendrocytes, signaling between different cell types and/or other mechanisms for formation, wrapping, or compaction of the myelin membrane itself). Thus, without knowledge on the cellular pathophysiology, it is to be determined whether correcting cells after birth and the initial waves of OPC production/migration during the *in utero* period of myelin development would be applicable. Knowledge of disease pathogenesis would help predict the probability of success for delivery of gene editing constructs at certain stages of the disease progression, and whether myelination is possible.

CONCLUSION: THE FUTURE OF POLR3-HLD THERAPIES

Along the front of therapy development for rare inherited neurological disorders, advances in cell therapy, gene therapy, and gene editing techniques have all presented exciting results in recent years. Combination approaches have also been considered, including the use of gene transfer or editing of stem cells for transplantation to improve disease phenotypes (Ricca et al., 2015; Meneghini et al., 2017). In considering POLR3-HLD, much information remains to be uncovered regarding the pathophysiology of the disease and whether myelin restoration is possible. As pathological studies demonstrate that oligodendrocytes are primarily affected in POLR3-HLD, this review provided a cell-specific approach to the consideration of therapies. However, disease pathogenesis may involve other cell types, which could also be targeted in combination. The described therapies offer potential options for exploration, and future studies in both cellular and animal models to investigate their effectiveness and mechanisms would prove to be beneficial. Moreover, developing disease biomarkers and tangible clinical outcome measures are of utmost importance to evaluate therapeutic efficacy and successfully translate pre-clinical findings into the clinical setting. Ongoing research on POLR3-HLD pathophysiology will surely provide a backbone for ascertaining which therapy approaches could provide the most beneficial results, and ultimately uncover the avenues for potential clinical trial development to improve patient outcomes.

AUTHOR CONTRIBUTIONS

SP, MMR, and GB conceived the design of the article, reviewed the literature, and contributed to the writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the Canadian Institutes of Health Research (426534, 201610PJT-377869), Fondation Les Amis d'Elliot, Leuco-Action, and Fondation le Tout pour Loo. SP is supported by the Fonds de Recherche du Québec en Santé (FRQS) Doctoral Scholarship, the Fondation du Grand défi Pierre Lavoie Doctoral Scholarship, the McGill Faculty of Medicine F.S.B. Miller Fellowship, and the Research Institute of the McGill University Health Centre Desjardins Studentship in Child Health Research. MMR is supported by the Vanier Canada Graduate Scholarship and the McGill University Faculty of Medicine MD/Ph.D. Program. GB has received a Research Scholar Junior 1 award from the Fonds de Recherche du Québec en Santé (FRQS; 2012-2016) and the New Investigator Salary Award from the Canadian Institutes of Health Research (CIHR; 2017-2022).

ACKNOWLEDGMENTS

We wish to acknowledge the patients and families involved in POLR3-related leukodystrophy research. Figures included in this article were created using BioRender (http://Biorender.com).

REFERENCES

Adang, L. A., Sherbini, O., Ball, L., Bloom, M., Darbari, A., Amartino, H., et al. (2017). Revised consensus statement on the preventive and symptomatic care of patients with leukodystrophies. *Mol. Genet. Metab.* 122, 18–32. doi: 10.1016/j.ymgme.2017.08.006

Ader, M., Schachner, M., and Bartsch, U. (2001). Transplantation of neural precursor cells into the dysmyelinated CNS of mutant mice deficient in the myelin-associated glycoprotein and Fyn tyrosine kinase. *Eur. J. Neurosci.* 14, 561–566. doi: 10.1046/j.0953-816x.2001.01673.x

Ader, M., Schachner, M., and Bartsch, U. (2004). Integration and differentiation of neural stem cells after transplantation into the dysmyelinated central nervous system of adult mice. *Eur. J. Neurosci.* 20, 1205–1210. doi: 10.1111/j.1460-9568.2004.03577.x

Al-Gharaibeh, A., Culver, R., Stewart, A. N., Srinageshwar, B., Spelde, K., Frollo, L., et al. (2017). Induced pluripotent stem cell-derived neural stem cell transplantations reduced behavioral deficits and ameliorated neuropathological changes in YAC128 mouse model of Huntington's disease. *Front. Neurosci.* 11:628. doi: 10.3389/fnins.2017.00628

Al Yazidi, G., Tran, L. T., Guerrero, K., Vanderver, A., Schiffmann, R., Wolf, N. I., et al. (2019). Dystonia in RNA polymerase III-related leukodystrophy. *Mov. Disord. Clin. Pract.* 6, 155–159. doi: 10.1002/mdc3.12715

Asheuer, M., Pflumio, F., Benhamida, S., Dubart-Kupperschmitt, A., Fouquet, F., Imai, Y., et al. (2004). Human $CD34^+$ cells differentiate into microglia and express recombinant therapeutic protein. *Proc. Natl. Acad. Sci. U S A* 101, 3557–3562. doi: 10.1073/pnas.0306431101

Atrouni, S., Daraze, A., Tamraz, J., Cassia, A., Caillaud, C., and Megarbane, A. (2003). Leukodystrophy associated with oligodontia in a large inbred family: fortuitous association or new entity? *Am. J. Med. Genet. A* 118A, 76–81. doi: 10.1002/ajmg.a.10019

Aung, W. Y., Mar, S., and Benzinger, T. L. (2013). Diffusion tensor MRI as a biomarker in axonal and myelin damage. *Imaging Med.* 5, 427–440. doi: 10.2217/iim.13.49

Azmanov, D. N., Siira, S. J., Chamova, T., Kaprelyan, A., Guergueltcheva, V., Shearwood, A. J., et al. (2016). Transcriptome-wide effects of a POLR3A gene mutation in patients with an unusual phenotype of striatal involvement. *Hum. Mol. Genet.* 25, 4302–4314. doi: 10.1093/hmg/ddw263

Barkovich, A. J., and Deon, S. (2016). Hypomyelinating disorders: an MRI approach. *Neurobiol. Dis.* 87, 50–58. doi: 10.1016/j.nbd.2015.10.015

Baron, W., and Hoekstra, D. (2010). On the biogenesis of myelin membranes: sorting, trafficking and cell polarity. *FEBS Lett.* 584, 1760–1770. doi: 10.1016/j.febslet.2009.10.085

Bennett, C. F. (2019). Therapeutic antisense oligonucleotides are coming of age. *Ann. Rev. Med.* 70, 307–321. doi: 10.1146/annurev-med-041217-010829

Bernard, G., Chouery, E., Putorti, M. L., Tétreault, M., Takanohashi, A., Carosso, G., et al. (2011). Mutations of POLR3A encoding a catalytic subunit of RNA polymerase Pol III cause a recessive hypomyelinating leukodystrophy. *Am. J. Hum. Genet.* 89, 415–423. doi: 10.1016/j.ajhg.2011.07.014

Bernard, G., Thiffault, I., Tetreault, M., Putorti, M. L., Bouchard, I., Sylvain, M., et al. (2010). Tremor-ataxia with central hypomyelination (TACH) leukodystrophy maps to chromosome 10q22.3-10q23.31. *Neurogenetics* 11, 457–464. doi: 10.1007/s10048-010-0251-8

Bernard, G., and Vanderver, A. (2017). "POLR3-related leukodystrophy," in *GeneReviews*, eds M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, et al. (Seattle, WA: University of Washington), 1993–2020.

Biffi, A., Montini, E., Lorioli, L., Cesani, M., Fumagalli, F., Plati, T., et al. (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 341:1233158. doi: 10.1126/science.1233158

Bonhoure, N., Praz, V., Moir, R. D., Willemin, G., Mange, F., Moret, C., et al. (2020). MAF1 is a chronic repressor of RNA polymerase III transcription in the mouse. *Sci. Rep.* 10:11956. doi: 10.1038/s41598-020-68665-0

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, 718–725. doi: 10.1212/WNL.0b013e3181eee46b

Boucher, A. A., Miller, W., Shanley, R., Ziegler, R., Lund, T., Raymond, G., et al. (2015). Long-term outcomes after allogeneic hematopoietic stem cell transplantation for metachromatic leukodystrophy: the largest single-institution cohort report. *Orphanet J. Rare Dis.* 10:94. doi: 10.1186/s13023-015-0313-y

Boycott, K. M., Dyment, D. A., Sawyer, S. L., Vanstone, M. R., and Beaulieu, C. L. (2014). Identification of genes for childhood heritable diseases. *Annu. Rev. Med.* 65, 19–31. doi: 10.1146/annurev-med-101712-122108

Bradbury, A. M., Bagel, J. H., Nguyen, D., Lykken, E. A., Pesayco Salvador, J., Jiang, X., et al. (2020). Krabbe disease successfully treated *via* monotherapy of intrathecal gene therapy. *J. Clin. Invest.* 130, 4906–4920. doi: 10.1172/JCI133953

Brüstle, O., Spiro, A. C., Karram, K., Choudhary, K., Okabe, S., and Mckay, R. D. (1997). *In vitro*-generated neural precursors participate in mammalian brain development. *Proc. Natl. Acad. Sci. U S A* 94, 14809–14814. doi: 10.1073/pnas.94.26.14809

Buchet, D., Garcia, C., Deboux, C., Nait-Oumesmar, B., and Baron-Van Evercooren, A. (2011). Human neural progenitors from different foetal forebrain regions remyelinate the adult mouse spinal cord. *Brain* 134, 1168–1183. doi: 10.1093/brain/awr030

Burger, C., Gorbatyuk, O. S., Velardo, M. J., Peden, C. S., Williams, P., Zolotukhin, S., et al. (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2 and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol. Ther.* 10, 302–317. doi: 10.1016/j.ymthe.2004.05.024

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Bougnères, P., Schmidt, M., Kalle, C. V., et al. (2012). Lentiviral hematopoietic cell gene therapy for X-linked adrenoleukodystrophy. *Methods Enzymol.* 507, 187–198. doi: 10.1016/B978-0-12-386509-0.00010-7

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutschera, I., et al. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 326, 818–823. doi: 10.1126/science.1171242

Cearley, C. N., Vandenberghe, L. H., Parente, M. K., Carnish, E. R., Wilson, J. M., and Wolfe, J. H. (2008). Expanded repertoire of AAV vector serotypes mediate unique patterns of transduction in mouse brain. *Mol. Ther.* 16, 1710–1718. doi: 10.1038/mt.2008.166

Cearley, C. N., and Wolfe, J. H. (2006). Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9 and Rh10 in the mouse brain. *Mol. Ther.* 13, 528–537. doi: 10.1016/j.ymthe.2005.11.015

Chanoumidou, K., Mozafari, S., Baron-Van Evercooren, A., and Kuhlmann, T. (2020). Stem cell derived oligodendrocytes to study myelin diseases. *Glia* 68, 705–720. doi: 10.1002/glia.23733

Chen, H., Mccarty, D. M., Bruce, A. T., Suzuki, K., and Suzuki, K. (1998). Gene transfer and expression in oligodendrocytes under the control of myelin basic protein transcriptional control region mediated by adeno-associated virus. *Gene Ther.* 5, 50–58. doi: 10.1038/sj.gt.3300547

Choquet, K., Forget, D., Meloche, E., Dicaire, M. J., Bernard, G., Vanderver, A., et al. (2019a). Leukodystrophy-associated POLR3A mutations down-regulate the RNA polymerase III transcript and important regulatory RNA BC200. *J. Biol. Chem.* 294, 7445–7459. doi: 10.1074/jbc.RA118.006271

Choquet, K., Pinard, M., Yang, S., Moir, R. D., Poitras, C., Dicaire, M. J., et al. (2019b). The leukodystrophy mutation POLR3b R103H causes homozygote mouse embryonic lethality and impairs RNA polymerase III biogenesis. *Mol. Brain* 12:59. doi: 10.1186/s13041-019-0479-7

Choquet, K., Yang, S., Moir, R. D., Forget, D., Lariviere, R., Bouchard, A., et al. (2017). Absence of neurological abnormalities in mice homozygous for the POLR3a G672E hypomyelinating leukodystrophy mutation. *Mol. Brain* 10:13. doi: 10.1186/s13041-017-0294-y

Chouery, E., Delague, V., Jalkh, N., Salem, N., Kfoury, J., Rodriguez, D., et al. (2011). A whole-genome scan in a large family with leukodystrophy and oligodontia reveals linkage to 10q22. *Neurogenetics* 12, 73–78. doi: 10.1007/s10048-010-0256-3

Cloutier, P., and Coulombe, B. (2010). New insights into the biogenesis of nuclear RNA polymerases? *Biochem. Cell Biol.* 88, 211–221. doi: 10.1139/o09-173

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. doi: 10.1126/science.1231143

Costello, D. J., Eichler, A. F., and Eichler, F. S. (2009). Leukodystrophies: classification, diagnosis and treatment. *Neurologist* 15, 319–328. doi: 10.1097/NRL.0b013e3181b287c8

Cota-Coronado, A., Diaz-Martinez, N. F., Padilla-Camberos, E., and Diaz-Martinez, N. E. (2019). Editing the central nervous system through CRISPR/Cas9 systems. *Front. Mol. Neurosci.* 12:110. doi: 10.3389/fnmol.2019.00110

Cristofanilli, M., Harris, V. K., Zigelbaum, A., Goossens, A. M., Lu, A., Rosenthal, H., et al. (2011). Mesenchymal stem cells enhance the engraftment and myelinating ability of allogeneic oligodendrocyte progenitors in dysmyelinated mice. *Stem Cells Dev.* 20, 2065–2076. doi: 10.1089/scd.2010.0547

Daoud, H., Tetreault, M., Gibson, W., Guerrero, K., Cohen, A., Gburek-Augustat, J., et al. (2013). Mutations in POLR3A and POLR3B are a major cause of hypomyelinating leukodystrophies with or without dental abnormalities and/or hypogonadotropic hypogonadism. *J. Med. Genet.* 50, 194–197. doi: 10.1136/jmedgenet-2012-101357

De Feo, D., Merlini, A., Laterza, C., and Martino, G. (2012). Neural stem cell transplantation in central nervous system disorders: from cell replacement to neuroprotection. *Curr. Opin. Neurol.* 25, 322–333. doi: 10.1097/WCO.0b013e328352ec45

Degasperis, S. M., Bernard, G., Wolf, N. I., Miller, E., and Pohl, D. (2020). 4H leukodystrophy: mild clinical phenotype and comorbidity with multiple sclerosis. *Neurol. Genet.* 6:e409. doi: 10.1212/NXG.0000000000000409

Dieci, G., Conti, A., Pagano, A., and Carnevali, D. (2013). Identification of RNA polymerase III-transcribed genes in eukaryotic genomes. *Biochim. Biophys. Acta* 1829, 296–305. doi: 10.1016/j.bbagrm.2012.09.010

Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M., and Pagano, A. (2007). The expanding RNA polymerase III transcriptome. *Trends Genet.* 23, 614–622. doi: 10.1016/j.tig.2007.09.001

Dietrich, R. B., Bradley, W. G., Zaragoza, E. J. T., Otto, R. J., Taira, R. K., Wilson, G. H., et al. (1988). MR evaluation of early myelination patterns in normal and developmentally delayed infants. *Am. J. Roentgenol.* 150, 889–896. doi: 10.2214/ajr.150.4.889

Dorboz, I., Dumay-Odelot, H., Boussaid, K., Bouyacoub, Y., Barreau, P., Samaan, S., et al. (2018). Mutation in *POLR3K* causes hypomyelinating leukodystrophy and abnormal ribosomal RNA regulation. *Neurol. Genet.* 4:e289. doi: 10.1212/NXG.0000000000000289

Duncan, I. D. (2005). Oligodendrocytes and stem cell transplantation: their potential in the treatment of leukoencephalopathies. *J. Inherit. Metab. Dis.* 28, 357–368. doi: 10.1007/s10545-005-7058-z

Duncan, I. D., Kondo, Y., and Zhang, S.-C. (2011). The myelin mutants as models to study myelin repair in the leukodystrophies. *Neurotherapeutics* 8, 607–624. doi: 10.1007/s13311-011-0080-y

Eftekharpour, E., Karimi-Abdolrezaee, S., Wang, J., El Beheiry, H., Morshead, C., and Fehlings, M. G. (2007). Myelination of congenitally dysmyelinated spinal cord axons by adult neural precursor cells results in formation of nodes of ranvier and improved axonal conduction. *J. Neurosci.* 27, 3416–3428. doi: 10.1523/JNEUROSCI.0273-07.2007

Ehrlich, M., Mozafari, S., Glatza, M., Starost, L., Velychko, S., Hallmann, A. L., et al. (2017). Rapid and efficient generation of oligodendrocytes from human induced pluripotent stem cells using transcription factors. *Proc. Natl. Acad. Sci. U S A* 114, E2243–E2252. doi: 10.1073/pnas.1614412114

Eichler, F., Duncan, C., Musolino, P. L., Orchard, P. J., De Oliveira, S., Thrasher, A. J., et al. (2017). Hematopoietic stem-cell gene therapy for cerebral adrenoleukodystrophy. *N. Engl. J. Med.* 377, 1630–1638. doi: 10.1056/NEJMoa1700554

Elbaz, B., and Popko, B. (2019). Molecular control of oligodendrocyte development. *Trends Neurosci.* 42, 263–277. doi: 10.1016/j.tins.2019.01.002

Elitt, M. S., Barbar, L., Shick, H. E., Powers, B. E., Maeno-Hikichi, Y., Madhavan, M., et al. (2020). Suppression of proteolipid protein rescues

Pelizaeus-Merzbacher disease. *Nature* 585, 397–403. doi: 10.1038/s41586-020-2494-3

Emery, B. (2010). Regulation of oligodendrocyte differentiation and myelination. *Science* 330, 779–782. doi: 10.1126/science.1190927

Escolar, M. L., Poe, M. D., Provenzale, J. M., Richards, K. C., Allison, J., Wood, S., et al. (2005). Transplantation of umbilical-cord blood in babies with infantile Krabbe's disease. *N. Engl. J. Med.* 352, 2069–2081. doi: 10.1056/NEJMoa042604

Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M., and Kaspar, B. K. (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat. Biotechnol.* 27, 59–65. doi: 10.1038/nbt.1515

Fox, I. J., Daley, G. Q., Goldman, S. A., Huard, J., Kamp, T. J., and Trucco, M. (2014). Stem cell therapy. Use of differentiated pluripotent stem cells as replacement therapy for treating disease. *Science* 345:1247391. doi: 10.1126/science.1247391

Franklin, R. J., and Ffrench-Constant, C. (2008). Remyelination in the CNS: from biology to therapy. *Nat. Rev. Neurosci.* 9, 839–855. doi: 10.1038/nrn2480

Friedmann, T. (2001). Stanfield rogers: insights into virus vectors and failure of an early gene therapy model. *Mol. Ther.* 4, 285–288. doi: 10.1006/mthe.2001.0454

Gao, G., Alvira, M. R., Somanathan, S., Lu, Y., Vandenberghe, L. H., Rux, J. J., et al. (2003). Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc. Natl. Acad. Sci. U S A* 100, 6081–6086. doi: 10.1073/pnas.0937739100

Gao, G., Vandenberghe, L. H., Alvira, M. R., Lu, Y., Calcedo, R., Zhou, X., et al. (2004). Clades of adeno-associated viruses are widely disseminated in human tissues. *J. Virol.* 78, 6381–6388. doi: 10.1128/JVI.78.12.6381-6388.2004

Gao, G.-P., Alvira, M. R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J. M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. U S A* 99, 11854–11859. doi: 10.1073/pnas.182412299

Garbern, J. Y. (2007). Pelizaeus-Merzbacher disease: genetic and cellular pathogenesis. *Cell. Mol. Life Sci.* 64, 50–65. doi: 10.1007/s00018-006-6182-8

Gauquelin, L., Cayami, F. K., Sztriha, L., Yoon, G., Tran, L. T., Guerrero, K., et al. (2019). Clinical spectrum of POLR3-related leukodystrophy caused by biallelic POLR1C pathogenic variants. *Neurol. Genet.* 5:e369. doi: 10.1212/NXG.0000000000000369

Gessler, D. J., Li, D., Xu, H., Su, Q., Sanmiguel, J., Tuncer, S., et al. (2017). Redirecting *N*-acetylaspartate metabolism in the central nervous system normalizes myelination and rescues Canavan disease. *JCI Insight* 2:e90807. doi: 10.1172/jci.insight.90807

Gessler, D. J., Tai, P. W. L., Li, J., and Gao, G. (2019). Intravenous infusion of AAV for widespread gene delivery to the nervous system. *Methods Mol. Biol.* 1950, 143–163. doi: 10.1007/978-1-4939-9139-6_8

Giedd, J. N., Blumenthal, J., Jeffries, N. O., Castellanos, F. X., Liu, H., Zijdenbos, A., et al. (1999). Brain development during childhood and adolescence: a longitudinal MRI study. *Nat. Neurosci.* 2, 861–863. doi: 10.1038/13158

Girbig, M., Misiaszek, A. D., Vorländer, M. K., Lafita, A., Grötsch, H., Baudin, F., et al. (2020). Cryo-EM structures of human RNA polymerase III in its unbound and transcribing states. *BioRxiv* [Preprint]. doi: 10.1101/2020.06.29.177642

Goldman, S. A. (2011). Progenitor cell-based treatment of the pediatric myelin disorders. *Arch. Neurol.* 68, 848–856. doi: 10.1001/archneurol.2011.46

Goldman, S. A. (2017). Progenitor cell-based treatment of glial disease. *Prog. Brain Res.* 231, 165–189. doi: 10.1016/bs.pbr.2017.02.010

Gonzalez-Perez, O. (2012). Neural stem cells in the adult human brain. *Biol. Biomed. Rep.* 2, 59–69. Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3505091/

Gordon-Lipkin, E., and Fatemi, A. (2018). Current therapeutic approaches in leukodystrophies: a review. *J. Child Neurol.* 33, 861–868. doi: 10.1177/0883073818792313

Greene, C. S., and Voight, B. F. (2016). Pathway and network-based strategies to translate genetic discoveries into effective therapies. *Hum. Mol. Genet.* 25, R94–R98. doi: 10.1093/hmg/ddw160

Groeschel, S., Kuhl, J.-S., Bley, A. E., Kehrer, C., Weschke, B., Doring, M., et al. (2016). Long-term outcome of allogeneic hematopoietic stem cell transplantation in patients with juvenile metachromatic leukodystrophy compared with nontransplanted control patients. *JAMA Neurol.* 73, 1133–1140. doi: 10.1001/jamaneurol.2016.2067

Gruenenfelder, F. I., Mclaughlin, M., Griffiths, I. R., Garbern, J., Thomson, G., Kuzman, P., et al. (2020). Neural stem cells restore myelin in a demyelinating model of Pelizaeus-Merzbacher disease. *Brain* 143, 1383–1399. doi: 10.1093/brain/awaa080

Gupta, N., Henry, R. G., Kang, S.-M., Strober, J., Lim, D. A., Ryan, T., et al. (2019). Long-term safety, immunologic response and imaging outcomes following neural stem cell transplantation for Pelizaeus-Merzbacher disease. *Stem Cell Reports* 13, 254–261. doi: 10.1016/j.stemcr.2019.07.002

Gupta, N., Henry, R. G., Strober, J., Kang, S.-M., Lim, D. A., Bucci, M., et al. (2012). Neural stem cell engraftment and myelination in the human brain. *Sci. Transl. Med.* 4:155ra137. doi: 10.1126/scitranslmed.3004373

Gutierrez, M., Thiffault, I., Guerrero, K., Martos-Moreno, G. Á., Tran, L. T., Benko, W., et al. (2015). Large exonic deletions in POLR3B gene cause POLR3-related leukodystrophy. *Orphanet J. Rare Dis.* 10:69. doi: 10.1186/s13023-015-0279-9

Harting, I., Al-Saady, M., Krageloh-Mann, I., Bley, A., Hempel, M., Bierhals, T., et al. (2020). POLR3A variants with striatal involvement and extrapyramidal movement disorder. *Neurogenetics* 21, 121–133. doi: 10.1007/s10048-019-00602-4

Helman, G., Van Haren, K., Bonkowsky, J. L., Bernard, G., Pizzino, A., Braverman, N., et al. (2015). Disease specific therapies in leukodystrophies and leukoencephalopathies. *Mol. Genet. Metab.* 114, 527–536. doi: 10.1016/j.ymgme.2015.01.014

Hiraide, T., Kubota, K., Kono, Y., Watanabe, S., Matsubayashi, T., Nakashima, M., et al. (2020a). POLR3A variants in striatal involvement without diffuse hypomyelination. *Brain Dev.* 42, 363–368. doi: 10.1016/j.braindev.2019.12.012

Hiraide, T., Nakashima, M., Ikeda, T., Tanaka, D., Osaka, H., and Saitsu, H. (2020b). Identification of a deep intronic POLR3A variant causing inclusion of a pseudoexon derived from an alu element in Pol III-related leukodystrophy. *J. Hum. Genet.* 65, 921–925. doi: 10.1038/s10038-020-0786-y

Hofman, M. A. (2014). Evolution of the human brain: when bigger is better. *Front. Neuroanat.* 8:15. doi: 10.3389/fnana.2014.00015

Horvath, P., and Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327, 167–170. doi: 10.1126/science.1179555

Hsu, H. L., Brown, A., Loveland, A. B., Lotun, A., Xu, M., Luo, L., et al. (2020). Structural characterization of a novel human adeno-associated virus capsid with neurotropic properties. *Nat. Commun.* 11:3279. doi: 10.1038/s41467-020-17047-1

Izrael, M., Zhang, P., Kaufman, R., Shinder, V., Ella, R., Amit, M., et al. (2007). Human oligodendrocytes derived from embryonic stem cells: effect of noggin on phenotypic differentiation *in vitro* and on myelination *in vivo*. *Mol. Cell. Neurosci.* 34, 310–323. doi: 10.1016/j.mcn.2006.11.008

Janson, C., Mcphee, S., Bilaniuk, L., Haselgrove, J., Testaiuti, M., Freese, A., et al. (2002). Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. *Hum. Gene. Ther.* 13, 1391–1412. doi: 10.1089/104303402760128612

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., and Doudna, J. (2013). RNA-programmed genome editing in human cells. *eLife* 2:e00471. doi: 10.7554/eLife.00471

Johnson, S. S., Zhang, C., Fromm, J., Willis, I. M., and Johnson, D. L. (2007). Mammalian Maf1 is a negative regulator of transcription by all three nuclear RNA polymerases. *Mol. Cell* 26, 367–379. doi: 10.1016/j.molcel.2007.03.021

Jurkiewicz, E., Dunin-Wasowicz, D., Gieruszczak-Bialek, D., Malczyk, K., Guerrero, K., Gutierrez, M., et al. (2017). Recessive mutations in POLR3B encoding RNA polymerase III subunit causing diffuse hypomyelination in patients with 4H leukodystrophy with polymicrogyria and cataracts. *Clin. Neuroradiol.* 27, 213–220. doi: 10.1007/s00062-015-0472-1

Kolli, N., Lu, M., Maiti, P., Rossignol, J., and Dunbar, G. L. (2017). CRISPR-Cas9 mediated gene-silencing of the mutant huntingtin gene in an *in vitro* model of Huntington's disease. *Int. J. Mol. Sci.* 18:754. doi: 10.3390/ijms18040754

Koob, M., Rousseau, F., Laugel, V., Meyer, N., Armspach, J. P., Girard, N., et al. (2016). Cockayne syndrome: a diffusion tensor imaging and volumetric study. *Br. J. Radiol.* 89:20151033. doi: 10.1259/bjr.20151033

Kowalski, P. S., Rudra, A., Miao, L., and Anderson, D. G. (2019). Delivering the messenger: advances in technologies for therapeutic mRNA delivery. *Mol. Ther.* 27, 710–728. doi: 10.1016/j.ymthe.2019.02.012

Krivit, W. (2004). Allogeneic stem cell transplantation for the treatment of lysosomal and peroxisomal metabolic diseases. *Springer Semin. Immunopathol.* 26, 119–132. doi: 10.1007/s00281-004-0166-2

Krivit, W., Peters, C., and Shapiro, E. G. (1999). Bone marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, hurler, maroteaux-lamy and sly syndromes and gaucher disease type III. *Curr. Opin. Neurol.* 12, 167–176. doi: 10.1097/00019052-199904000-00007

Krivit, W., Sung, J. H., Shapiro, E. G., and Lockman, L. A. (1995). Microglia: the effector cell for reconstitution of the central nervous system following bone marrow transplantation for lysosomal and peroxisomal storage diseases. *Cell Transplant.* 4, 385–392. doi: 10.1016/0963-6897(95)00021-o

La Piana, R., Cayami, F. K., Tran, L. T., Guerrero, K., Van Spaendonk, R., Ounap, K., et al. (2016). Diffuse hypomyelination is not obligate for POLR3-related disorders. *Neurology* 86, 1622–1626. doi: 10.1212/WNL.0000000000002612

La Piana, R., Tonduti, D., Gordish Dressman, H., Schmidt, J. L., Murnick, J., Brais, B., et al. (2014). Brain magnetic resonance imaging (MRI) pattern recognition in pol III-related leukodystrophies. *J. Child Neurol.* 29, 214–220. doi: 10.1177/0883073813503902

Laule, C., Vavasour, I. M., Shahinfard, E., Mädler, B., Zhang, J., Li, D. K. B., et al. (2018). Hematopoietic stem cell transplantation in late-onset krabbe disease: no evidence of worsening demyelination and axonal loss 4 years post-allograft. *J. Neuroimaging* 28, 252–255. doi: 10.1111/jon.12502

Lawlor, P. A., Bland, R. J., Mouravlev, A., Young, D., and During, M. J. (2009). Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol. Ther.* 17, 1692–1702. doi: 10.1038/mt.2009.170

Lee, Y., Morrison, B. M., Li, Y., Lengacher, S., Farah, M. H., Hoffman, P. N., et al. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 487, 443–448. doi: 10.1038/nature11314

Leone, P., Shera, D., Mcphee, S. W. J., Francis, J. S., Kolodny, E. H., Bilaniuk, L. T., et al. (2012). Long-term follow-up after gene therapy for canavan disease. *Sci. Transl. Med.* 4:165ra163. doi: 10.1126/scitranslmed.3003454

Lesniewska, E., and Boguta, M. (2017). Novel layers of RNA polymerase III control affecting tRNA gene transcription in eukaryotes. *Open Biol.* 7:170001. doi: 10.1098/rsob.170001

Li, B., Niu, Y., Ji, W., and Dong, Y. (2020). Strategies for the CRISPR-based therapeutics. *Trends Pharmacol. Sci.* 41, 55–65. doi: 10.1016/j.tips.2019.11.006

Long, C., Mcanally, J. R., Shelton, J. M., Mireault, A. A., Bassel-Duby, R., and Olson, E. N. (2014). Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 345, 1184–1188. doi: 10.1126/science.1254445

Low, H. P., Gréco, B., Tanahashi, Y., Gallant, J., Jones, S. N., Billings-Gagliardi, S., et al. (2009). Embryonic stem cell rescue of tremor and ataxia in myelin-deficient shiverer mice. *J. Neurol. Sci.* 276, 133–137. doi: 10.1016/j.jns.2008.09.037

Maeder, M. L., Stefanidakis, M., Wilson, C. J., Baral, R., Barrera, L. A., Bounoutas, G. S., et al. (2019). Development of a gene-editing approach to restore vision loss in leber congenital amaurosis type 10. *Nat. Med.* 25, 229–233. doi: 10.1038/s41591-018-0327-9

Mahmood, A., Raymond, G. V., Dubey, P., Peters, C., and Moser, H. W. (2007). Survival analysis of haematopoietic cell transplantation for childhood cerebral X-linked adrenoleukodystrophy: a comparison study. *Lancet Neurol.* 6, 687–692. doi: 10.1016/S1474-4422(07)70177-1

Makarova, K. S., and Koonin, E. V. (2015). Annotation and classification of CRISPR-Cas systems. *Methods Mol. Biol.* 1311, 47–75. doi: 10.1007/978-1-4939-2687-9_4

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., Dicarlo, J. E., et al. (2013). RNA-guided human genome engineering *via* Cas9. *Science* 339, 823–826. doi: 10.1126/science.1232033

Mariani, J. N., Zou, L., and Goldman, S. A. (2019). Human glial chimeric mice to define the role of glial pathology in human disease. *Methods Mol. Biol.* 1936, 311–331. doi: 10.1007/978-1-4939-9072-6_18

Marteyn, A., Sarrazin, N., Yan, J., Bachelin, C., Deboux, C., Santin, M. D., et al. (2016). Modulation of the innate immune response by human neural precursors prevails over oligodendrocyte progenitor remyelination to rescue a severe model of Pelizaeus-Merzbacher disease. *Stem Cells* 34, 984–996. doi: 10.1002/stem.2263

Martin, H. R., Poe, M. D., Provenzale, J. M., Kurtzberg, J., Mendizabal, A., and Escolar, M. L. (2013). Neurodevelopmental outcomes of umbilical cord blood transplantation in metachromatic leukodystrophy. *Biol. Blood Marrow Transplant.* 19, 616–624. doi: 10.1016/j.bbmt.2013.01.010

Matalon, R., Surendran, S., Rady, P. L., Quast, M. J., Campbell, G. A., Matalon, K. M., et al. (2003). Adeno-associated virus-mediated aspartoacylase gene transfer to the brain of knockout mouse for canavan disease. *Mol. Ther.* 7, 580–587. doi: 10.1016/s1525-0016(03)00066-2

Matsukawa, T., Yamamoto, T., Honda, A., Toya, T., Ishiura, H., Mitsui, J., et al. (2020). Clinical efficacy of haematopoietic stem cell transplantation for adult adrenoleukodystrophy. *Brain Commun.* 2:fcz048. doi: 10.1093/braincomms/fcz048

Mcphee, S. W., Francis, J., Janson, C. G., Serikawa, T., Hyland, K., Ong, E. O., et al. (2005). Effects of AAV-2-mediated aspartoacylase gene transfer in the tremor rat model of Canavan disease. *Mol. Brain Res.* 135, 112–121. doi: 10.1016/j.molbrainres.2004.12.007

Mendell, J. R., Al-Zaidy, S., Shell, R., Arnold, W. D., Rodino-Klapac, L. R., Prior, T. W., et al. (2017). Single-dose gene-replacement therapy for spinal muscular atrophy. *N. Engl. J. Med.* 377, 1713–1722. doi: 10.1056/NEJMoa1706198

Mendes, M. I., Gutierrez Salazar, M., Guerrero, K., Thiffault, I., Salomons, G. S., Gauquelin, L., et al. (2018). Bi-allelic Mutations in EPRS, encoding the glutamyl-prolyl-aminoacyl-tRNA synthetase, cause a hypomyelinating leukodystrophy. *Am. J. Hum. Genet.* 102, 676–684. doi: 10.1016/j.ajhg.2018.02.011

Meneghini, V., Frati, G., Sala, D., De Cicco, S., Luciani, M., Cavazzin, C., et al. (2017). Generation of human induced pluripotent stem cell-derived bona fide neural stem cells for *ex vivo* gene therapy of metachromatic leukodystrophy. *Stem Cells Transl. Med.* 6, 352–368. doi: 10.5966/sctm.2015-0414

Merheb, E., Cui, M.-H., Dubois, J. C., Branch, C. A., Gulinello, M., Shafit-Zagardo, B., et al. (2020). Defective oligodendrocyte development and function in an RNA polymerase III mutant leukodystrophic mouse. *BioRxiv* [Preprint]. doi: 10.1101/2020.12.09.418657

Michalski, J.-P., and Kothary, R. (2015). Oligodendrocytes in a Nutshell. *Front. Cell. Neurosci.* 9:340. doi: 10.3389/fncel.2015.00340

Minnerop, M., Kurzwelly, D., Wagner, H., Soehn, A. S., Reichbauer, J., Tao, F., et al. (2017). Hypomorphic mutations in POLR3A are a frequent cause of sporadic and recessive spastic ataxia. *Brain* 140, 1561–1578. doi: 10.1093/brain/awx095

Mitew, S., Hay, C. M., Peckham, H., Xiao, J., Koenning, M., and Emery, B. (2014). Mechanisms regulating the development of oligodendrocytes and central nervous system myelin. *Neuroscience* 276, 29–47. doi: 10.1016/j.neuroscience.2013.11.029

Moir, R. D., Lavados, C., Lee, J., and Willis, I. M. (2020). Functional characterization of Polr3a hypomyelinating leukodystrophy mutations in the *S. cerevisiae* homolog, RPC160. *Gene* doi: 10.1016/j.gene.2020.145259. [Online ahead of print].

Monaco, M. C., Maric, D., Bandeian, A., Leibovitch, E., Yang, W., and Major, E. O. (2012). Progenitor-derived oligodendrocyte culture system from human fetal brain. *J. Vis. Exp.* doi: 10.3791/4274

Monteys, A. M., Ebanks, S. A., Keiser, M. S., and Davidson, B. L. (2017). CRISPR/Cas9 editing of the mutant huntingtin allele *in vitro* and *in vivo*. *Mol. Ther.* 25, 12–23. doi: 10.1016/j.ymthe.2016.11.010

Mothe, A. J., and Tator, C. H. (2008). Transplanted neural stem/progenitor cells generate myelinating oligodendrocytes and Schwann cells in spinal cord demyelination and dysmyelination. *Exp. Neurol.* 213, 176–190. doi: 10.1016/j.expneurol.2008.05.024

Musolino, P. L., Lund, T. C., Pan, J., Escolar, M. L., Paker, A. M., Duncan, C. N., et al. (2014). Hematopoietic stem cell transplantation in the leukodystrophies: a

systematic review of the literature. *Neuropediatrics* 45, 169–174. doi: 10.1055/s-0033-1364179

Neofytou, E., O'brien, C. G., Couture, L. A., and Wu, J. C. (2015). Hurdles to clinical translation of human induced pluripotent stem cells. *J. Clin. Invest.* 125, 2551–2557. doi: 10.1172/JCI80575

Nunes, M. C., Roy, N. S., Keyoung, H. M., Goodman, R. R., Mckhann, G. II, Jiang, L., et al. (2003). Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat. Med.* 9, 439–447. doi: 10.1038/nm837

Ognjenović, J., and Simonović, M. (2018). Human aminoacyl-tRNA synthetases in diseases of the nervous system. *RNA Biol.* 15, 623–634. doi: 10.1080/15476286.2017.1330245

Okano, H., and Temple, S. (2009). Cell types to order: temporal specification of CNS stem cells. *Curr. Opin. Neurobiol.* 19, 112–119. doi: 10.1016/j.conb.2009.04.003

Osorio, M. J., and Goldman, S. A. (2016). Glial progenitor cell-based treatment of the childhood leukodystrophies. *Exp. Neurol.* 283, 476–488. doi: 10.1016/j.expneurol.2016.05.010

Osterman, B., Sylvain, M., Chouinard, S., and Bernard, G. (2012). Tremor-ataxia with central hypomyelination (TACH): dystonia as a new clinical feature. *Mov. Disord.* 27, 1829–1830. doi: 10.1002/mds.25270

Parikh, S., Bernard, G., Leventer, R. J., Van Der Knaap, M. S., Van Hove, J., Pizzino, A., et al. (2015). A clinical approach to the diagnosis of patients with leukodystrophies and genetic leukoencephelopathies. *Mol. Genet. Metab.* 114, 501–515. doi: 10.1016/j.ymgme.2014.12.434

Park, S. G., Schimmel, P., and Kim, S. (2008). Aminoacyl tRNA synthetases and their connections to disease. *Proc. Natl. Acad. Sci. U S A* 105, 11043–11049. doi: 10.1073/pnas.0802862105

Pelletier, F., Perrier, S., Cayami, F. K., Mirchi, A., Saikali, S., Tran, L. T., et al. (2020). Endocrine and growth abnormalities in 4H leukodystrophy caused by variants in *POLR3A*, *POLR3B* and *POLR1C*. *J. Clin. Endocrinol. Metab.* doi: 10.1210/clinem/dgaa700. [Online ahead of print].

Perrier, S., Gauquelin, L., Fallet-Bianco, C., Dishop, M. K., Michell-Robinson, M. A., Tran, L. T., et al. (2020). Expanding the phenotypic and molecular spectrum of RNA polymerase III-related leukodystrophy. *Neurol. Genet.* 6:e425. doi: 10.1212/NXG.0000000000000425

Peters, C., Charnas, L. R., Tan, Y., Ziegler, R. S., Shapiro, E. G., Defor, T., et al. (2004). Cerebral X-linked adrenoleukodystrophy: the international hematopoietic cell transplantation experience from 1982 to 1999. *Blood* 104, 881–888. doi: 10.1182/blood-2003-10-3402

Pfeiffer, S. E., Warrington, A. E., and Bansal, R. (1993). The oligodendrocyte and its many cellular processes. *Trends Cell Biol.* 3, 191–197. doi: 10.1016/0962-8924(93)90213-k

Piguet, F., Sondhi, D., Piraud, M., Fouquet, F., Hackett, N. R., Ahouansou, O., et al. (2012). Correction of brain oligodendrocytes by AAVrh.10 intracerebral gene therapy in metachromatic leukodystrophy mice. *Hum. Gene. Ther.* 23, 903–914. doi: 10.1089/hum.2012.015

Poretti, A., Meoded, A., and Fatemi, A. (2016). Diffusion tensor imaging: a biomarker of outcome in Krabbe's disease. *J. Neurosci. Res.* 94, 1108–1115. doi: 10.1002/jnr.23769

Potic, A., Brais, B., Choquet, K., Schiffmann, R., and Bernard, G. (2012). 4H syndrome with late-onset growth hormone deficiency caused by POLR3A mutations. *Arch. Neurol.* 69, 920–923. doi: 10.1001/archneurol.2011.1963

Pouwels, P. J., Vanderver, A., Bernard, G., Wolf, N. I., Dreha-Kulczewksi, S. F., Deoni, S. C., et al. (2014). Hypomyelinating leukodystrophies: translational research progress and prospects. *Ann. Neurol.* 76, 5–19. doi: 10.1002/ana.24194

Pouya, A., Satarian, L., Kiani, S., Javan, M., and Baharvand, H. (2011). Human induced pluripotent stem cells differentiation into oligodendrocyte progenitors and transplantation in a rat model of optic chiasm demyelination. *PLoS One* 6:e27925. doi: 10.1371/journal.pone.0027925

Powell, S. K., Khan, N., Parker, C. L., Samulski, R. J., Matsushima, G., Gray, S. J., et al. (2016). Characterization of a novel adeno-associated viral vector with preferential oligodendrocyte tropism. *Gene Ther.* 23, 807–814. doi: 10.1038/gt.2016.62

Priller, J., Flugel, A., Wehner, T., Boentert, M., Haas, C. A., Prinz, M., et al. (2001). Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. *Nat. Med.* 7, 1356–1361. doi: 10.1038/nm1201-1356

Privat, A., Jacque, C., Bourre, J. M., Dupouey, P., and Baumann, N. (1979). Absence of the major dense line in myelin of the mutant mouse "shiverer". *Neurosci. Lett.* 12, 107–112. doi: 10.1016/0304-3940(79)91489-7

Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A., et al. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 28, 69–80. doi: 10.1016/s0896-6273(00)00086-6

Ramsay, E. P., Abascal-Palacios, G., Daiß, J. L., King, H., Gouge, J., Pilsl, M., et al. (2020). Structure of human RNA polymerase III. *Nat. Commun.* 11:6409. doi: 10.1038/s41467-020-20262-5

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308. doi: 10.1038/nprot.2013.143

Reina, J. H., Azzouz, T. N., and Hernandez, N. (2006). Maf1, a new player in the regulation of human RNA polymerase III transcription. *PLoS One* 1:e134. doi: 10.1371/journal.pone.0000134

Ricca, A., Rufo, N., Ungari, S., Morena, F., Martino, S., Kulik, W., et al. (2015). Combined gene/cell therapies provide long-term and pervasive rescue of multiple pathological symptoms in a murine model of globoid cell leukodystrophy. *Hum. Mol. Genet.* 24, 3372–3389. doi: 10.1093/hmg/ddv086

Richards, M. R., Plummer, L., Chan, Y. M., Lippincott, M. F., Quinton, R., Kumanov, P., et al. (2017). Phenotypic spectrum of POLR3B mutations: isolated hypogonadotropic hypogonadism without neurological or dental anomalies. *J. Med. Genet.* 54, 19–25. doi: 10.1136/jmedgenet-2016-104064

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., and Hood, L. (1985). Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. *Cell* 42, 149–155. doi: 10.1016/s0092-8674(85)80110-0

Rodríguez-Rodríguez, D. R., Ramírez-Solís, R., Garza-Elizondo, M. A., Garza-Rodríguez, M. L., and Barrera-Saldaña, H. A. (2019). Genome editing: a perspective on the application of CRISPR/Cas9 to study human diseases (review). *Int. J. Mol. Med.* 43, 1559–1574. doi: 10.3892/ijmm.2019.4112

Rogers, S. (1959). Induction of arginase in rabbit epithelium by the shope rabbit papilloma virus. *Nature* 183, 1815–1816. doi: 10.1038/1831815b0

Rogers, S. (1966). Shope papilloma virus: a passenger in man and its significance to the potential control of the host genome. *Nature* 212, 1220–1222. doi: 10.1038/2121220a0

Rogers, S. (1971). Change in the structure of shope papilloma virus-induced arginase associated with mutation of the virus. *J. Exp. Med.* 134, 1442–1452. doi: 10.1084/jem.134.6.1442

Rogers, S., and Pfuderer, P. (1968). Use of viruses as carriers of added genetic information. *Nature* 219, 749–751. doi: 10.1038/219749a0

Rosenblum, D., Gutkin, A., Dammes, N., and Peer, D. (2020). Progress and challenges towards CRISPR/Cas clinical translation. *Adv. Drug Deliv. Rev.* 154-155, 176–186. doi: 10.1016/j.addr.2020.07.004

Roth, A. D., Ivanova, A., and Colman, D. R. (2006). New observations on the compact myelin proteome. *Neuron Glia Biol.* 2, 15–21. doi: 10.1017/S1740925X06000068

Saitsu, H., Osaka, H., Sasaki, M., Takanashi, J.-I., Hamada, K., Yamashita, A., et al. (2011). Mutations in POLR3A and POLR3B encoding RNA polymerase III subunits cause an autosomal-recessive hypomyelinating leukoencephalopathy. *Am. J. Hum. Genet.* 89, 644–651. doi: 10.1016/j.ajhg.2011.10.003

Sampaio-Baptista, C., and Johansen-Berg, H. (2017). White matter plasticity in the adult brain. *Neuron* 96, 1239–1251. doi: 10.1016/j.neuron.2017.11.026

San Sebastian, W., Samaranch, L., Heller, G., Kells, A. P., Bringas, J., Pivirotto, P., et al. (2013). Adeno-associated virus type 6 is retrogradely transported in the non-human primate brain. *Gene Ther.* 20, 1178–1183. doi: 10.1038/gt.2013.48

Sandoval, A. Jr., Elahi, H., and Ploski, J. E. (2020). Genetically engineering the nervous system with CRISPR-cas. *eNeuro* 7:ENEURO.0419-19.2020. doi: 10.1523/ENEURO.0419-19.2020

Sarret, C., Lemaire, J.-J., Sontheimer, A., Coste, J., Savy, N., Pereira, B., et al. (2018). Brain diffusion imaging and tractography to distinguish clinical severity of human PLP1-related disorders. *Dev. Neurosci.* 40, 301–311. doi: 10.1159/000492218

Sasaki, M., Takanashi, J.-I., Tada, H., Sakuma, H., Furushima, W., and Sato, N. (2009). Diffuse cerebral hypomyelination with cerebellar atrophy and

hypoplasia of the corpus callosum. *Brain Dev.* 31, 582–587. doi: 10.1016/j.braindev.2008.09.003

Schiffmann, R., and Van Der Knaap, M. S. (2009). Invited article: an MRI-based approach to the diagnosis of white matter disorders. *Neurology* 72, 750–759. doi: 10.1212/01.wnl.0000343049.00540.c8

Schmidt, J. L., Pizzino, A., Nicholl, J., Foley, A., Wang, Y., Rosenfeld, J. A., et al. (2020). Estimating the relative frequency of leukodystrophies and recommendations for carrier screening in the era of next-generation sequencing. *Am. J. Med. Genet. A* 182, 1906–1912. doi: 10.1002/ajmg.a.61641

Schoenemann, P. T., Sheehan, M. J., and Glotzer, L. D. (2005). Prefrontal white matter volume is disproportionately larger in humans than in other primates. *Nat. Neurosci.* 8, 242–252. doi: 10.1038/nn1394

Sessa, M., Lorioli, L., Fumagalli, F., Acquati, S., Redaelli, D., Baldoli, C., et al. (2016). Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an *ad hoc* analysis of a non-randomised, open-label, phase 1/2 trial. *Lancet* 388, 476–487. doi: 10.1016/S0140-6736(16)30374-9

Setten, R. L., Rossi, J. J., and Han, S.-P. (2019). The current state and future directions of RNAi-based therapeutics. *Nat. Rev. Drug Discov.* 18, 421–446. doi: 10.1038/s41573-019-0017-4

Sevin, C., Benraiss, A., Van Dam, D., Bonnin, D., Nagels, G., Verot, L., et al. (2006). Intracerebral adeno-associated virus-mediated gene transfer in rapidly progressive forms of metachromatic leukodystrophy. *Hum. Mol. Genet.* 15, 53–64. doi: 10.1093/hmg/ddi425

Sevin, C., Verot, L., Benraiss, A., Van Dam, D., Bonnin, D., Nagels, G., et al. (2007). Partial cure of established disease in an animal model of metachromatic leukodystrophy after intracerebral adeno-associated virus-mediated gene transfer. *Gene Ther.* 14, 405–414. doi: 10.1038/sj.gt.3302883

Shin, J. W., Kim, K.-H., Chao, M. J., Atwal, R. S., Gillis, T., Macdonald, M. E., et al. (2016). Permanent inactivation of Huntington's disease mutation by personalized allele-specific CRISPR/Cas9. *Hum. Mol. Genet.* 25, 4566–4576. doi: 10.1093/hmg/ddw286

Sim, F. J., Mcclain, C. R., Schanz, S. J., Protack, T. L., Windrem, M. S., and Goldman, S. A. (2011). CD140a identifies a population of highly myelinogenic, migration-competent and efficiently engrafting human oligodendrocyte progenitor cells. *Nat. Biotechnol.* 29, 934–941. doi: 10.1038/nbt.1972

Srivastava, S., Cohen, J. S., Vernon, H., Baranano, K., Mcclellan, R., Jamal, L., et al. (2014). Clinical whole exome sequencing in child neurology practice. *Ann. Neurol.* 76, 473–483. doi: 10.1002/ana.24251

Steenweg, M. E., Vanderver, A., Blaser, S., Bizzi, A., De Koning, T. J., Mancini, G. M., et al. (2010). Magnetic resonance imaging pattern recognition in hypomyelinating disorders. *Brain* 133, 2971–2982. doi: 10.1093/brain/awq257

Sun, J. M., and Kurtzberg, J. (2018). Cell therapy for diverse central nervous system disorders: inherited metabolic diseases and autism. *Pediatr. Res.* 83, 364–371. doi: 10.1038/pr.2017.254

Taft, R. J., Vanderver, A., Leventer, R. J., Damiani, S. A., Simons, C., Grimmond, S. M., et al. (2013). Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am. J. Hum. Genet.* 92, 774–780. doi: 10.1016/j.ajhg.2013.04.006

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872. doi: 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, 663–676. doi: 10.1016/j.cell.2006.07.024

Takanashi, J.-I., Osaka, H., Saitsu, H., Sasaki, M., Mori, H., Shibayama, H., et al. (2014). Different patterns of cerebellar abnormality and hypomyelination between POLR3A and POLR3B mutations. *Brain Dev.* 36, 259–263. doi: 10.1016/j.braindev.2013.03.006

Temple, S. (2001). The development of neural stem cells. *Nature* 414, 112–117. doi: 10.1038/35102174

Terao, Y., Saitsu, H., Segawa, M., Kondo, Y., Sakamoto, K., Matsumoto, N., et al. (2012). Diffuse central hypomyelination presenting as 4H syndrome caused by compound heterozygous mutations in POLR3A encoding the catalytic subunit of polymerase III. *J. Neurol. Sci.* 320, 102–105. doi: 10.1016/j.jns.2012.07.005

Terheggen, H. G., Lowenthal, A., Lavinha, F., Colombo, J. P., and Rogers, S. (1975). Unsuccessful trial of gene replacement in arginase deficiency. *Z. Kinderheilkd.* 119, 1–3. doi: 10.1007/BF00464689

Tétreault, M., Choquet, K., Orcesi, S., Tonduti, D., Balottin, U., Teichmann, M., et al. (2011). Recessive mutations in POLR3B, encoding the second largest subunit of pol III, cause a rare hypomyelinating leukodystrophy. *Am. J. Hum. Genet.* 89, 652–655. doi: 10.1016/j.ajhg.2011.10.006

Tetreault, M., Putorti, M. L., Thiffault, I., Sylvain, M., Venderver, A., Schiffmann, R., et al. (2012). TACH leukodystrophy: locus refinement to chromosome 10q22.3-23.1. *Can. J. Neurol. Sci.* 39, 122–123. doi: 10.1017/s0317167100022174

Thiffault, I., Wolf, N. I., Forget, D., Guerrero, K., Tran, L. T., Choquet, K., et al. (2015). Recessive mutations in POLR1C cause a leukodystrophy by impairing biogenesis of RNA polymerase III. *Nat. Commun.* 6:7623. doi: 10.1038/ncomms8623

Uchida, N., Chen, K., Dohse, M., Hansen, K. D., Dean, J., Buser, J. R., et al. (2012). Human neural stem cells induce functional myelination in mice with severe dysmyelination. *Sci. Transl. Med.* 4:155ra136. doi: 10.1126/scitranslmed.3004371

Uddin, F., Rudin, C. M., and Sen, T. (2020). CRISPR gene therapy: applications, limitations and implications for the future. *Front. Oncol.* 10:1387. doi: 10.3389/fonc.2020.01387

Van Den Broek, B. T. A., Page, K., Paviglianiti, A., Hol, J., Allewelt, H., Volt, F., et al. (2018). Early and late outcomes after cord blood transplantation for pediatric patients with inherited leukodystrophies. *Blood Adv.* 2, 49–60. doi: 10.1182/bloodadvances.2017010645

Van Der Knaap, M. S., and Bugiani, M. (2017). Leukodystrophies: a proposed classification system based on pathological changes and pathogenetic mechanisms. *Acta Neuropathol.* 134, 351–382. doi: 10.1007/s00401-017-1739-1

Van Der Knaap, M. S., and Valk, J. (2005). *Magnetic Resonance Imaging of Myelination and Myelin Disorders.* Berlin, Heidelberg: Springer.

Van Rappard, D. F., Konigs, M., Steenweg, M. E., Boelens, J. J., Oosterlaan, J., Van Der Knaap, M. S., et al. (2018). Diffusion tensor imaging in metachromatic leukodystrophy. *J. Neurol.* 265, 659–668. doi: 10.1007/s00415-018-8765-3

Vanderver, A., Prust, M., Tonduti, D., Mochel, F., Hussey, H. M., Helman, G., et al. (2015). Case definition and classification of leukodystrophies and leukoencephalopathies. *Mol. Genet. Metab.* 114, 494–500. doi: 10.1016/j.ymgme.2015.01.006

Vanderver, A., Simons, C., Helman, G., Crawford, J., Wolf, N. I., Bernard, G., et al. (2016). Whole exome sequencing in patients with white matter abnormalities. *Ann. Neurol.* 79, 1031–1037. doi: 10.1002/ana.24650

Vanderver, A., Tonduti, D., Bernard, G., Lai, J., Rossi, C., Carosso, G., et al. (2013). More than hypomyelination in Pol-III disorder. *J. Neuropathol. Exp. Neurol.* 72, 67–75. doi: 10.1097/NEN.0b013e31827c99d2

Vorländer, M. K., Baudin, F., Moir, R. D., Wetzel, R., Hagen, W. J. H., Willis, I. M., et al. (2020). Structural basis for RNA polymerase III transcription repression by Maf1. *Nat. Struct. Mol. Biol.* 27, 229–232. doi: 10.1038/s41594-020-0383-y

Vrij-Van Den Bos, S., Hol, J. A., La Piana, R., Harting, I., Vanderver, A., Barkhof, F., et al. (2017). 4H leukodystrophy: a brain magnetic resonance imaging scoring system. *Neuropediatrics* 48, 152–160. doi: 10.1055/s-0037-1599141

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., et al. (2013). Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell* 12, 252–264. doi: 10.1016/j.stem.2012.12.002

White, R. J. (2011). Transcription by RNA polymerase III: more complex than we thought. *Nat. Rev. Genet.* 12, 459–463. doi: 10.1038/nrg3001

Willis, I. M., and Moir, R. D. (2018). Signaling to and from the RNA polymerase III transcription and processing machinery. *Annu. Rev. Biochem.* 87, 75–100. doi: 10.1146/annurev-biochem-062917-012624

Windrem, M. S., Nunes, M. C., Rashbaum, W. K., Schwartz, T. H., Goodman, R. A., Mckhann, G., et al. (2004). Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. *Nat. Med.* 10, 93–97. doi: 10.1038/nm974

Windrem, M. S., Schanz, S. J., Guo, M., Tian, G.-F., Washco, V., Stanwood, N., et al. (2008). Neonatal chimerization with human glial progenitor cells can both remyelinate and rescue the otherwise lethally hypomyelinated shiverer mouse. *Cell Stem Cell* 2, 553–565. doi: 10.1016/j.stem.2008.03.020

Windrem, M. S., Schanz, S. J., Morrow, C., Munir, J., Chandler-Militello, D., Wang, S., et al. (2014). A competitive advantage by neonatally engrafted

human glial progenitors yields mice whose brains are chimeric for human glia. *J. Neurosci.* 34, 16153–16161. doi: 10.1523/JNEUROSCI.1510-14.2014

Windrem, M. S., Schanz, S. J., Zou, L., Chandler-Militello, D., Kuypers, N. J., Nedergaard, M., et al. (2020). Human glial progenitor cells effectively remyelinate the demyelinated adult brain. *Cell Rep.* 31:107658. doi: 10.1016/j.celrep.2020.107658

Wirth, T., Parker, N., and Ylä-Herttuala, S. (2013). History of gene therapy. *Gene* 525, 162–169. doi: 10.1016/j.gene.2013.03.137

Wolf, N. I., Harting, I., Innes, A. M., Patzer, S., Zeitler, P., Schneider, A., et al. (2007). Ataxia, delayed dentition and hypomyelination: a novel leukoencephalopathy. *Neuropediatrics* 38, 64–70. doi: 10.1055/s-2007-985137

Wolf, N. I., Salomons, G. S., Rodenburg, R. J., Pouwels, P. J., Schieving, J. H., Derks, T. G., et al. (2014a). Mutations in RARS cause hypomyelination. *Ann. Neurol.* 76, 134–139. doi: 10.1002/ana.24167

Wolf, N. I., ffrench-Constant, C., and van der Knaap, M. S. (2020). Hypomyelinating leukodystrophies - unravelling myelin biology. *Nat. Rev. Neurol.* doi: 10.1038/s41582-020-00432-1. [Online ahead of print].

Wolff, A., Koch, M. J., Benzinger, S., Van Waes, H., Wolf, N. I., Boltshauser, E., et al. (2010). Rare dental peculiarities associated with the hypomyelinating leukoencephalopathy 4H syndrome/ADDH. *Pediatr. Dent.* 32, 386–392. Available online at: https://aapd.publisher.ingentaconnect.com/contentone/aapd/pd/2010/00000032/00000005/art00003#

Wolf, N. I., Vanderver, A., Van Spaendonk, R. M., Schiffmann, R., Brais, B., Bugiani, M., et al. (2014b). Clinical spectrum of 4H leukodystrophy caused by POLR3A and POLR3B mutations. *Neurology* 83, 1898–1905. doi: 10.1212/WNL.0000000000001002

Wolpert, L. (1994). Positional information and pattern formation in development. *Dev. Genet.* 15, 485–490. doi: 10.1002/dvg.1020150607

Wright, M. D., Poe, M. D., Derenzo, A., Haldal, S., and Escolar, M. L. (2017). Developmental outcomes of cord blood transplantation for Krabbe disease: A 15-year study. *Neurology* 89, 1365–1372. doi: 10.1212/WNL.0000000000004418

Wu, C.-C., Herzog, F., Jennebach, S., Lin, Y.-C., Pai, C.-Y., Aebersold, R., et al. (2012). RNA polymerase III subunit architecture and implications for open promoter complex formation. *Proc. Natl. Acad. Sci. U S A* 109, 19232–19237. doi: 10.1073/pnas.1211665109

Wu, S., Bai, Z., Dong, X., Yang, D., Chen, H., Hua, J., et al. (2019). Novel mutations of the POLR3A gene caused POLR3-related leukodystrophy in a chinese family: a case report. *BMC Pediatr.* 19:289. doi: 10.1186/s12887-019-1656-7

Yandava, B. D., Billinghurst, L. L., and Snyder, E. Y. (1999). "Global" cell replacement is feasible *via* neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. *Proc. Natl. Acad. Sci. U S A* 96, 7029–7034. doi: 10.1073/pnas.96.12.7029

Yang, L., Mali, P., Kim-Kiselak, C., and Church, G. (2014). CRISPR-Cas-mediated targeted genome editing in human cells. *Methods Mol. Biol.* 1114, 245–267. doi: 10.1007/978-1-62703-761-7_16

Yang, S., Chang, R., Yang, H., Zhao, T., Hong, Y., Kong, H. E., et al. (2017). CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease. *J. Clin. Invest.* 127, 2719–2724. doi: 10.1172/JCI92087

Yin, H., Xue, W., Chen, S., Bogorad, R. L., Benedetti, E., Grompe, M., et al. (2014). Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat. Biotechnol.* 32, 551–553. doi: 10.1038/nbt.2884

Zerah, M., Piguet, F., Colle, M.-A., Raoul, S., Deschamps, J.-Y., Deniaud, J., et al. (2015). Intracerebral gene therapy using aavrh.10-harsa recombinant vector to treat patients with early-onset forms of metachromatic leukodystrophy: preclinical feasibility and safety assessments in nonhuman primates. *Hum. Gene Ther. Clin. Dev.* 26, 113–124. doi: 10.1089/humc.2014.139

Zhao, X., and Moore, D. L. (2018). Neural stem cells: developmental mechanisms and disease modeling. *Cell Tissue Res.* 371, 1–6. doi: 10.1007/s00441-017-2738-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 17 March 2021
doi: 10.3389/fncel.2021.631548

Rapid and Efficient Generation of Myelinating Human Oligodendrocytes in Organoids

*Mohammed R. Shaker[1], Giovanni Pietrogrande[1], Sally Martin[1,2], Ju-Hyun Lee[3], Woong Sun[3] and Ernst J. Wolvetang[1]**

[1] Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, QLD, Australia, [2] School of Biomedical Sciences, The University of Queensland, Brisbane, QLD, Australia, [3] Department of Anatomy, Brain Korea 21 Plus Program for Biomedical Science, Korea University College of Medicine, Seoul, South Korea

Human stem cell derived brain organoids are increasingly gaining attention as an ideal model system for investigating neurological diseases, particularly those that involve myelination defects. However, current protocols for generating brain organoids with sufficiently mature oligodendrocytes that deposit myelin on endogenously produced neurons are lengthy and complicated. Taking advantage of a human pluripotent stem cell line that reports on SOX10 expression, we developed a protocol that involves a 42 day exposure of neuroectoderm-derived organoids to a cocktail of growth factors and small molecules that collectively foster oligodendrocyte specification and survival. Importantly, the resulting day 42 brain organoids contain both myelinating oligodendrocytes, cortical neuronal cells and astrocytes. These oligodendrocyte brain organoids therefore constitute a valuable and tractable platform for functional neurogenomics and drug screening for white matter diseases.

Keywords: oligodendrocyte, myelination, induced pluripotent stem cells, organoid, reporter cell line

OPEN ACCESS

Edited by:
Dominic J. Gessler, University of Minnesota Twin Cities, United States

Reviewed by:
Fernando C. Ortiz, Autonomous University of Chile, Chile
Adelaide Fernandes, University of Lisbon, Portugal

***Correspondence:**
Ernst J. Wolvetang
e.wolvetang@uq.edu.au

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *20 November 2020*
Accepted: *23 February 2021*
Published: *17 March 2021*

Citation:
Shaker MR, Pietrogrande G, Martin S, Lee J-H, Sun W and Wolvetang EJ (2021) Rapid and Efficient Generation of Myelinating Human Oligodendrocytes in Organoids. Front. Cell. Neurosci. 15:631548. doi: 10.3389/fncel.2021.631548

INTRODUCTION

Oligodendrocytes (OL) are cells of the central nervous system (CNS) that generate the multilayered myelin membrane sheath around vertebrate axons, enhancing the propagation of action potentials. OL provide metabolic support to neurons and are important for maintaining axonal integrity (Fünfschilling et al., 2012). In multiple sclerosis, OL generation, turnover and dysfunction have been linked to demyelination, axonal damage and disease progression (Domingues et al., 2016). Similarly, a loss of myelination and a consequent breakdown of OL-axon communication are increasingly linked to neurodegenerative diseases, including amyotrophic lateral sclerosis (Salameh et al., 2015), Parkinson (Ferrer, 2018), and Alzheimer disease (McKenzie et al., 2017). Hypomyelination also occurs in a range of neurodevelopmental diseases such as Down syndrome (Reiche et al., 2019) and is typical of leukodystrophies (Wasseff and Scherer, 2015). Therapies aimed at modulating OL function and survival, as well as delivery of exogenous healthy OL progenitors (OPCs) are promising avenues for restoring axonal integrity and neurological function.

Our current understanding of OL development and function is predominantly based on rodent studies, and to date therapies for treating OL dysfunction have not translated well to human clinical practice (Mak et al., 2014). Recent advances in human brain organoid systems provide an opportunity to study human OL function in health and disease under conditions that more closely mimic the *in vivo* 3D make-up of the brain (Fatehullah et al., 2016). OL fail

to be appropriately and efficiently specified in brain organoids subjected to conventional cortical differentiation conditions (Quadrato et al., 2016), and only recently relatively robust induction of oligodendrogenesis and myelination was achieved in organoids, albeit only after a prolonged glial differentiation protocol for 210 days (Madhavan et al., 2018). Since such extended differentiation timelines make experimentation difficult and impractical, Kim et al. pioneered a protocol that accelerates the specification of myelinating OL in organoids and shortened the protocol to 105 days of culture (Kim et al., 2019). Despite this advance rapid high throughput investigation of demyelinating diseases and screening of therapeutics remains cumbersome, even with these reduced timelines. To address this bottleneck, we created a hiPSC line that reports on the expression of the early oligodendroglial gene SOX10 and used this cell line to develop a facile and rapid 42 day protocol for the generation of human brain organoids that contain OL. We show that these OL brain organoids contain OL that myelinate endogenously co-specified cortical neurons and support astrocyte differentiation. Therefore, our efficient and rapid protocol for generating OL brain organoids represents a promising and versatile platform for modeling CNS diseases associated with hypo-myelination or demyelination as well as for drug screening.

RESULTS

Generation of Human Brain Organoids With Oligodendrocyte and Neural Progenitors

SOX10 expression in the developing CNS is restricted to oligodendroglia (García-León et al., 2018). To track the emergence of oligodendroglia in the developing organoids, we utilized the WTC hiPSCs line (UCSFi001-A) that was engineered to contain an IRES-mMaple fluorescent protein locus (McEvoy et al., 2012) 125 base pairs downstream of the translation stop site in the 3'-UTR of the SOX10 gene, similar to the previously reported Nano-lantern line (Horikiri et al., 2017). To generate brain organoids containing multiple CNS cell lineages, we first generated human neuroectodermal (hNEct) cells by subjecting the hiPSCs for 3 days to a dual SMAD inhibition protocol (Shaker et al., 2020a). These hNEct cells were next lifted off the culture plates and allowed to aggregate in low attachment dishes (**Supplementary Video 1**) while stimulating their growth with bFGF for 4 days (**Figure 1A**). Successful formation of the neuroectodermal layer at day 7 was evidenced by bright smooth edges of the spheroids (**Figure 1B**), and these spheroids were next embedded in Matrigel. From as early as day 3, we exposed the organoids to a cocktail of growth factors and small molecules (**Figure 1A**), that included thyroid hormone T3, neurotrophin NT3, hepatocytes growth factor (HGF), insulin growth factor (IGF), and platelet derived growth factor (PDGF-AA) to promote differentiation toward the oligodendroglial lineage and to foster the proliferation of newly generated OPCs, as well as B27 without vitamin A and cAMP to drive the maturation of OPCs to myelinating OL (Douvaras and Fossati, 2015; Zhang et al., 2019). We further added biotin and BME to the OL differentiation media to increase the survival of the maturing OL. In addition, we added N2, insulin, and non-essential amino acids to promote the co-differentiation of neurons and astrocytes (**Figure 1A**).

To identify the proliferation and differentiation phases of OL brain organoid development, we measured the diameter of the organoids over time in culture. OL brain organoids gradually increased in size during the proliferative phase between day 7 and 35. The organoids then entered the differentiation phase where they maintained an average size of 1.5 mm until day 56 in culture (**Figure 1C**). In agreement with these data, immunostaining for the proliferation marker KI67 revealed 40% and 18% of cells expressed KI67 on day 7 and day 14, respectively, followed by a significant drop to about 5% by day 42 and day 56 (**Figures 1D,E**). Live imaging of OL brain organoids derived from the SOX10 reporter iPSCs subjected to our differentiation protocol revealed that SOX10 expression can be detected as early as day 7, as indicated by the presence of cells expressing photo-convertible mMaple (**Figure 1B**, RFP + UV in red, white arrows). Interestingly, we found <1% of SOX10+ cells expressed KI67 at days 7 and 14 which significantly dropped to <0.5% at days 42 and 56 (**Figure 1F**), consistent with recent single-cell transcriptomic analyses of human OL that revealed a cluster of OPCs are in s-phase and enriched for genes associated with cell cycle and division (Chamling et al., 2021).

The abundance of mMaple expressing cells substantially increased by day 14 and immunofluorescent labeling of organoid sections confirmed that mMaple expressing cells were labeled with SOX10 antibodies (**Figure 1G**, white arrows). We detected clusters of mMaple/SOX10 expressing cells at the periphery of the organoids by day 14, that subsequently dispersed over the periphery of the organoids by day 42 (**Figure 1G**). At this timepoint the mMaple labeled cells displayed a predominantly bipolar appearance with long processes, suggestive of OL maturation (**Figure 1B**, yellow box). This cell distribution pattern did not change with further maturation in culture (**Supplementary Figure 1A**).

To assess the abundance of oligodendroglia in the organoids, we quantified SOX10 expressing cells at day 14 and found ∼11% of OL brain organoid cells expressed SOX10 and that this did not significantly increase by day 42 (**Figures 1G,H**). In agreement with these data, we detected robust upregulation of *SOX10* mRNA by day 14 and no significant increase by day 42 (**Figure 1I**). We next assessed whether our optimized media cocktail would allow the co-differentiation of neural stem cells (NSCs) from hNEct cells. Our data show that 45% of cells in the OL brain organoids expressed the NSCs marker PAX6 at day 14 (**Figures 1J,K**), and that this number dropped to 8% in day 42 OL brain organoids (**Figure 1K**), in agreement with the notion that these organoids had entered the differentiation phase. A similar pattern was observed for *PAX6* mRNA levels (**Figure 1L**). Importantly, OL brain organoids exhibited a significant upregulation of the forebrain marker genes *EMX2* and *OTX1* between day 14 and day 42 (**Supplementary Figure 1B**), indicative of forebrain cortical neuron differentiation in the OL brain organoids. Collectively these data led us to conclude that our OL brain organoid differentiation protocol promotes the rapid specification of OPCs as well as NSCs with the potential to generate forebrain OL and cortical neurons, respectively.

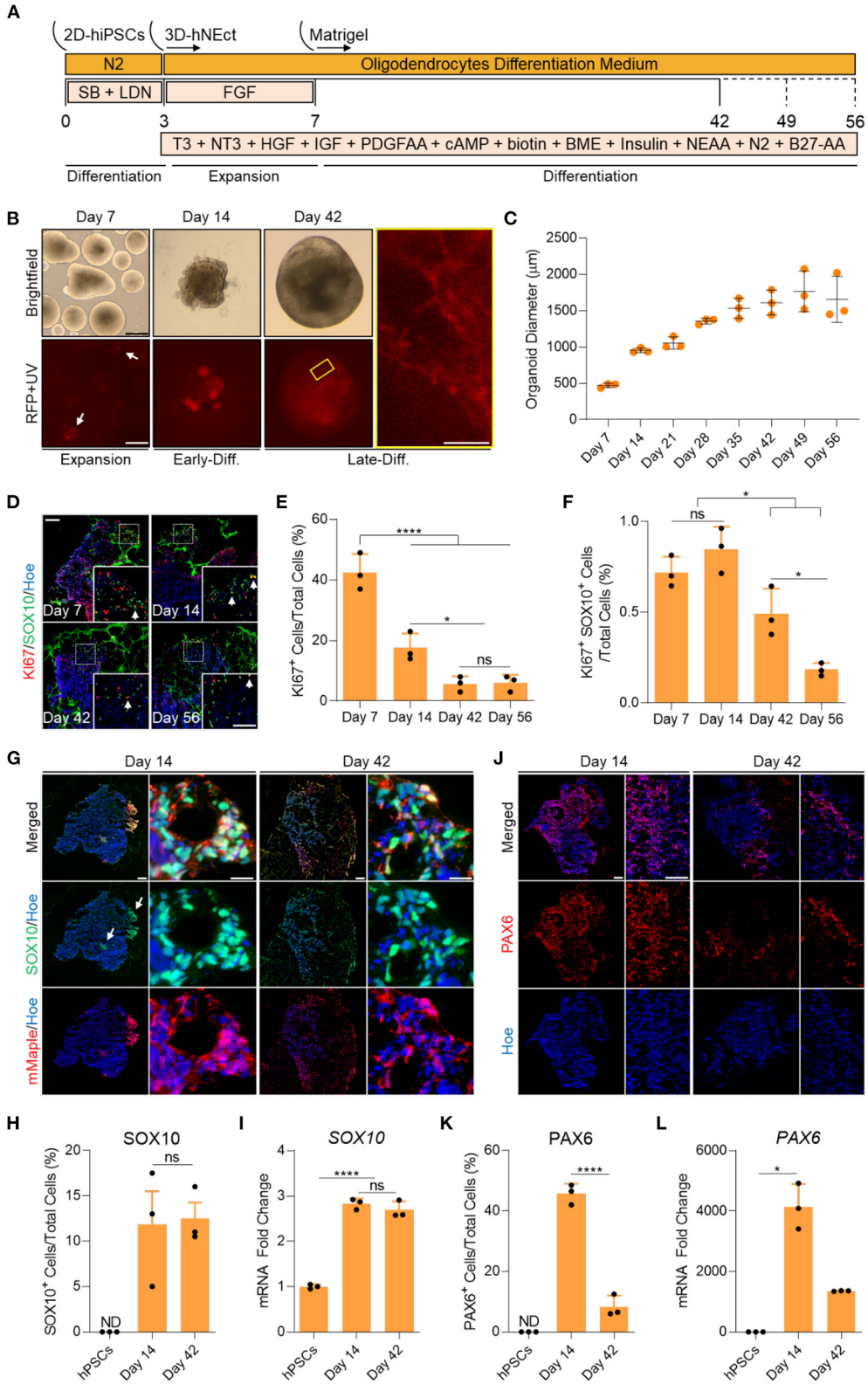


FIGURE 1 | Generation of human brain organoids containing OPCs and NSCs. **(A)** Schematic representation of the strategy used to generate OL brain organoids from hiPSCs. **(B)** Images showing the developmental stages of OL brain organoids over time in culture *in vitro* under brightfield, and red fluorescence protein (RFP).

(Continued)

FIGURE 1 | RFP indicates mMaple after UV photoconversion (emission 600–630 nm). Yellow box is a magnified image of a day 42 OL brain organoid. Scale bar is 125 μm, scale bar of magnified image is 15 μm. A total of 24 organoids was analyzed. **(C)** Graph showing the growth of OL brain organoids (based on the average diameter) at different stages of *in vitro* culture. Data are presented as the mean ± standard deviation ($n = 3$). The total number of analyzed organoids is 88. **(D)** Representative images of immunostained sectioned organoids at days 7, 14, 42, and 56 showing protein expression of KI67 (Red) and SOX10 (Green). All sections were counterstained with Hoechst 33342 (Blue). Dotted boxes indicate the magnified images. White arrows indicate cells co-expressing KI67 and SOX10. Scale bar = 200 and 100 μm in magnified images. A total of 48 organoids was analyzed. **(E)** Quantification of the percentage of cells expressing the proliferation marker (KI67) relative to the total number of cells per sample. Data are presented as mean ± standard deviation. Number of independent experiments = 3. $^{*}P < 0.05$, $^{****}P < 0.001$ via One Way ANOVA. NS is not significant. Normality Test (Shapiro-Wilk) Passed ($P = 0.834$). A total of 48 organoids was analyzed. **(F)** Quantification of the percentage of cell co-expressing the proliferation marker (KI67-Red) and OPCs (SOX10-Green) relative to the total number of cells per sample. Data are presented as mean ± standard deviation. Number of independent experiments = 3. $^{*}P < 0.05$ via One Way ANOVA. NS is not significant. Normality Test (Shapiro-Wilk) Passed ($P = 0.879$). A total of 48 organoids was analyzed. **(G)** Representative images of immunostained day 14 and 42 organoid sections showing the protein expression of SOX10 (Green) and mMaple (Red). All sections were counterstained with Hoechst 33342 (Blue). Scale bar = 200 μm. Scale bar of magnified images 25 μm. A total of 24 organoids was analyzed. **(H)** Quantification of the percentage of total $SOX10^{+}$ cells relative to the total number of cells in organoid sections (six sections, three biological replicates). Data are presented as the mean ± standard deviation. NS indicates the non-significant differences between day 35 and day 56 via One Way ANOVA. ND indicates not detected. Normality Test (Shapiro-Wilk) Passed ($P = 0.666$). A total of 24 organoids was analyzed. **(I)** qRT-PCR of OPC marker (*SOX10*). All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSC values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed. $^{****}P < 0.0001$ via One Way ANOVA. Normality Test (Shapiro-Wilk) Passed ($P = 0.542$). **(J)** Representative images of immunostained day 14 and 42 organoid sections showing the protein expression of PAX6 (Red). All sections were counterstained with Hoechst 33342 (Blue). Scale bar = 200 μm. scale bar of magnified images 75 μm. A total of 24 organoids was analyzed. **(K)** Quantification of the percentage of total $PAX6^{+}$ cells relative to the total number of cells per sample (six sections, three biological replicates). Data are presented as mean ± standard deviation. Number of independent experiments = 3. $^{****}P < 0.0001$ via One Way ANOVA. A total of 24 organoids was analyzed. Normality Test (Shapiro-Wilk) Passed ($P = 0.808$). **(L)** qRT-PCR of NSCs marker (*PAX6*). All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSC values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed. $^{*}P < 0.05$ via Kruskal–Wallis One Way Analysis of Variance on Ranks. Normality Test (Shapiro-Wilk) Failed ($P < 0.05$).

Progressive Maturation of Oligodendrocytes in OL Brain Organoids

To assess when and to what extent SOX10 expressing cells differentiate into pre-OL, immature OL, and mature myelinating OL, we first investigated the mRNA expression of several genes that mark these different stages of oligodendrogenesis. We found that *O4* expression, which marks pre- and immature-OL, was significantly increased by day 21 of differentiation (**Supplementary Figure 2**). By day 42, OL brain organoids showed significantly increased expression of *GALC* and *MBP,* genes that are expressed in mature and myelinating OL, respectively (**Supplementary Figure 2**). We also examined the expression of *OLIG2*, *NG2* and *NKX2.1* which are known to be expressed in OPCs and regulate oligodendrogenesis (Kessaris et al., 2006; Rivers et al., 2008). Interestingly, we found a significant induction of *OLIG2* and *NG2* at day 42 of differentiation (**Supplementary Figure 2**), while *NKX2.1*, a transcription factor known to regulate OL lineage specification, was significantly expressed at day 21 and persisted until day 42 of differentiation (**Supplementary Figure 2**).

Immunofluorescent staining of organoid sections confirmed the expression of O4, CNPase and MBP protein in these organoids (**Figure 2A**), and automated quantification of these markers in day 42 OL brain organoids revealed that 20% of total cells expressed O4 (**Figure 2B**), and that 40.7% and 39.4% of all cells were stained with CNPase and MBP antibodies, respectively (**Figure 2B**). Among these, only 8% of O4-labeled cells, 12% of CNPase-labeled cells and 10% of MBP-labeled cells expressed SOX10 (**Figure 2C**). Around 90% of all $SOX10^{+}$ cells co-expressed O4, CNPase or MBP, indicating that the vast majority of SOX10 expressing cells were differentiated into the oligodendrocyte lineage (**Figure 2D**). We speculate that the small number of SOX10 expressing cells that do not express these markers are either immature OPC progenitors or cells of the neural crest lineage that are co-specified.

Multiple single-cell transcriptomic studies of human OL revealed that not all oligodendroglial cells express SOX10 (Chamling et al., 2021), and that other neural cells can act as an additional source of human cortical OPCs (Huang et al., 2020), in agreement with the observation that not all OL generated in our OL brain organoids express SOX10 (**Figure 2C**). To further examine this possibility, we quantified the expression of several genes that are expressed in SOX10 negative OPCs progeny as well as genes expressed in the SOX10 positive OPCs population, as identified by Chamling et al. (2021). Interestingly, our qPCR analysis revealed that *RTN1*, *ELAVL4*, and *HS3ST1,* genes expressed in the SOX10 negative OPCs population, commenced at day 14 and persisted throughout subsequent maturation steps, whereas *CRABP1* and *CENPJ* were only expressed at day 14 and subsequently downregulated when mature OL are specified (**Figure 2E**). Furthermore, we also validated the presence of oligodendroglial genes associated with SOX10 positive OPCs (**Figure 2F**). Collectively, these data first indicate that the vast majority of OPCs successfully differentiated into mature OL within 42 days of OL brain organoid differentiation and that OL brain organoids contain both SOX10 positive and SOX10 negative OPCs that both contribute to the generation of OL.

OL Brain Organoids Contain Astrocytes and Oligodendrocytes That Myelinate Endogenously Produced Neurons

CNS myelination *in vivo* requires mature oligodendrocytes, astrocytes and sufficiently mature neurons (Simons and Nave, 2015). To assess whether the OL brain organoids generated with our protocol would be able to recapitulate this process *in vitro,* we first assessed the specification and maturation of cortical neurons.

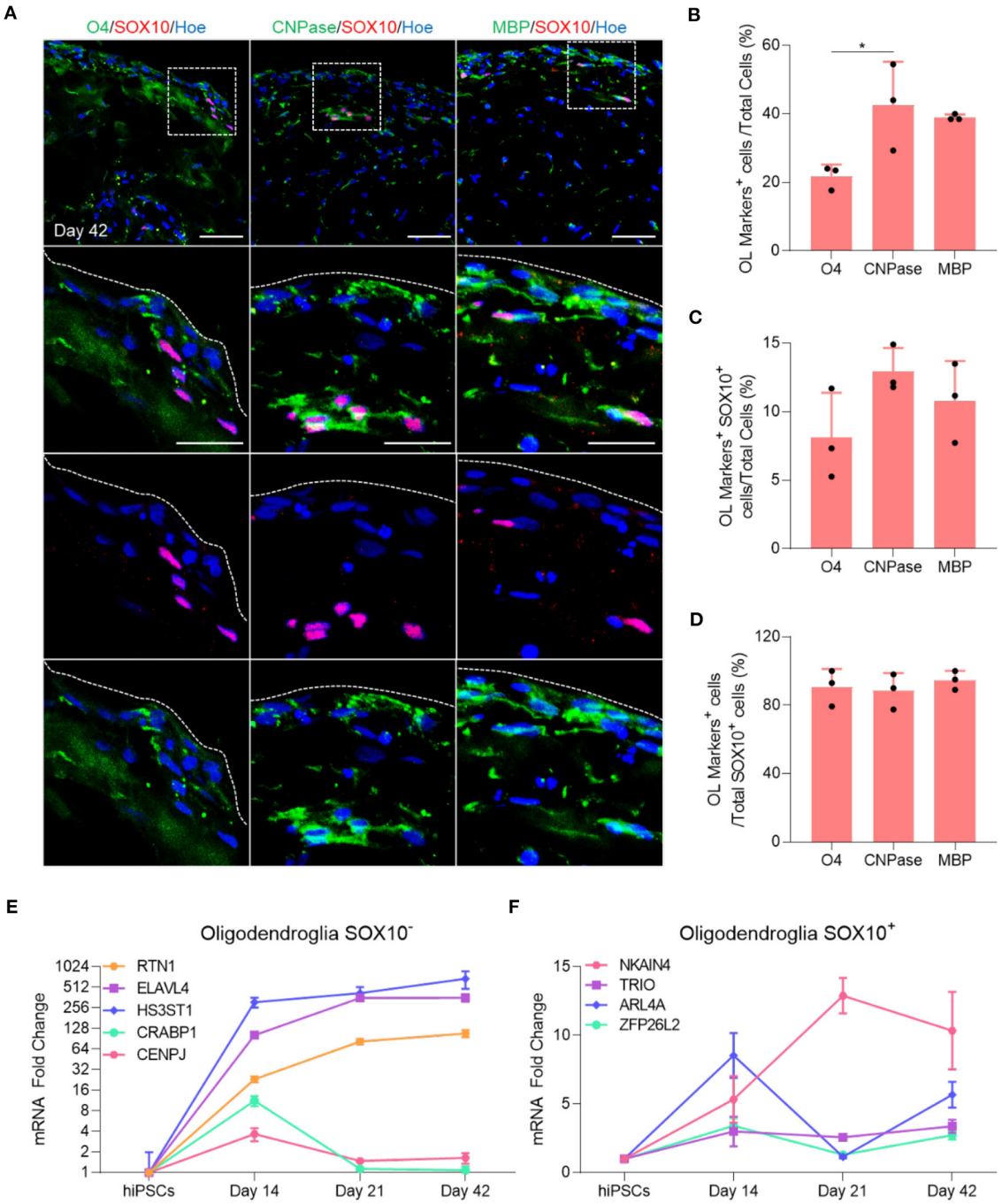


FIGURE 2 | Maturation of oligodendrocytes in brain organoids. **(A)** Representative images of immunostained day 42 organoid sections showing the protein expression of SOX10 (Red) and the oligodendrocytes markers of different stages of maturation labeled with O4 (Green), CNPase (Green), and MBP (Green). All sections were counterstained with Hoechst 33342 (Blue). Dotted boxes indicate the magnified area. Dotted lines indicate the peripheral boundary of the organoid. Scale bar = 50 μm. A total of 48 organoids was analyzed. **(B)** Quantification of the percentage of total oligodendrocyte cells labeled with O4, CNPase and MBP relative to the total number of cells in organoid sections (seven sections, three biological replicates). Data are presented as the mean ± standard deviation. Number of independent experiments = 3. *$P < 0.05$ via One Way ANOVA. A total of 48 organoids was analyzed. Normality Test (Shapiro-Wilk) Passed ($P = 0.179$). **(C)** Quantification of the percentage of total SOX10 positive oligodendrocyte cells that are labeled with O4, CNPase and MBP relative to the total number of cells in organoid sections (seven sections, three biological replicates). Data are presented as the mean ± standard deviation. Number of independent experiments = 3. A total of 48 organoids was analyzed. Normality Test (Shapiro-Wilk) Passed ($P = 0.584$). **(D)** Quantification of the percentage of total SOX10$^+$ oligodendrocyte cells labeled with O4, CNPase and MBP relative to the total number of SOX10$^+$ cells in organoid sections (seven sections, three biological replicates). Data are presented as the mean ± standard deviation. Number of independent experiments = 3. Normality Test (Shapiro-Wilk) Passed ($P = 0.372$). **(E)** qRT-PCR of several markers expressed in

(Continued)

FIGURE 2 | SOX10 negative oligodendroglial cells. All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSC values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed. Y axis is adjusted to log 2 value. **(F)** qRT-PCR of several markers expressed in SOX10 positive oligodendroglial cells. All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSC values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed.

Automated quantification of the mature post-mitotic neuronal marker NEUN in sections from OL brain organoids cultured for increasing amounts of time (**Figure 3A**) revealed a progressive increase in the number of NEUN positive cells relative to the total cell number from 8% at day 14, 16% at day 28 to 29% at 42 day (**Figure 3B**). Immunostaining with TUJ1 and MAP2 antibodies revealed labeling of axonal projections and dendrites of cells that were mainly localized to the periphery of OL brain organoid where the majority of mature OL were also localized (**Figure 3C**). We next assessed whether mature OL would interact with these neurons and engage in myelin deposition. Immunostaining of sectioned OL brain organoids with MBP and TUJ1 revealed that MBP is detected in close proximity to axons, and orthogonally cut sections further demonstrated the sheath-like morphology of MBP encompassing axons, indicative of myelination (**Figure 3D**). To further verify the presence of myelination in these organoids, we next performed transmission electron microscopy on OL brain organoid sections at day 42 and found a successful formation of compacting myelin sheaths that encircled neuronal axons (**Figure 3E**). Unlike spinal cord or optic nerve, the axons within the organoids are not aligned and sectioning invariable resulted in oblique cross-sections of the myelinated axons (**Supplementary Figure 3A**). Quantification of the number of myelin lamellae per axon over 10 randomly chosen axons of OL brain organoid at day 42 revealed a broad variation in myelin lamellae between axons (**Supplementary Figure 3B**), suggesting progressive events of early myelination in OL brain organoids.

Finally, we assessed the specification of astrocytes in the organoids cultured for progressive periods of time by immunostaining for glial fibrillary acidic protein (GFAP) and via qPCR analysis, revealing upregulation of GFAP mRNA from day 28 onwards (**Figure 3F**) and the emergence of immature GFAP positive cells at day 28. The number of GFAP expressing cells increased by day 42 and these cells displayed a more mature appearance (**Figure 3G**). We conclude that OL brain organoids contain OL capable of myelinating endogenously produced cortical neurons and contain progressively maturing astrocytes.

DISCUSSION

Myelin is produced by OL and is essential for enabling saltatory nerve conduction as well as normal brain function, and myelin disturbance is therefore often associated with neuropathologies and neurodevelopmental disorders. For instance, loss of myelin is a pivotal event in autoimmune disorders such as multiple sclerosis, whereas a failure in myelin formation occurs in leukodystrophies, such as in Pelizaeus-Merzbacher disease (Garbern et al., 1999). OL defects in specific leukodystrophies may be due to defects in OL differentiation, in altered MBP production, or impairment of the mechanism for myelin deposition (Nevin et al., 2017). An increasing body of literature suggests that effective myelination or inappropriate demyelination defects are not only due to defects in oligodendrocytes but can also be caused by suboptimal function of other brain cell types. For example, astrocytes are required for supporting myelination, microglia can trigger demyelination, and neurons themselves need to present appropriate signaling molecules and mechanical cues to trigger axon myelination (Matejuk and Ransohoff, 2020). It is not unlikely that the selective loss or deposition of myelin in specific brain regions, such as occurs in Hypomyelination with Brain Stem and Spinal Cord Involvement and Leg Spasticity (HBSL) (Taft et al., 2013; Fröhlich et al., 2018), is driven by a dysfunction of multiple brain cell types. For these reasons brain organoids in which these critical cooperative cell types are appropriately produced are valuable tools for studying (de)-myelination in health and disease states and constitute potential drug testing platforms for diseases that affect myelination, such as Pelizaeus-Merzbacher disease (Elitt et al., 2018; Nobuta et al., 2019) and HBLS.

To monitor the specification of putative oligodendroglia in brain organoids in real time we engineered the WTC human iPSC cell line to express the photo-convertible fluorophore mMaple under the control of the endogenous SOX10 regulatory system through knock-in into the 3'-UTR, since SOX10 is a pivotal transcription factor that drives oligodendroglial development. Using this cell line, we tested and optimized the composition of a single medium cocktail that is capable of inducing the specification of SOX10 positive OPCs in only 4 days following hNEct differentiation. Importantly, we show that this medium composition allows concurrent cortical development, astrocytes differentiation and neuronal maturation. Our data show that SOX10 expressing OPCs in the OL brain organoids predominantly differentiate into cells of the oligodendrocyte lineage, as indicated by the co-expression of MBP, CNPase and O4 in these cells, and that these cells associate with and deposit myelin on endogenous neurons, as indicated by co-localization of MBP with the neuronal marker TUJ1. In our protocol, the exposure of hNEct to a cocktail of growth factor allowed the emergence of mMaple/SOX10 clusters of cells which later dispersed around the OL brain organoids. Immunostaining of sectioned organoids revealed the peripheral distribution of oligodendroglial cells. We cannot exclude that this peripheral location is, at least in part, attributable to a lack of nutrition and oxygen penetration into the core of the organoids (Mansour et al., 2018), as human OL are known to be particularly sensitive to hypoxic conditions (Gautier et al., 2015).

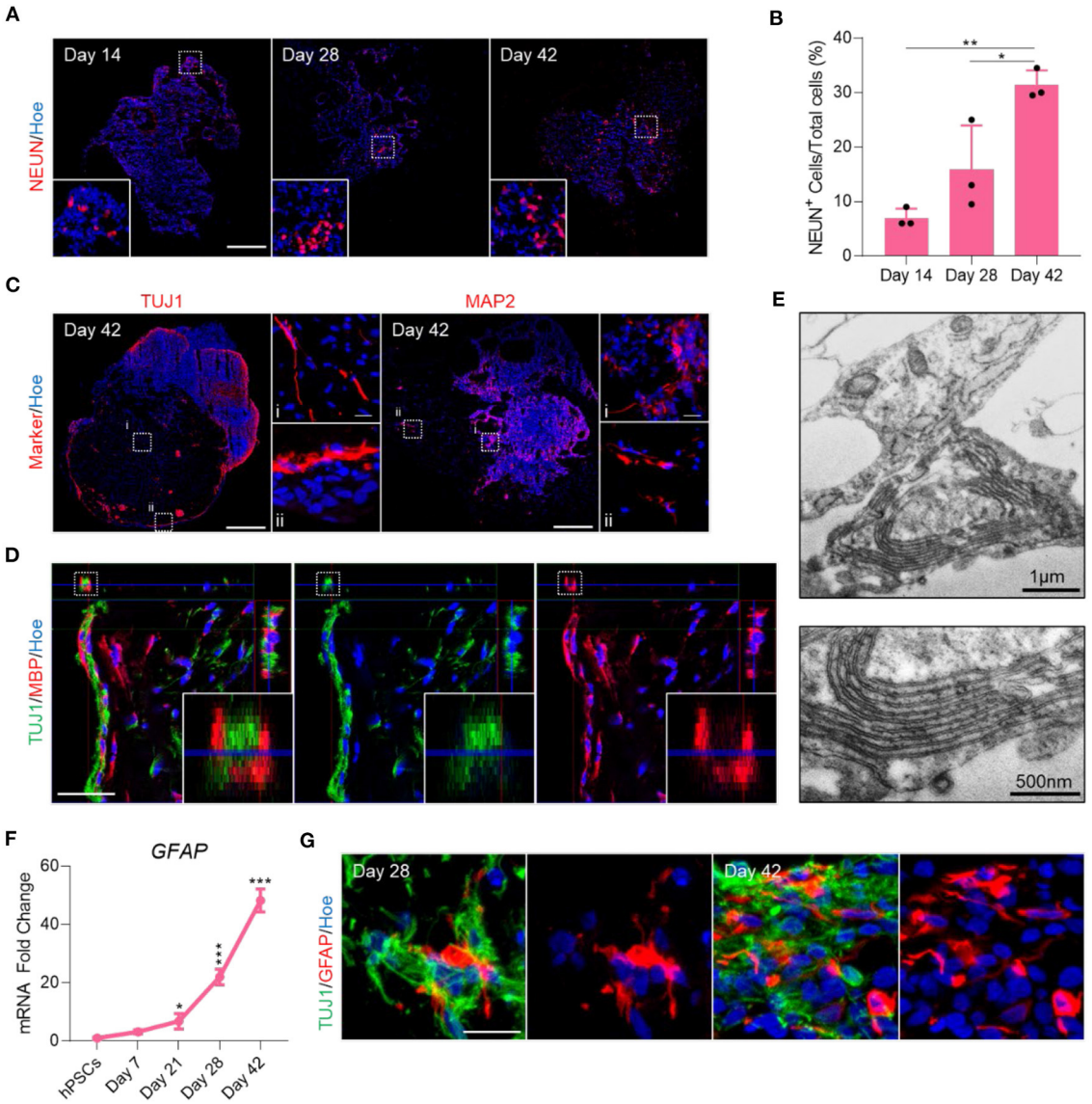


FIGURE 3 | Endogenous generation of myelinated neurons and astrocytes in OL brain organoids. **(A)** Representative images of immunostained day 14, 28, and 42 organoid sections showing the protein expression of NEUN (Red). All sections were counterstained with Hoechst 33342 (Blue). Scale bar = 200 µm. A total of 50 organoids was analyzed. **(B)** Quantification of the percentage of total NEUN$^+$ cells relative to the total number of cells in organoid sections (six sections, three biological replicates). Data are presented as the mean ± standard deviation. Number of independent experiments = 3. *$P < 0.05$. **$P < 0.01$ via One Way ANOVA. A total of 45 organoids was analyzed. Normality Test (Shapiro-Wilk) Passed ($P = 0.372$). **(C)** Representative images of immunostained day 42 organoid sections showing the protein expression of TUJ1 and MAP2 (Red). All sections were counterstained with Hoechst 33342 (Blue). Dotted boxes indicate the magnified images. Scale bar = 200 µm, magnified scale bar = 17 µm. A total of 50 organoids was analyzed. **(D)** Orthogonally cut images of immunostained day 42 organoid sections showing expression of TUJ1 (Green) and MBP (Red) protein. Dotted boxes indicate the magnified images. All sections were counterstained with Hoechst 33342 (Blue). Dotted boxes indicate the magnified images. Scale bar = 40 µm. **(E)** Transmission electron microscopy of OL brain organoids at day 42 showing evidence of myelin sheath layer formation by the oligodendrocytes. Sections were performed at the edge of organoids where previous immunostaining indicates the interaction between neurons and oligodendrocytes. Scale bar = 1 µm, scale bar of magnified images = 500 nm. **(F)** qRT-PCR of astrocyte marker (GFAP). All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSC values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed. ***$P < 0.001$, *$P < 0.05$ via One Way ANOVA. Normality Test (Shapiro-Wilk) Passed ($P = 0.646$). **(G)** Representative images of immunostained day 28 and 42 organoid sections showing the protein expression of TUJ1 (Green) and GFAP (Red). All sections were counterstained with Hoechst 33342 (Blue). Scale bar = 20 µm. A total of 48 organoids was analyzed.

We noted that there are a substantial fraction of cells that do not express SOX10 yet are labeled with CNPase and MBP antibodies. Since these proteins are also present in myelin sheaths it is likely that these represent neuronal cells that have started to undergo myelination (as shown in **Figure 3**). Intriguingly, we also detected a proportion of cells that were O4 positive yet

SOX10 negative, suggesting that in our human organoids not all oligodendroglial progenitor-derived cells express SOX10. During oligodendrogenesis, oligodendroglial cells progress through multiple stages before specification into mature OL that produce myelin proteins and myelinate neurons. Although many markers have been identified that mark oligodendroglia at different stages of differentiation, there are no unique markers that uniquely distinguish these different stages of oligodendrogenesis (Goldman and Kuypers, 2015). For instance, oligodendroglia at pre-OL stage express SOX10, PLP, O4, O1 and CNPase. These proteins persist until the mature stage when OL begin to express additional markers such as GalC, MBP, MAG, and MOG (Kuhn et al., 2019). In this study, we found that in day 42 OL brain organoids ∼90% of SOX10 positive cells express O4, as well as CNPase and MBP. These data suggest that oligodendroglial cells and OL produced in our protocol mimic the *in vivo* events of oligodendrogenesis. It is not unlikely that the remaining 10% of SOX10 positive cells may require additional time *in vitro* to differentiate into mature OL. In our hands, the OL brain organoid differentiation protocol described in this paper is robust as we find similar consistent specification of myelinating oligodendrocytes between different cell lines as well as between different clones of the same line (data not shown).

Oligodendroglial cells mature to deposit myelin around neurons that progressively develop electrophysiological as organoids mature over time. It will be interesting to find out whether the accelerated deposition of myelin as reported here results in faster neuronal maturation or more mature electrophysiological properties of the neurons, and whether this is different in OL organoids established from iPSC derived from patients with hypomyelination disorders. While we observe clear evidence of myelin sheaths wrapping around axons, we acknowledge that in day 42 and day 56 organoids we detect only very few axons with compacted myelin sheaths that are observed *in vivo*, similar to previous reports (Madhavan et al., 2018; Kim et al., 2019). It remains to be established whether more prolonged culture or addition of myelination enhancing molecules such as lanosterole, ketoconazole, and clemastine can further enhance neuronal myelination and the development of more compact myelin sheaths in our OL brain organoids.

Previously the pioneering work by Madhavan et al. (2018) demonstrated that generating brain organoids with myelinating oligodendrocytes and elucidation of myelination associated disease processes is indeed possible, however, the organoid differentiation process required 210 days and timed addition of various growth factor cocktails. Kim et al. (2019) subsequently showed that this could be accelerated and observed MBP expressing mature OL as early as 9 weeks and myelination of axons by 15 weeks. In this paper, we now describe an innovative and simplified one step protocol for generating human cortical brain organoids that contain neurons and astrocytes as well as oligodendrocytes that are able to myelinate endogenous neuronal axons by 42 days, making this protocol to our knowledge the fastest method to date for generating mature oligodendrocytes within a human cortical organoid.

MATERIALS AND METHODS

Engineering Human Induced Pluripotent Stem Cells

For gene targeting WTC cells at passage 22 were dissociated in 1 mL Accutase, collected into a 15-mL Falcon, centrifuged (160 × g, 3 min, RT) and counted manually. Three million cells were resuspended in 100 μL of Lonza Amaxa Primary P3 nucleofection solution with 3 μg vector DNA for mMaple transgene insertion and 1 μg of a plasmid expressing the guide RNA and CRISPR-Cas9 (pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang, Addgene plasmid # 62988) and subjected to nucleofection following the manufacturer's instructions for the Lonza Amaxa Primary P3 Kit (V4XP-3024, Lonza). After nucleofection cells were replated onto a 6 well plate in mTser Plus supplemented with 10 μM ROCKi Y-27632 (Lonza-PeproTech, 1293823-B) for 24 h. After 72 h cells were incubated with mTeSR Plus containing 0.5 ug/ml of Puromycin (Life Technologies, A1113803) for 72 h. After further 5 days of culture cells were detached with Accutase and single cell cloned. After 2 weeks of expansion individual clones were screened and frozen in Synth-a-Freeze (Thermofisher, A1254201).

Human iPSCs Culture and OL Brain Organoid Generation

Engineered mMaple WTC line was cultured according to Stem Cell Technologies protocols (https://www.stemcell.com/maintenance-of-human-pluripotent-stem-cells-in-mtesr1.html) on Matrigel (StemCell Technologies, Cat. #354277) in mTeSR (Stem Cell Technologies, Cat. #85851) (Shaker et al., 2020a). To generate OL organoids, iPSCs were first treated with 10 uM SB 431542 (Sapphire Biosciences, A10826) and 0.1 uM LDN193189 Dihydrochloride (Sigma, SML0559) for 3 days in N2 medium: DMEM/F12 (Gibco, Cat. #11320-33), 2% B-27 supplement (Gibco, Cat. # 17504044), 1% N-2 supplement (Gibco, Cat. #17502-048), 1% MEM Non-Essential Amino Acids (Gibco, Cat. #11140-050), 1% penicillin/streptomycin (Gibco, Cat. #15140148), 0.1% β-mercaptoethanol (Gibco, Cat. #21985-023) to induce hNEct cells (Shaker et al., 2020c). These cells were then detached gently using dispase enzyme for 2o min at 37°C and allowed to aggregate in low-attachment culture plates (Sigma, CLS3473) overnight (Lee et al., 2020b), and expanded for 4 days in OL differentiation medium (OLDM) consisting of: DMEM/F12 (Gibco, Cat. #11320-33), 2% B-27 minus vitamin A supplement (Gibco, Cat. # 17504044), 1% N-2 supplement (Gibco, Cat. #17502-048), 1% MEM Non-Essential Amino Acids (Gibco, Cat. #11140-050), 1% penicillin/streptomycin (Gibco, Cat. #15140148), 0.1% β-mercaptoethanol (Gibco, Cat. #21985-023), Human IGF-I (Lonza-PeproTech, 100-11-100), Insulin (LifeTechnologies, 12585014), Human NT-3 (Peprotech, 450-03-50), 3,3',5-Triiodo-L-thyronine (Sapphire Bioscience, 000-23845), HGF (Lonza-PeproTech, 100-39H) Biotin (Sigma, B4639) cAMP (Sigma, D0627), PDGF-AA (Lonza-PeproTech, 100-13A). Induced neuroepithelial spheroids were then embedded in Matrigel (StemCell Technologies, Cat. #354277) and maintained in OLDM medium. Fresh media was replaced three times a week. To verify the induction of

SOX10 in OL organoids, organoids were exposed to UV light for 30 s for efficient mMaple photoconversion. Brightfield images were automatically acquired with 582 JuLi^{TM}Stage (NanoEntek) to capture stages of hNEct 2D sheet conversion into 3D. All experiments were carried out in accordance with the ethical guidelines of the University of Queensland and with the approval by the University of Queensland Human Research Ethics Committee (Approval number-2019000159).

Immunohistochemistry

Tissue processing was performed as described in Lee et al. (2019) and immunohistochemistry (IHC) was performed as described in Kim et al. (2017). In brief, organoids were fixed in 4% PFA for 1 to 3 h at room temperature (RT) or overnight at 4°C. Fixed organoids were then washed three times for 10 min at RT with 1× phosphate buffered saline (PBS) before immersing in 30% sucrose in 1× PBS at 4 °C. Organoids were allowed to sink before embedding in a solution containing 3:2 ratio of Optimal Cutting Temperature (O.C.T) and 30% sucrose on dry ice. Mounted blocks of tissues were then sectioned into 14-μm thick serial sections, and collected onto Superfrost slides (Thermo Scientific, cat. #SF41296) (Shaker et al., 2020b). For IHC (Lee et al., 2020a), organoid sections were washed with 1× PBS three time for 10 min at RT before blocking for 1 to 6 h at RT with 3% bovine serum albumin (BSA) (Sigma, Cat. A9418-50G) and 0.1% triton X-100 in 1x PBS. After blocking, the sectioned organoids were incubated with primary antibodies overnight at 4°C, followed by washing with 1× PBS three times for 10 min each at RT. Labeled tissues were then incubated with appropriate secondary antibodies for 1 h at RT before mounting and imaging. All samples were counterstained with Hoechst 33342 (Invitrogen, Cat. #H3570). All images were acquired using confocal microscopy (Leica TCS SP8). The primary antibodies used in this study are listed in **Supplementary Table 1**. Alexa-488, Alexa-546, and Alexa-633-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratory.

qRT-PCR

Total RNA was isolated from 3 pooled organoids using an RNA extraction kit (Qiagen, 79256) according to the manufacturer's instructions (Shaker et al., 2015). cDNA was synthesized starting from 500 ng of RNA using VILO cDNA Synthesis Kit. qPCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) on a Bio-Rad CFX96 Touch Real-Time PCR detection system. Each reaction was performed in triplicate. GAPDH was used for normalization. Primers sequences are listed in **Supplementary Table 2**.

Transmission Electron Microscopy

Organoids were fixed in 2.5% glutaraldehyde for 24 h. Fixed organoids were contrasted with 1% osmium tetroxide and 2% uranyl acetate prior to dehydration through a series of ethanol solutions (50–100%) and embedded in EPON resin using a PELCO BioWave (Ted Pella Inc). Following polymerisation at 60°C for 24 h, ultrathin sections (∼60 nm) were cut using an ultramicrotome (UC64, Leica). Sections were visualized on a transmission electron microscope (model 1011; JEOL) equipped with cooled charge-coupled device camera (Morada; Olympus) and images acquired using iTEM software (Olympus Soft Imaging Solutions).

Statistical Analysis

All data were expressed as the mean ± standard deviation of the mean of the indicated number of independent experiments. The number of biological replicates as well as the sample size are indicated in the figure legends. Three is the minimum number of replicates used in this study. Image J was utilized to quantify the number of positive cells from confocal images. One-way ANOVA was applied for comparing more than two groups for normally distributed data that passed the normality test (Shapiro-Wilk). Kruskal–Wallis H test or one-way ANOVA on ranks was applied for comparing more than two groups for non-normally distributed data that failed to pass the normality test (Shapiro-Wilk). Tukey's *post-hoc* analysis was applied for comparisons to a single control. Statistical analysis was performed using GraphPad Prism 8.3.1® software. Minimal statistical significance was defined at $P < 0.05$.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All experiments were carried out in accordance with the ethical guidelines of the University of Queensland and with the approval by the University of Queensland Human Research Ethics Committee (Approval number-2019000159).

AUTHOR CONTRIBUTIONS

MS: performed, analyzed and designed experiments, interpreted the results, and wrote the manuscript. GP: performed additional experiments and wrote the manuscript. SM: performed additional experiments. J-HL: performed additional experiment. WS: resources. EW: conceived and supervised the study, interpreted results, and wrote the manuscript. All authors: final version of the manuscript was approved.

FUNDING

MS and GP are supported by the Medical Research Future Fund- Accelerated Research, Leukodystrophy flagship Massimo's Mission EPCD000034. SM is supported by the Perry Cross Spinal Research Foundation. EW is supported by the Australian National Health and Medical Research Council (applications 1138795, 1127976, 1144806, and 1130168), BrAshA-T foundation, Perry Cross Spinal Research Foundation and the Australian Research Council.

ACKNOWLEDGMENTS

Bruce Conklin (Department of Medicine Gladstone Institute of Cardiovascular Disease) is greatly acknowledged for providing the original WTC iPSC. The authors acknowledge the facilities, and the scientific and technical assistance, of the Microscopy Australia Facility at the Center for Microscopy and Microanalysis (CMM) of The University of Queensland. This work was performed in part at the Queensland node of the Australian National Fabrication Facility, a company established under the National Collaborative Research Infrastructure Strategy to provide nano- and micro-fabrication facilities for Australia's researchers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.631548/full#supplementary-material

Supplementary Figure 1 | Characterization of mMaple+ cells and regional markers in OL brain organoids, related to **Figure 1**. **(A)** Analysis of sectioned organoids at days 7, 14, 42, and 56 showing mMaple after UV photoconversion (emission 600–630 nm). All sections were counterstained with Hoechst 33342 (Blue). Scale bar = 200 μm. Total of 48 organoids were analyzed. **(B)** qRT-PCR of forebrain markers (*EMX2* and *OTX1*). All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSCs values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed. $*P < 0.05$ via Kruskal–Wallis One Way Analysis of Variance on Ranks. Normality Test (Shapiro-Wilk): Failed.

Supplementary Figure 2 | Characterization of oligodendrocytes marker expression in OL brain organoids, related to **Figure 2**. qRT-PCR of different stages of oligodendrocyte specification markers (O4, GALC, MBP, OLIG2, NG2, and NKX2.1). All values were normalized to GAPDH levels of their respective samples and expressed relative to hiPSC values to obtain the fold change. Data are shown as mean ± standard deviation; Number of independent experiments = 3. Total of 18 organoids were analyzed. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ via One Way ANOVA, Normality Test (Shapiro-Wilk): Passed ($P = 0.667$). For MBP, Kruskal-Wallis One Way Analysis of Variance on Ranks was applied. Normality Test (Shapiro-Wilk): Failed ($P < 0.050$).

Supplementary Figure 3 | Progress of myelination in OL brain organoids, related to **Figure 3**. **(A)** Transmission electron microscopy analysis demonstrates the presence of multiple myelin sheaths in day 42 OL brain organoids. Scale bar = 500 nm. **(B)** Plot graph demonstrates the variation in number of myelin lamellae around axons across 42 days OL brain organoids.

Supplementary Table 1 | List of Antibodies used for immunohistochemistry.

Supplementary Table 2 | List of Primer Sequences used for RT-PCR (5′-3′ orientation).

Supplementary Video 1 | Live imaging of induced human NEct 2D sheet conversion to 3D. Induced colonies of hNEct were detached from the dish and transferred to ultra-low attachment culture dish. 2D colonies were converted to 3D hNEct spheroids within 12 h. Serial images were captured every 5 min.

REFERENCES

Chamling, X., Kallman, A., Fang, W., Berlinicke, C. A., Mertz, J. L., Devkota, P., et al. (2021). Single-cell transcriptomic reveals molecular diversity and developmental heterogeneity of human stem cell-derived oligodendrocyte lineage cells. *Nat. Commun.* 12, 1–20. doi: 10.1038/s41467-021-20892-3

Domingues, H. S., Portugal, C. C., Socodato, R., and Relvas, J. B. (2016). Oligodendrocyte, astrocyte, and microglia crosstalk in myelin development, damage, and repair. *Front. Cell Dev. Biol.* 4:71. doi: 10.3389/fcell.2016.00079

Douvaras, P., and Fossati, V. (2015). Generation and isolation of oligodendrocyte progenitor cells from human pluripotent stem cells. *Nat. Protoc.* 10:1143. doi: 10.1038/nprot.2015.075

Elitt, M. S., Shick, H. E., Madhavan, M., Allan, K. C., Clayton, B. L. L., Weng, C., et al. (2018). Chemical screening identifies enhancers of mutant oligodendrocyte survival and unmasks a distinct pathological phase in pelizaeus-merzbacher disease. *Stem Cell Reports* 11, 711–726. doi: 10.1016/j.stemcr.2018.07.015

Fatehullah, A., Tan, S. H., and Barker, N. (2016). Organoids as an *in vitro* model of human development and disease. *Nat. Cell Biol.* 18, 246–254. doi: 10.1038/ncb3312

Ferrer, I. (2018). Oligodendrogliopathy in neurodegenerative diseases with abnormal protein aggregates: the forgotten partner. *Progress Neurobiol.* 169, 24–54. doi: 10.1016/j.pneurobio.2018.07.004

Fröhlich, D., Suchowerska, A. K., Voss, C., He, R., Wolvetang, E., Von Jonquieres, G., et al. (2018). Expression pattern of the Aspartyl-tRNA Synthetase DARS in the human brain. *Front. Mol. Neurosci.* 11:81. doi: 10.3389/fnmol.2018.00081

Fünfschilling, U., Supplie, L. M., Mahad, D., Boretius, S., Saab, A. S., Edgar, J., et al. (2012). Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* 485, 517–521. doi: 10.1038/nature11007

Garbern, J., Cambi, F., Shy, M., and Kamholz, J. (1999). The molecular pathogenesis of Pelizaeus-Merzbacher disease. *Arch. Neurol.* 56, 1210–1214. doi: 10.1001/archneur.56.10.1210

García-León, J. A., Kumar, M., Boon, R., Chau, D., Wolfs, E., Eggermont, K., et al. (2018). SOX10 single transcription factor-based fast and efficient generation of oligodendrocytes from human pluripotent stem cells. *Stem Cell Reports* 10, 655–672. doi: 10.1016/j.stemcr.2017.12.014

Gautier, H. O., Evans, K. A., Volbracht, K., James, R., Sitnikov, S., Lundgaard, I., et al. (2015). Neuronal activity regulates remyelination via glutamate signalling to oligodendrocyte progenitors. *Nat. Commun.* 6, 1–15. doi: 10.1038/ncomms9518

Goldman, S. A., and Kuypers, N. J. (2015). How to make an oligodendrocyte. *Development* 142, 3983–3995. doi: 10.1242/dev.126409

Horikiri, T., Ohi, H., Shibata, M., Ikeya, M., Ueno, M., Sotozono, C., et al. (2017). SOX10-nano-lantern reporter human iPS cells; a versatile tool for neural crest research. *PLoS ONE* 12:e0170342. doi: 10.1371/journal.pone.0170342

Huang, W., Bhaduri, A., Velmeshev, D., Wang, S., Wang, L., Rottkamp, C. A., et al. (2020). Origins and proliferative states of human oligodendrocyte precursor cells. *Cell* 182, 594–608. e511. doi: 10.1016/j.cell.2020.06.027

Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., and Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat. Neurosci.* 9, 173–179. doi: 10.1038/nn1620

Kim, H., Xu, R., Padmashri, R., Dunaevsky, A., Liu, Y., Dreyfus, C. F., et al. (2019). Pluripotent stem cell-derived cerebral organoids reveal human oligodendrogenesis with dorsal and ventral origins. *Stem Cell Reports* 12, 890–905. doi: 10.1016/j.stemcr.2019.04.011

Kim, J. Y., Shaker, M. R., Lee, J.-H., Lee, B., Kim, H., and Sun, W. (2017). Identification of molecular markers distinguishing adult neural stem cells in the subventricular and subcallosal zones. *Animal Cells Syst.* 21, 152–159. doi: 10.1080/19768354.2017.1324522

Kuhn, S., Gritti, L., Crooks, D., and Dombrowski, Y. (2019). Oligodendrocytes in development, myelin generation and beyond. *Cells* 8:1424. doi: 10.3390/cells8111424

Lee, E., Kim, H. J., Shaker, M. R., Ryu, J. R., Ham, M. S., Seo, S. H., et al. (2019). High-performance acellular tissue scaffold combined with hydrogel polymers for regenerative medicine. *ACS Biomaterials Sci. Eng.* 5, 3462–3474. doi: 10.1021/acsbiomaterials.9b00219

Lee, J.-H., Shaker, M. R., Lee, E., Lee, B., and Sun, W. (2020a). NeuroCore formation during differentiation of neurospheres of mouse embryonic neural stem cells. *Stem Cell Res.* 34:101691. doi: 10.1016/j.scr.2019.101691

Lee, J.-H., Shin, H., Shaker, M. R., Kim, H. J., Kim, J. H., Lee, N., et al. (2020b). Human spinal cord organoids exhibiting neural tube morphogenesis for a quantifiable drug screening system of neural tube defects. *bioRxiv*. doi: 10.1101/2020.12.02.409177

Madhavan, M., Nevin, Z. S., Shick, H. E., Garrison, E., Clarkson-Paredes, C., Karl, M., et al. (2018). Induction of myelinating oligodendrocytes in human cortical spheroids. *Nat. Methods* 15, 700–706. doi: 10.1038/s41592-018-0081-4

Mak, I. W., Evaniew, N., and Ghert, M. (2014). Lost in translation: animal models and clinical trials in cancer treatment. *Am. J. Transl. Res.* 6:114. doi: 10.2147/DDDT.S49584

Mansour, A. A., Gonçalves, J. T., Bloyd, C. W., Li, H., Fernandes, S., Quang, D., et al. (2018). An in vivo model of functional and vascularized human brain organoids. *Nat. Biotechnol.* 36, 432–441. doi: 10.1038/nbt.4127

Matejuk, A., and Ransohoff, R. M. (2020). Crosstalk between astrocytes and microglia: an overview. *Front. Immunol.* 11:1416. doi: 10.3389/fimmu.2020.01416

McEvoy, A. L., Hoi, H., Bates, M., Platonova, E., Cranfill, P. J., Baird, M. A., et al. (2012). mMaple: a photoconvertible fluorescent protein for use in multiple imaging modalities. *PLoS ONE* 7:e51314. doi: 10.1371/journal.pone.0051314

McKenzie, A. T., Moyon, S., Wang, M., Katsyv, I., Song, W.-M., Zhou, X., et al. (2017). Multiscale network modeling of oligodendrocytes reveals molecular components of myelin dysregulation in Alzheimer's disease. *Mol. Neurodegener.* 12:82. doi: 10.1186/s13024-017-0219-3

Nevin, Z. S., Factor, D. C., Karl, R. T., Douvaras, P., Laukka, J., Windrem, M. S., et al. (2017). Modeling the mutational and phenotypic landscapes of pelizaeus-merzbacher disease with human iPSC-derived oligodendrocytes. *Am. J. Hum. Genet.* 100, 617–634. doi: 10.1016/j.ajhg.2017.03.005

Nobuta, H., Yang, N., Ng, Y. H., Marro, S. G., Sabeur, K., Chavali, M., et al. (2019). Oligodendrocyte death in pelizaeus-merzbacher disease is rescued by iron chelation. *Cell Stem Cell* 25, 531–541.e536. doi: 10.1016/j.stem.2019.09.003

Quadrato, G., Brown, J., and Arlotta, P. (2016). The promises and challenges of human brain organoids as models of neuropsychiatric disease. *Nat. Med.* 22, 1220–1228. doi: 10.1038/nm.4214

Reiche, L., Küry, P., and Göttle, P. (2019). Aberrant oligodendrogenesis in Down syndrome: shift in gliogenesis? *Cells* 8:1591. doi: 10.3390/cells8121591

Rivers, L. E., Young, K. M., Rizzi, M., Jamen, F., Psachoulia, K., Wade, A., et al. (2008). PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. *Nat. Neurosci.* 11, 1392–1401. doi: 10.1038/nn.2220

Salameh, J. S., Brown, R. H. Jr., and Berry, J. D. (2015). "Amyotrophic lateral sclerosis," in: *Seminars in Neurology*, (Thieme Medical *Publishers)*, 469–476. doi: 10.1055/s-0035-1558984

Shaker, M. R., Cooper-White, J., and Wolvetang, E. J. (2020a). Self-organizing 3D human choroid plexus-ventricle-cortical organoids. *BioRxiv*. doi: 10.1101/2020.09.30.321554

Shaker, M. R., Kim, J. Y., Kim, H., and Sun, W. (2015). Identification and characterization of secondary neural tube-derived embryonic neural stem cells *in vitro*. *Stem Cells Dev.* 24, 1171–1181. doi: 10.1089/scd.2014.0506

Shaker, M. R., Lee, J.-H., Kim, K. H., Kim, V. J., Kim, J. Y., Lee, J. Y., et al. (2020b). Spatiotemporal contribution of neuromesodermal progenitor-derived neural cells in the elongation of developing mouse spinal cord. *bioRxiv*. doi: 10.1101/2020.05.03.075382

Shaker, M. R., Lee, J.-H., Park, S.-H., Kim, J. Y., Son, G. H., Son, J. W., et al. (2020c). Anteroposterior Wnt-RA gradient defines adhesion and migration properties of neural progenitors in developing spinal cord. *Stem Cell Reports* 15, 898–911. doi: 10.1016/j.stemcr.2020.08.016

Simons, M., and Nave, K. A. (2015). Oligodendrocytes: myelination and axonal support. *Cold Spring Harb. Perspect. Biol.* 8:a020479. doi: 10.1101/cshperspect.a020479

Taft, R. J., Vanderver, A., Leventer, R. J., Damiani, S. A., Simons, C., Grimmond, S. M., et al. (2013). Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am. J. Hum. Genet.* 92, 774–780. doi: 10.1016/j.ajhg.2013.04.006

Wasseff, S. K., and Scherer, S. S. (2015). Activated immune response in an inherited leukodystrophy disease caused by the loss of oligodendrocyte gap junctions. *Neurobiol. Dis.* 82, 86–98. doi: 10.1016/j.nbd.2015.05.018

Zhang, Y., Lu, X.-Y., Casella, G., Tian, J., Ye, Z.-Q., Yang, T., et al. (2019). Generation of oligodendrocyte progenitor cells from mouse bone marrow cells. *Front. Cell. Neurosci.* 13:247. doi: 10.3389/fncel.2019.00247

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 23 April 2021
doi: 10.3389/fncel.2021.661928

Canavan Disease as a Model for Gene Therapy-Mediated Myelin Repair

Anoushka Lotun[1,2], *Dominic J. Gessler*[1,2,3]* *and Guangping Gao*[1,2,4]*

[1] Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA, United States, [2] Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, United States, [3] Department of Neurosurgery, University of Minnesota, Minneapolis, MN, United States, [4] Li Weibo Institute for Rare Diseases Research, University of Massachusetts Medical School, Worcester, MA, United States

In recent years, the scientific and therapeutic fields for rare, genetic central nervous system (CNS) diseases such as leukodystrophies, or white matter disorders, have expanded significantly in part due to technological advancements in cellular and clinical screenings as well as remedial therapies using novel techniques such as gene therapy. However, treatments aimed at normalizing the pathological changes associated with leukodystrophies have especially been complicated due to the innate and variable effects of glial abnormalities, which can cause large-scale functional deficits in developmental myelination and thus lead to downstream neuronal impairment. Emerging research in the past two decades have depicted glial cells, particularly oligodendrocytes and astrocytes, as key, regulatory modulators in constructing and maintaining myelin function and neuronal viability. Given the significance of myelin formation in the developing brain, myelin repair in a time-dependent fashion is critical in restoring homeostatic functionality to the CNS of patients diagnosed with white matter disorders. Using Canavan Disease (CD) as a leukodystrophy model, here we review the hypothetical roles of N-acetylaspartate (NAA), one of the brain's most abundant amino acid derivatives, in Canavan disease's CNS myelinating pathology, as well as discuss the possible functions astrocytes serve in both CD and other leukodystrophies' time-sensitive disease correction. Through this analysis, we also highlight the potential remyelinating benefits of gene therapy for other leukodystrophies in which alternative CNS cell targeting for white matter disorders may be an applicable path for reparative treatment.

Keywords: Canavan disease, NAA, myelination, oligodendrocyte, astrocyte, white matter, leukodystrophy, gene therapy

OPEN ACCESS

Edited by:
Paola Leone,
Rowan University School of Osteopathic Medicine, United States

Reviewed by:
Jeremy Francis,
Rowan University School of Osteopathic Medicine, United States
Bilal Ersen Kerman,
Istanbul Medipol University, Turkey

***Correspondence:**
Dominic J. Gessler
dominic.gessler@umassmed.edu;
gessl013@umn.edu
Guangping Gao
guangping.gao@umassmed.edu

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *31 January 2021*
Accepted: *23 March 2021*
Published: *23 April 2021*

Citation:
Lotun A, Gessler DJ and Gao G (2021) Canavan Disease as a Model for Gene Therapy-Mediated Myelin Repair. Front. Cell. Neurosci. 15:661928. doi: 10.3389/fncel.2021.661928

INTRODUCTION

Leukodystrophies, also known as myelin or white matter (WM) disorders, result from functional mutations in glia or downstream metabolic divergence in neuronal and/or non-neuronal populations (Seitelberger, 1984; Powers and Rubio, 1995; Kevelam et al., 2016). These genetically rare myelin diseases are often characterized in early childhood with several clinical presenting forms, namely hypotonia, ataxia, and seizures (Traeger and Rapin, 1998; Barkovich, 2005; van der Knaap and Bugiani, 2017). Some symptoms have also been observed of advancing into more severe stages such as hydrocephaly and megalencephaly (Matalon et al., 1988; Bugiani et al., 2010; Desai and Grimm, 2013; Sánchez et al., 2020). Consequently, many diagnosed patients can

progressively develop significant debilitation and cognitive impairment, thus negatively affecting their quality of life and contributing to emotional and financial burdens on their families. Abating and conceivably eradicating leukodystrophies remains a central focus of the scientific and healthcare communities. Recent studies have aimed to target glial cells, which appear to be the primary affected cell types, as an avenue for prospective leukodystrophy therapy (Muramatsu et al., 2007; Piguet et al., 2012; Kagiava et al., 2014; Francis et al., 2016; Georgiou et al., 2017; Gessler et al., 2017; Jonquieres et al., 2018). However, several key challenges are posed in this course of treatment. The first lies in tailoring therapy specifically toward the type of myelin abnormality in the leukodystrophy; i.e., either hypomyelination, a deficit of myelin; demyelination, a degradation of myelin; or dysmyelination, defective myelin (Naidu, 1999; van der Knaap and Bugiani, 2017), which may have relevance in the strategies used for therapeutic treatment. The second concerns the current limitations and challenges of efficiently and selectively targeting certain glial populations, particularly oligodendrocytes, in the brain to enable normalization of the myelin disorder. A third takes into consideration the potential contribution of peripheral organs in treatment strategies for leukodystrophies as little is currently known about how other organs play a role in its disease etiology. Irrespective of these challenges however, treatment is thought to be geared toward correcting for myelin deficits observed in affected individuals. Here we review the demyelinating progression of Canavan disease (CD) as an example of prospective avenues for leukodystrophy therapies, as well as the current approaches, setbacks, and successes of gene therapy as a time-dependent corrective measure for this lethal demyelinating leukodystrophy.

ETIOLOGY OF CANAVAN DISEASE

Characterized as a rare leukodystrophy, Canavan disease makes for an applicable and apt disease to examine the inner workings between demyelination and gene therapy application in a basic and translational setting. CD is caused by a loss of function of the aspartoacylase (ASPA) gene primarily in ASPA-expressing mature oligodendrocytes of the Central Nervous System (CNS) (Kirmani et al., 2002; Moffett et al., 2007). The resulting dysfunctional ASPA enzyme inhibits oligodendrocytes from hydrolyzing neuronally derived N-acetylaspartate (NAA), one of the brain's most abundant amino acid derivatives, into acetate and L-aspartate. This leads to the disease's characteristic accumulation of NAA in the CNS (Matalon et al., 1988; Toft et al., 1993), detectable by elevated NAA peaks in proton magnetic resonance spectroscopy (H-MRS) (Toft et al., 1993). The increased NAA concentrations observed in CD contrasts that of other leukodystrophies, such as Vanishing White Matter (VWM) disease (Bugiani et al., 2010) and Alexander disease (AD) (Moffett et al., 2007; Vázquez et al., 2008), which have been reported to instead present with a reduction of NAA (Moffett et al., 2007; Ariyannur et al., 2013). Nonetheless, in relation to its potential myelinating relevance, NAA levels in the CNS indirectly serves as a biomarker for neuronal health as well as a potential building block for CNS metabolism (Moffett et al., 2007; Francis et al., 2016; Jonquieres et al., 2018).

HYPOTHETICAL CNS FUNCTIONS OF NAA

To date there is limited understanding of the connection between non-functional NAA hydrolysis and myelin deterioration in Canavan disease. Perhaps the closest, indirect relationship has been seen phenotypically wherein NAA levels increase upon degradation of the WM (Leone et al., 2012; Francis et al., 2016). Although no conclusive consensus on the role of NAA currently exists, five leading hypotheses have been proposed in recent years to better understand its function in the CNS as well as its potential myelinating relevance in CD (Baslow, 2002; Moffett et al., 2007, 2013; Pederzolli et al., 2007, 2009; Kołodziejczyk et al., 2009; Jonquieres et al., 2018).

NAA-Derived Acetate Used for Lipid Synthesis

The first hypothesis suggests that in a healthy brain, NAA-derived acetate is used as a building block for fatty acid synthesis. *In vivo* research performed by Benuck and D'Amado et al. demonstrated supporting evidence for this hypothesis in which they traced radiolabeled NAA in rat brains, revealing that the liberated acetate from catabolized NAA was used in generating long chain fatty acids (Benuck and D'Adamo, 1968). Supplemental *in vitro* research performed on immortalized brown adipogenic cells (iBACs) over-expressing the NAA synthesizing enzyme, N-acetyltransferase 8-like (NAT8L), depicted a 4-fold increase of incorporated labeled-glucose into lipids such as triacylglycerols and free fatty acids (Pessentheiner et al., 2012; Huber et al., 2018), thus further reinforcing this hypothesis. In the context of Canavan disease, wherein NAA cannot be broken down into its subcomponents, NAA accumulation leads to an acetate deficiency and thus a decrease in lipid production (Madhavarao et al., 2005). This hypothesis in part suggests that the generated lipids from NAA catabolism could be used for myelin formation, which therefore would account for the demyelination in CD (Namboodiri et al., 2006).

NAA-Derived Acetate Used in Energy Metabolism

Consequently, the acetate provided from NAA breakdown has also been hypothesized to be involved in energy production. In this hypothesis, acetate would be synthesized into acetyl-CoA *via* acetyl-CoA synthase for its subsequential use in the citric acid cycle (D'Adamo et al., 1968; Namboodiri et al., 2006). In CD however, acetate production from NAA is compromised, which therefore would lead to an insufficient acetate pool for acetyl-CoA generation (Ariyannur et al., 2010). It could be hypothesized that acetate supplementation could possibly correct this feature, however, acetate replacement studies, by way of oral glyceryl-triacetate (GTA) administration in the CD tremor rat model and the traumatic brain injury rat model, showed limited therapeutic benefits; although improvements in motor performance and vacuolization were observed (Mathew et al., 2005; Arun et al.,

2010; Moffett et al., 2013), complete normalization was not achieved, suggesting that other pathological factors may be at play. Accordingly, in the acetate deficient environment of CD, the affected CNS may require an alternate source of energy, which could potentially be provided from lipid degradation. Supporting i*n vitro* studies using iBACs over-expressing NAT8L demonstrated a 40% elevation in the basal oxygen consumption rate compared to control brown adipocytes (Pessentheiner et al., 2012), suggesting that NAT8L overexpression, and therefore NAA accumulation, increases cellular respiration and consequently energy production potentially through lipolysis (Pessentheiner et al., 2012; Huber et al., 2018). An extension of this hypothesis would also indicate that the expenditure of fatty acids for energy production results in a reduction of myelinating lipids.

NAA Toxicity Affects Myelination

The third hypothesis proposes that NAA is toxic and therefore its elevated concentration in the CNS may lead to downstream progressive demyelination and vacuolization. Several groups have generated supporting data for this hypothesis in that increased oxidative stress on proteins, impaired non-enzymatic antioxidant defenses (Pederzolli et al., 2007, 2009), and absence-like seizure activity (Kitada et al., 2002) were reported following direct stereotaxic intracerebroventricular injections of NAA into rat brains. Patch-clamp studies further back these findings by demonstrating that NAA and its anabolic counterpart N-acetyl-aspartyl-glutamate (NAAG) depolarize gray matter granule cells *via* N-methyl-D-aspartate (NMDA) receptors in a calcium induced manner, but to a much lesser extent in white matter oligodendrocytes (Kołodziejczyk et al., 2009). Thus, NAA-mediated stimulation of neuronal NMDA receptors could serve to explain the seizure activity reflected in the previous study. Correspondingly, if elevated NAA concentrations are considered to be cytotoxic, one could hypothesize that NAA, or likewise NAAG, could result in NMDA receptor overstimulation in either neurons or oligodendrocytes and therefore cause cell death, thus accounting for demyelination in CD. However, the same group, Kolodziejczyk et al., has shown contrasting data on this front, depicting that upon incubation of cerebellar slices in the presence of NAAG, non-significant differences in cell death were observed in comparison to the control group.

In addition to *in vitro* experiments, *in vivo* studies have also depicted a toxic vs. non-toxic duality role of NAA. Rodent models in which both alleles for the NAA synthesizing enzyme, NAT8L, were knocked down revealed restorative pathology with a lack of CNS vacuolization (Maier et al., 2015) and a visible reduction of astrogliosis (Guo et al., 2015), contrasting that of CD rodent models expressing NAT8L. However, the survival and motor performance of these animals remained untherapeutic (Guo et al., 2015; Maier et al., 2015), suggesting that complete knockdown of NAA synthesis is not sustainable long-term. Likewise, other studies have shown that ASPA deficiency in rats causes seizure activity, reminiscent of the CD phenotype, which is subsequently reduced upon ASPA restoration *via* gene therapy (Klugmann et al., 2005), thus further supporting this hypothesis. On the contrary, heterozygous NAT8L expression in mice resulted in almost fully sustained survival comparable to wildtype (WT) animals, however, complete remedial reversal of CNS spongy degeneration was not observed (Maier et al., 2015). Other groups such as Jonquieres et al. have used animal lines overexpressing NAT8L to demonstrate that NAA elevation does not cause neuropathology, and thus does not intrinsically contribute to neurotoxicity, hypothesizing that it is rather the compartmentalization of NAA in oligodendrocytes, specifically the lack of oligodendroglial NAA hydrolysis and intolerance to NAA buildup in oligodendrocytes, which contributes to CD pathology (Jonquieres et al., 2018). Interestingly, concurrent research using cultured stomach cells extracted from rat tissue was found to exhibit nitric oxide toxicity through dose-dependent NAA incubation, in which upregulation of nuclear factor-kappa B (NF-kB) and mitogen activated protein kinases (MAPKs) were observed, leading to activation of nitric oxide synthase (iNOS) and cell death mediation, respectively (Surendran, 2009, 2010). Although the duality of whether NAA is toxic or non-toxic is still unclear, if the finding of nitric oxide toxicity holds true for the CNS, it could be hypothesized that elevated NAA in ASPA deficient oligodendrocytes causes toxicity through a potentially similar pathway.

NAA-Derived Acetate Used for Epigenetic Regulation

The fourth theory bridges the utilization of NAA-derived acetate for energy production to epigenetic regulation (Prokesch et al., 2016) and considers a potential role of peripheral organs in CD pathology. The hypothesis follows that acetyl-CoA generated from acetate precursors are used in transcriptional control *via* acetylation of histone groups (Takahashi et al., 2006; Moffett et al., 2013). Auxiliary research done by Prokesch et al. demonstrated that *in vitro* ASPA knock-down in brown adipocytes led to a significant reduction of histone lysine acetylation, particularly of H3K27ac and H3K9ac (Prokesch et al., 2016), raising the question if the periphery plays a role in CD gene regulation and/or if the CNS undergoes similar epigenetic changes which may lead to downstream pathological effects. In this manner, Canavan disease which presents with an inadequate amount of NAA-derived acetate, could result in altered epigenetic regulation given that acetyl-CoA levels would be reduced due to a lack of available acetate pools, thus consequently altering gene expression.

NAA Acts as an Osmolyte in the CNS

Lastly, according to the molecular water pump (MWP) theory, NAA has also been hypothesized to function as a neuronal osmolyte involved in osmoregulation (Baslow, 2002). Given the CNS vacuolization observed in some severe forms of CD, the MWP hypothesis aims to provide an association between CNS edema and the role of NAA in maintaining water homeostasis (Baslow and Guilfoyle, 2013). Studies using microdialysis have previously shown NAA levels increase in the extracellular fluid (ECF) of rat brains in a stepwise manner following perfusion with increasingly hyposmotic media, thus suggesting that NAA is released as a result of osmotic changes which contribute to downstream extracellular swelling (Taylor et al., 1995). However,

this would suggest that NAA is potentially transported out of neurons and subsequently into the extracellular matrix, which has not yet been determined by scientific research (Moffett et al., 2007).

NAA'S FUNCTIONAL CORRELATION IN ASTROCYTES

In the main hypotheses presented for NAA's myelinating role in the CNS, we see a common thread of liberated acetate from NAA catabolism acting as a precursor for lipid synthesis and energy production. This is interesting because as evidenced by radiolabeled tracing of acetate, astrocytes have previously been identified as acetate transporters from the extracellular space into synaptosomes (Waniewski and Martin, 1998). In addition, they have been noted to be one of the major utilizers of acetate, oxidizing it for downstream use in the TCA cycle (Waniewski and Martin, 1998; Moffett et al., 2013). Therefore, following the hypothetical role of NAA-derived acetate in lipid production for myelin synthesis as well as the role of astrocytes in regulating acetate, it would be possible that astrocytes are fundamental for remyelination in the context of Canavan disease therapy.

Interestingly, the MWP theory also proposes a dynamic and possibly metabolic use of astrocytes, rather than oligodendrocytes, in normalizing NAA levels (Baslow and Guilfoyle, 2009). In this regard, astrocytes, which are known to form critical, regulatory gap junctions with oligodendrocytes (Benfenati and Ferroni, 2010; van der Knaap and Bugiani, 2017), could be the key to restoring myelination in CD. The premise follows that elevated NAA levels in the CNS are in part due to catabolism of NAAG *via* NAAG peptidase (Baslow and Guilfoyle, 2009) located on astrocytes. This subsequently increases osmotic pressure following water influx toward regions of amplified NAA concentration, which is hypothesized to result in the hallmarked WM edema observed in CD (Baslow, 2002; Baslow and Guilfoyle, 2009). In addition, water transport proteins such as aquaporin 4 (AQP4) have been found to be localized in astrocyte foot processes and play important regulatory roles in maintaining water homeostasis and osmolality in the CNS (Baslow and Guilfoyle, 2009; Papadopoulos and Verkman, 2013; Clarner et al., 2014). Electron microscopy (EM) studies of CD patients further reinforces these scientific findings by depicting large vacuole formation in cortical astrocytes (Adachi et al., 1972), effectively characterized as astrocytic swelling (Moffett et al., 2007; Baslow and Guilfoyle, 2009). Collectively, the NAAG-derived NAA and myelinic edema in CD alludes to the partaking of astrocytes in NAA metabolism and the resulting downstream pathology of vacuolization. This observation is markedly significant because like oligodendrocytes, astrocytes also contribute to CNS myelination (Nash et al., 2011).

PATHOBIOLOGY OF CANAVAN DISEASE

The phenotypic changes attributed with hallmarked demyelination in CD, such as ataxia, muscle weakness, and motor impairment, are shared amongst other leukodystrophies (Moers et al., 1991; Traeger and Rapin, 1998; van der Knaap and Bugiani, 2017). Notably, these changes are representative of the downstream effects of non-functioning myelin and thus neuronal impairment. In CD, magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) testing has shown ASPA deficient brains to have white matter abnormalities, which can histologically present as cerebral and cerebellar spongiform vacuolization (Adachi et al., 1973; Toft et al., 1993; Matalon and Michals-Matalon, 2000; Barkovich, 2005; Traka et al., 2008; Sommer and Sass, 2012; Gessler et al., 2017). Because myelin constitutes an integral role in the CNS, its degeneration can cause lasting adverse effects such as a delay in or loss of neuronal function due to decreased axonal conductivity and/or cellular metabolism, both of which are critical for neuronal survival (Philips and Rothstein, 2017). This in turn is supported by brain biopsies of CD patients which exhibit significant neuronal loss in deep cortical regions (Adachi et al., 1973; Matalon and Michals-Matalon, 2000). The consequential, and observed, neuronal debt can clinically present in the form of seizures, cognitive impairment, and weakened psychomotor responses. Thus, to better understand the downstream pathology of neuronal deterioration, we must first discern the upstream effects of demyelination in Canavan disease.

GLIAL COMPOSITION OF WHITE MATTER

Collective pathological features between CD and other leukodystrophies requires a brief account into the nature of CNS myelination. An appreciation for the complexity of cell-type connectivity throughout the brain is central in further characterizing the framework of myelin and the supportive role of glial populations in its maintenance. As research has shown, WM is constructed from an array of glia, mainly comprised of but not limited to oligodendrocytes, oligodendrocyte precursors cells (OPCs), and astrocytes (Back et al., 2002; Young et al., 2013; van der Knaap and Bugiani, 2017). Wherein oligodendrocytes construct myelin, astrocytes are responsible for maintaining oligodendrocyte and neuronal homeostasis (Philips and Rothstein, 2017; Jorge and Bugiani, 2019). One particular example of this is the astrocyte-neuron lactate shuttle, in which, upon the presence of glutamate binding, astrocytes uptake circulating blood glucose and convert it to pyruvate (through glycolysis), and subsequently into lactate by lactate dehydrogenase (LDH). The generated lactate is then shuttled, *via* monocarboxylate transporters (MCT), into neurons and oligodendrocytes where it is converted back into pyruvate for energy production through the Kreb's cycle, thus maintaining the functional, energic needs of those respective cell populations (Pellerin and Magistretti, 1994; Kasischke et al., 2004; Philips and Rothstein, 2017). This close-knit homeostasis between the major CNS cell-types is indicative that WM integrity, and therefore neuronal health, is contingent upon a coordinated equilibrium of glial cell types (Casper and McCarthy, 2005). This suggests that leukodystrophy treatments in the case of CD may not be entirely reliant on simply rescuing oligodendrocytes, but rather

may involve a re-balancing of overall glial function to restore or correct for myelin loss.

GENETIC AND TIME-DEPENDENT SIGNIFICANCE OF MYELINATION IN CANAVAN DISEASE

Myelination in the developing brain is a concerted effort of differentiating oligodendrocyte precursor cells (OPCs) into mature, myelinating oligodendrocytes (Barres et al., 1992; Trapp et al., 1997). However, the rate of OPCs differentiation varies during the human lifespan, with turnover into myelination peaking within the first 2 years of life and slowing down soon after (Barkovich, 2005). In the human forebrain, oligodendrocyte transcription factor 1 (Olig1) and platelet-derived growth factor receptor alpha (PDGFRα) positive cells can appear as early as 6 weeks into gestational development and begin differentiating into myelin basic protein (MBP)-expressing mature oligodendrocytes at 8 weeks of gestational age (Jakovcevski et al., 2009). Past neonatal age however, OPC's rate of differentiation decreases (Back et al., 2002), potentially as a result of increased brain size and the need for more focused regulation of glial proliferation (van der Knaap and Bugiani, 2017). This distinction is noteworthy because it highlights the complexity of myelin formation during development. In addition, characterization of CD neuropathology suggests that the demyelinating pattern that contributes to the disease progression is time dependent (Smith, 1973).

Originally characterized by Adachi et al. (1973), the clinical onset of Canavan disease was classified into one of three major forms; infantile, congenital, and juvenile. Although genetically, based on current scientific knowledge, all forms of Canavan disease are congenital (Mendes et al., 2017), the infantile and juvenile classifications serve to demonstrate the variability in clinical presentations of the disease. The infantile form, which is most common type and often results in the most severe progression (Adachi et al., 1973; Matalon et al., 1988; Traeger and Rapin, 1998), displays no phenotypical abnormalities at birth. However, within the first months of life, patients begin to present with hypotonia and delayed cognitive responses (Matalon and Michals-Matalon, 1999). These symptoms can progressively develop into more severe, potentially lethal, forms of psychomotor arrest and hydrocephaly (Matalon et al., 1988; Matalon and Michals-Matalon, 2000). The juvenile type presents later in the patient's lifespan, at roughly around 5 years of age (Adachi et al., 1973). Patients with this form typically survive into late adolescence and exhibit a milder phenotype of the disease (Traeger and Rapin, 1998).

Notably, the varying degrees of clinical severity in Canavan disease patients, either severe or mild, are, respectively, contingent upon the genetic makeup and developmental stage of the disease. This is further backed by a recent study of a group of 14 patients with novel ASPA missense mutations which demonstrated that ASPA mutations exhibiting <1% of enzymatic activity tended to correlate with a more severe disease development and an early onset of phenotypic presentations including increased mental delay, increased motor delay, and lack of speech (Mendes et al., 2017). On the contrary, patients with ASPA mutations that resulted in roughly 10% of wildtype ASPA's enzymatic activity were associated with a juvenile, milder presenting form of the disease (Mendes et al., 2017). However, this distinctive genotype and phenotype correlation did not pertain to all patients in the study. One patient, classified as a mild case, had normal language development and motor control, yet harbored an ASPA mutation that rendered <1% of enzymatic activity. A second mild patient had an ASPA mutation with ∼12% of enzymatic activity, which was considered in the study to be a relatively high functionality, yet presented with severe clinical phenotypes such as mental delays and language impairment within the first 3 months of life (Mendes et al., 2017). This observed independent phenomenon was also demonstrated in a separate clinical study in which two children were classified with juvenile, late onset forms of CD but similarly had low enzymatic ASPA activity, 0 and 5%, respectively (Leone et al., 1999). An analogous case was also seen in two additional CD patients who had the genetic A305E ASPA mutation associated with a severe form of CD, but presented with the juvenile course of the disease (Shaag et al., 1995). Interestingly, it has also been shown that the enzymatic activity of ASPA in four non-affected, heterozygous carrier parents of Canavan disease patients was 40% of the WT ASPA (Barash et al., 1991), further compounding efforts to properly correlate the genotypic and phenotypic relationship in Canavan disease. Nonetheless, the several cases of associated clinical presentation to enzymatic activity do suggest that, to a certain extent, an existing ASPA mutation in an individual Canavan patient may affect whether the disease will present in either an infantile or juvenile manner (Adachi et al., 1973; Barkovich, 2005; Mendes et al., 2017). In this regard, a component of genetics and time are essential in fostering a deeper understanding of the myelination pathogenesis and timeline for treatment intervention in Canavan disease. A distinction of the genetics, pathological onset, and severity of Canavan disease is critical because, like other leukodystrophies, therapeutic efforts, and success will in part rely on correcting for the developmental CNS progression of myelination in a time-sensitive manner (Lattanzi et al., 2010), which is further challenging due to the varying developmental forms of CD (Best et al., 2018; Knaap et al., 2019). Thus, conducted CD research would need to account for disease advancement in the context of successive CNS myelination.

RODENT MODELS FOR CANAVAN DISEASE

Several CD rodent models have been developed in recent years, allowing for further understanding of the demyelinating disease progression. By way of positional cloning techniques, the tremor rat model (*tm/tm*) was constructed by genomic deletion in the region containing the ASPA gene, resulting in lack of ASPA expression and presence of phenotypical Canavan disease traits such as CNS spongiform degeneration (Kitada et al., 2002). This rat model has also been used to study myelin lipid abnormalities

in CD (Wang et al., 2008). However, one caveat of the *tm/tm* rat model is that other genes, most notably the calcium-calmodulin-dependent protein kinase IV gene, which is involved in calcium signaling, are located in the same deletion region as the ASPA gene, thus potentially creating complications in studying single gene contribution to disease pathology. On the other hand, the *nur7* and CD KO mouse models, both of which only effect the ASPA gene, enable for research into more distinct gene-specific etiology. The *nur7* mouse model, first screened by Kile et al. and further classified by Traka et al., encodes a nonsense mutation, Q193X, in the ASPA gene resulting in a non-functional, truncated protein (Kile et al., 2003; Traka et al., 2008). The resulting $ASPA^{nur7}$ mutant mice parallel the mild form of Canavan disease in that they phenotypically exhibit elevated NAA levels, spongy degeneration, and vacuolization in the CNS, but do not develop early lethality from disease progression. Contrastingly, the ASPA knockout mouse model (CD KO), first constructed in a C57BL/6 background (Matalon et al., 2000) and, due to genetic instability, was later crossed into a SV129/Ev background (Ahmed and Gao, 2013; Ahmed et al., 2016; Gessler et al., 2017), presents with the severe form of Canavan disease, manifesting acute disease progression, such as reduced motor function, spasticity, and lethality as early as 4 weeks of age. Interestingly, CD KO mice in the C57BL/6 background did not consistently display the severe phenotype found in the SV129/Ey background. Both the CD KO and $ASPA^{nur7}$ mutant mice mirror the phenotypic nature of Canavan disease and thus use of these mouse models allows for more applicable readouts of CD gene therapy effectiveness. The $ASPA^{nur7}$ mutant mice, which exhibit milder phenotypes and longer lifespans, enable investigations into potential successes of long-term gene therapy, which is specifically beneficial to study adult CNS myelination. The CD KO mice, on the other hand, exhibit severe phenotypes consequently followed by a shortened lifespan and therefore permit research into immediate readout of gene therapy efficacy, particularly at neonatal, juvenile, and young adult timepoints. The time-dependent pathology onset in these CD mouse models can accordingly provide further insight into CD neuropathology which may aid in disease regression and prevent potential fatality associated with acute forms of the disease.

GLIAL CELL TARGETING FOR LEUKODYSTROPHIES USING GENE THERAPY

Combating the high lethality rates of leukodystrophies such as Canavan disease has been a challenging feat for the scientific community. Due to a lack of genetic screening, challenges for current prognoses are augmented (Lattanzi et al., 2010; Best et al., 2018; Knaap et al., 2019), thus further complicating effective diagnoses at early postnatal ages. Thus far, treatment for CD has mainly centered around symptomatic remedies rather than curative therapies, with the exception of several clinical trials, as later discussed. Nonetheless, recent implementations of CNS-directed gene therapy using Lentiviruses (LVs) and recombinant Adeno-Associated Viruses (rAAVs) have opened novel therapeutic paths, particularly for Canavan disease.

Lentiviral use for *in vivo* gene therapy is notable for its high transgene capacity (Kagiava et al., 2014), long-term sustainability of transgene expression, and ability to integrate into post-mitotic cells (Ashrafi et al., 2019). Previous research has illustrated its therapeutic application for leukodystrophies such as globoid cell leukodystrophy (GLD) (Lattanzi et al., 2014) and metachromatic leukodystrophy (MLD) (Piguet et al., 2012). Despite these successes however, LV has been reported in both adult rodents (Alisky et al., 2003; Lundberg et al., 2008) and in non-human primates (Kordower et al., 2000) for its limitations in non-neuronal capsid tropism. Intending to overcome these challenges, promoters such as myelin-basic protein (MBP) and 2,3-cyclic nucleotide 3-phosphodiesterase (CNP) have been used in mice for more directive oligodendrocyte expression, but transduction rates for this cell population remains as low as 20–30% past juvenile ages (Kagiava et al., 2014). Therefore, given its predominantly neuronal tropism and reduced glial transduction capabilities, LV application may not be as clinically relevant for leukodystrophies such as Canavan disease, which may require more robust glial expression at early developmental stages.

Adeno-associated virus has, in recent years, been the preferential gene delivery vector for CNS related diseases. Out of the now continuously growing library of serotypes present in the gene therapy field, several prominent AAVs have been thoroughly identified in displaying notable CNS transduction capabilities, contingent on the route of administration, in the mouse brain, mainly AAV2, 7, 8, 9, and rh.10 (Davidson et al., 2000; Burger et al., 2004; Taymans et al., 2007; Cearley et al., 2008; Yang et al., 2014). Of these serotypes, AAV2 exhibits the lowest transduction efficiency (Cearley et al., 2008; Yang et al., 2014) with a primarily neuron-tropic profile (Kaplitt et al., 1994; Xu et al., 2001). Contrastingly, AAV8 and AAV9 are established for having more widespread CNS transduction (Muramatsu et al., 2007; Cearley et al., 2008) particularly at neonatal stages, but nonetheless have a similar preferential neuronal selectivity to that of AAV2 (Lawlor et al., 2009).

Established clinical trials for CD patients first began through intraventricular delivery of liposome-encapsulated plasmid DNA (LPD) of pAAV-ASPA in a group of first two CD patients and then later in 14 CD patients (Leone et al., 1999, 2000). In this phase I trial, it was found that dose administration of 80 μg/mL in a total LPD-complex volume of 5 mL provided partial reversal of NAA levels, although hyperintensive T2-weighted imaging depicted that these patients still exhibited cases of CNS spongiform degeneration post-treatment (Leone et al., 2000). Advancements in gene therapy at the time subsequently allowed for utilization of AAV2, known for its neuronal tropism, as the new CNS transfer vector for the neuron-specific enolase (NSE) driven ASPA transgene (Janson et al., 2002; Leone et al., 2012). The 13 CD patients chosen for the study had six burr holes drilled intracranially to allow for placement of infusion cannulas for CNS AAV-ASPA delivery of 1×10^{12} GC/mL in a total volume of 150 μL per injection site (Leone et al., 2012). Longitudinal post-treatment studies over the course of 48

months revealed a broad decrease in NAA concentrations and an overall mass increase of subcortical brain regions in these patients (Leone et al., 2012). Immune response studies from the phase I CD trial showed 30% of subjects developed neutralizing antibodies (NAB) to AAV post-gene therapy, but nonetheless maintained a relatively basal and consistent, unchanging level of pro-inflammatory cytokines, suggesting the relative safety following rAAV administration (McPhee et al., 2006). Clinical monitoring of other CD patients has also shown a regional and partial decrease in previously elevated NAA concentrations as well as indications of slowdown of brain mass reduction upon gene therapy treatment (Hoshino and Kubota, 2014). Undoubtedly, the success of rAAV administration on these CD patients portrays the potential gene therapy has in the context of leukodystrophies. However, as discussed, clinical trials using the AAV2 vector presents limitations in CNS targeting of non-neuronal cell types. In the scope of leukodystrophy gene therapies, greater glial transducing AAVs for clinical trials would enable effective targeting of other glial cells. Although hypothetical, further research into widespread CNS tropic AAVs such as AAV9 may present with more advanced clinical progress for leukodystrophies.

Irrespective of vector tropism, promoters such as MBP, myelin-associated glycoprotein (MAG), and glial fibrillary acidic protein (GFAP) have been utilized to enhance oligodendrocyte and astrocyte targeting (Muramatsu et al., 2007; Lawlor et al., 2009; Jonquieres et al., 2013, 2016; Georgiou et al., 2017; Gessler et al., 2017). The difference in vector choice is evidenced by research done comparing EGFP expression levels in adult mice treated with MBP driven EGFP using a lentiviral vector, which yielded 20% oligodendrocyte transduction (Kagiava et al., 2014) vs. the same construct packaged in the AAV which yielded 25 and 95% transduction in neonates and juvenile mice, respectively (Jonquieres et al., 2013; Kagiava et al., 2014). Therefore, use of promoters such as MBP, MAG, and GFAP, in conjunction with AAV capsids expressing global CNS tropism, such as AAV8 and 9, provides a greater ability in targeting selective glial populations.

In the context of gene therapy, existing literature has thus far presented effective oligodendrocyte transduction in the brains of adult and juvenile murine and rodent models (Jonquieres et al., 2016; Georgiou et al., 2017). In CD, however, this oligodendrocyte approach presents with a major setback in that current gene therapies are limited in their ability to selectively target oligodendrocyte populations during neonatal development. This is showcased by MBP driven EGFP transgene expression in neonatal, juvenile, and adult mice in which the MBP promoter leads to high leaky astrocyte expression in neonates which is consequently reversed upon treatment in juvenile mice (Jonquieres et al., 2013). Given the progressive myelinating pattern in the developing brain as well as the increasing postnatal rate of myelin dysfunction in affected individuals, it may be imperative to consider therapies applicable at early ages, especially for leukodystrophies which can have an infantile or juvenile onset.

GENE THERAPY SUCCESS IN MOUSE MODELS FOR CANAVAN DISEASE

Interestingly, recent research suggests that ubiquitous targeting of CNS cell types using intravenous systemic delivery of rAAV9 packaged with the chicken beta-actin (CB) driven hASPA transgene in mice treated at postnatal day 1 (P1) is adequate for rescuing CD KO mice and furthermore leads to sustained motor and histological recovery into adulthood (Gessler et al., 2017). Likewise, use of the gene therapy vector AAV/Olig001 carrying the ASPA transgene has demonstrated effective reversal of Canavan disease pathology to wildtype levels, such as reduced thalamic and cerebellar vacuolization and increased myelination, as well as normalization of NAA levels upon predominately oligodendrocyte-specific targeting (Francis et al., 2016, 2021). Additional research as also shown promising disease rescue and remyelinating success in the cerebellum, hippocampus, and thalamic CNS regions of ASPA KO mice treated with an AAV cy5 serotype packaged with the oligodendrocyte-specific MBP promoter driven ASPA transgene (Jonquieres et al., 2018). Notably, both the AAV/Olig001 and AAV cy5 gene therapy successes for Canavan disease models were achieved *via* direct stereotaxic injections into the CNS (Francis et al., 2016, 2021; Jonquieres et al., 2018). In the context of route of administrations, systemic rAAV delivery to achieve widespread CNS transduction is challenged by the blood brain barrier, which affects dose requirements and thus the side-effect profile of therapeutic efficiency. On the other hand, direct intracranial injection allows for a more local delivery of rAAV which may change the intraparenchymal transduction spread. However, from a clinical and translation standpoint, direct brain injections can be invasive medical procedures and therefore further research is needed to address the therapeutic effects of alternate, perhaps more systemic, routes of administration for oligodendrocyte targeting in the context of Canavan disease reversal in clinical trials.

More surprisingly, current data has also shown that selectively expressing ASPA in astrocytes, *via* rAAV9 GFAP-driven hASPA, can phenotypically rescue CD KO mice, also treated at P1 by systemic injections, to wildtype levels (Gessler et al., 2017). In these treated animals, histological data and behavioral tests, respectively, confirm normalized CNS pathology, such as lack of spongiform degeneration, intact myelination, and complete motor restoration. In addition, use of the GFAP promoter, which has high astrocytic selectivity (Lawlor et al., 2009; Gessler et al., 2017), possesses the added advantage of robust transduction in the rodent brain. This is notable because astrocytes are the most abundant CNS cell type (Haydon and Carmignoto, 2006; Casper et al., 2007) and given their role in myelin maintenance and CNS metabolism (Baslow and Guilfoyle, 2009), they are a probable cell type for using gene therapy to effectively target the CNS on a global scale. For leukodystrophies such as Canavan disease this is important because the effects of demyelination can be far reaching. Thus, for late stage myelin deterioration which may require more robust disease turnaround, targeting astrocytes could be beneficial given their largescale CNS distribution.

PROSPECTIVE ASTROCYTIC ROLES IN PATHOBIOLOGY OF CANAVAN DISEASE

Astrocytes, which are known for their homeostatic role in the CNS, are critical participants in maintaining and regulating metabolic functions in the brain (Pellerin and Magistretti, 1994; Kasischke et al., 2004). As previously discussed, research pertinent to the role of NAA has depicted the involvement of astrocytes in acetate regulation and NAAG catabolism, thus linking a mechanistic feature of astrocytes to the CD pathomechanism. In addition to these components, astrocytes have also been reported to affect other CNS factors such as neuronal-glial signaling by way of glutamate-mediated NMDA receptors (Kasischke et al., 2004), and changes in myelinating capabilities of oligodendrocytes by modulating the extracellular matrix (ECM) (Pellerin and Magistretti, 1994; Nutma et al., 2020). Both of these aspects may hold relevance for CD therapies and help to rationalize the restorative and sustained success met with astrocyte-directed gene therapy in CD mouse models.

In fact, literature has shown that astrocytes encompass a duality function, both destructive and protective, toward oligodendrocyte pathology (Tilborg et al., 2016). Research suggests that the local environment of oligodendrocytes, in part controlled by mitogen release by astrocytes, affects the proliferation rate of myelinating oligodendrocytes (Barres and Raff, 1993; Young et al., 2013). For example, *in vitro* studies have shown that astrocytes operate as paracrine regulators by secreting factors such as platelet-derived growth factor (PDGF) in response to TNFα and IL-1β pro-inflammatory cytokines, which act to suppress oligodendrocyte differentiation (Gard et al., 1995; Silberstein et al., 1996; Zhang and An, 2007). On the contrary, *in vivo* studies in mouse models of other demyelinating diseases such as Multiple Sclerosis have demonstrated that reactive astrocytes secrete stromal cell-derived factor 1 chemokine 12 (CXCL12) to promote oligodendrocyte differentiation (Patel et al., 2012). In addition to cytokine signaling, glutamate signaling, which is required for proper oligodendrocyte myelination (Fannon et al., 2015), is regulated through expression of glutamate transporters (Goursaud et al., 2009) and NMDA receptors (Lalo et al., 2006) on astrocytes to prevent excitotoxicity. Furthermore, recent research using the cuprizone-induced demyelinating model has found that deletion of voltage gated Cav1.2 calcium channels in GFAP-positive astrocytes reduces astrogliosis and promotes remyelination, observable by a significant increase of Olig2-positive proliferating cells and a significant decrease in Iba1 and GFAP expressing cells (Zamora et al., 2020). Considering the downstream metabolism and catabolism of NAAG to NAA, as well as the importance of glutamate and calcium channels in astrocytes, it is possible that ASPA gene therapy in astrocytes somehow normalizes glutamate or calcium and/or cytokine pools in the CNS, thus correcting CD pathology; i.e., either directly by modulating glutamate or calcium levels to prevent hyperexcitability of neurons in an environment otherwise devoid of sufficient myelination, or indirectly by altering mitogen release necessary for oligodendrocyte growth.

Although there is not yet a clear explanation as to why and how astrocyte-targeted gene therapy can rescue CD pathogenesis and reverse demyelination in CD KO mice, it nonetheless raises into question how dependent Canavan disease treatment is on targeted oligodendrocyte populations. From a demyelination standpoint, the success of selective astrocytic therapy has noteworthy implications because it hints at the potential cellular crosstalk between either solely glial populations or inter neuronal-glial roles that allow for rescue of CD KO mice. This is supported through *in vivo* work that has shown a preferential ability of oligodendrocytes to remyelinate neuronal axons which are surrounded by astrocytes (Blakemore and Crang, 1989; Franklin et al., 1991; Talbott et al., 2005). *In vitro* research done by Nash et al. has also depicted that reactive astrocytes can induce myelination (Nash et al., 2011), thus reinforcing the notion that astrocytic restoration of ASPA could be beneficial in CD due to its ability to aid in remyelination, possibly through NAA-acetate homeostasis. Similarly, the hypothesized metabolic sink theory, in which NAA is conceivably redirected to astrocytes (Gessler et al., 2017) likewise supports the potential role astrocytes play in NAA metabolism, and thus in myelination. Interestingly, astrocytes have also been explored as therapeutic targets for other neurological diseases.

THERAPEUTIC BENEFITS OF ASTROCYTIC TARGETING IN OTHER NEURODEGENERATIVE DISEASES

As previously discussed, astrocytes have both a restorative and degenerative role in the CNS (Tilborg et al., 2016). Fine tuning of their metabolic and functional regulation, such as is the case of CD, has shown to be similarly favorable for neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS) and Rett Syndrome. Treatments for ALS, which is a progressively degenerative disease affecting motor neurons and neighboring astrocytes in the brain and spinal cord, are linked to mutations in the superoxide dismutase (SOD1) protein which has recently shown therapeutic promise from astrocyte targeting (Valori et al., 2019). In SOD1 transgenic mice, mutant SOD1 astrocytes which are prone to mediated cell death by way of glutamate-activated metabotropic type-5 receptor (mGluR5), depict a deceleration of disease progression upon mGluR5 inactivation (Rossi et al., 2008). This receptor blockage in astrocytes extends animal survival and decreases astrocytic degeneration, further prolonging ALS disease onset (Rossi et al., 2008). Similar ALS research has depicted that downregulation of intracellular calcium following mGluR5 activation in mutant SOD1 astrocytes can likewise extend survival and improve motor function of SOD1 mice (Martorana et al., 2012). Astrocytes' important neurological roles are also observed in Rett syndrome mouse models. Rett Syndrome is caused by mutations in the X-linked methyl CpG-binding protein 2 (Mecp2) gene, which primarily affects neurons and subsequently adjacent glia (Chahrour and Zoghbi, 2007). Research in the past decade has found that re-expression of Mecp2 in astrocytes of Mecp2 knockout

mice restores motor function, prolongs the mice lifespan, and improves phenotypic presentations of the disease (Lioy et al., 2011). As in the cases of ALS and Rett Syndrome, we observe a common functionality of astrocyte reprogramming leading to amelioration of disease progression, similar to the success of astrocyte-selective gene therapy in CD. These findings suggest that treatment for other neurological diseases and leukodystrophies, which can result from a variety of affected cell types (van der Knaap and Bugiani, 2017), may be achieved through alternative cell-type targeting.

ALTERNATIVE CELL TARGETING STRATEGIES FOR LEUKODYSTROPHIES

Advances in stem-cell therapies in recent years have also shown promising therapeutic results for Canavan disease. Using Canavan patient derived induced pluripotent stem cells (iPSCs) to differentiate into either neural progenitor cells (iNPCs) or oligodendrocyte progenitor cells (iOPCs), it has been elegantly demonstrated that upon stereotaxic injection of iNPCs or iOPCs into brains of *nur7* mice, reduced NAA, reversal of spongiform degeneration, prolonged motor function restoration, and significantly improved myelination are observed (Feng et al., 2020).

In addition to stem-cell therapies, gene therapy for leukodystrophies such as Metachromatic Leukodystrophy (MLD) and Krabbe's disease (KD) have also illustrated the therapeutic benefit of targeting unconventional cell types relative to the core of the pathology. MLD, which results from a deficiency of the lysosomal enzyme arylsulfatase A (ARSA), is a leukodystrophy which exhibits neurological damage with similar phenotypes to that of CD, such as ataxia and seizures, in addition to early infantile or juvenile death (Neufeld, 1991; Biffi et al., 2006). The ARSA enzyme is highly expressed in the white matter of the CNS and cerebellum as well as the glands of the endocrine system and is responsible for converting sulfatides, a sphingolipid, for its use in cell membranes. Notably, sulfatide has been shown to be involved in axon-glial signaling (Eckhardt, 2008). ARSA deficient MLD patients consequently lack sufficient sphingolipids, thus leading to downstream myelin deficits (Biffi et al., 2006). Original MLD therapies included hematopoietic cell or bone marrow transplantations (BMT) but were limited in their therapeutic success (Krivit et al., 1999; Peters and Steward, 2003). Since then, use of lentiviruses and AAVs have proven more promising; LV treatment of microglial populations in the tremor mouse model has exhibited restorative functions (Biffi et al., 2006) and AAVrh10 carrying the ARSA transgene has been found to correct for MLD by targeting up to 21% of oligodendrocytes (Piguet et al., 2012). Likewise, Krabbe's disease gene therapy has promoted use of alternative glial targeting for restorative function and remyelination. KD is caused by the loss of function of the lysosomal enzyme galactoceramidase (GALC). Comparable to MLD treatments, primary KD therapy involved BMTs which provided limited success (Marshall et al., 2018). Use of AAV9 in two separate studies have demonstrated that ubiquitous CNS expression of GALC reduces disease-associated astrogliosis and microgliosis and furthermore extends the lifespan of KD mice (Marshall et al., 2018; Pan et al., 2019).

Notably, leukodystrophies such as CD, MLD, and KD result from enzyme deficiencies and therefore disease mitigation could involve forms of metabolic restoration *via* cross-correction approaches, either through enzyme redistribution by substitute cells or by metabolite recompartmentalization and/or processing by neighboring cells. Although metabolic cross-correction mechanisms have not yet been established in Canavan disease, it is possible that such strategies may be therapeutic for non-metabolic leukodystrophies. Therefore, the therapeutic achievements using gene therapy, as well as potentially metabolic treatments, to target alternative glial cell-types, further highlight the advantages of non-traditional cell type targeting for leukodystrophies on a restorative front.

CONCLUSION

Leukodystrophies, which are largely classified according to the affected CNS cell-type in the respective white matter disorder (van der Knaap and Bugiani, 2017), remains elusive in concrete treatments for disease pathogenesis, in part due to variable disease onset, lack of prognosis screening, and neuropathological complexity associated with disease progression. These drawbacks can therefore lead to unsuccessful clinical intervention on a time-sensitive front, potentially resulting in significant, irreversible cognitive impairment and even fatality. For Canavan disease, in particular, a current absence of NAA's conclusive role in the CNS further compounds treatment applications for the leukodystrophy. In addition, complexities involved in selectively targeting oligodendrocyte populations limits the range of therapeutics for this disease which is thought to be predominately oligodendrocyte affected. Nonetheless, use of gene therapies, notably AAVs, have shown promising results in targeting other glial cell types, particularly astrocytes, as a restorative option for Canavan disease. Given the homeostatic role of astrocytes in the CNS, it is not completely unexpected that astrocyte-restricted ASPA expression leads to complete reversal of CD CNS pathology and sustained myelination and survival in CD mouse models. The implications of such research suggest that other leukodystrophies may benefit from gene therapy for alternative glial targeting, potentially non-oligodendrocyte focused, an area which is certainly worth delving into given the successes observed in Canavan disease research thus far.

AUTHOR CONTRIBUTIONS

AL outlined the review and wrote the manuscript. DG contributed to the outline of the review. DG and GG edited the manuscript. AL, DG, and GG prepared the paper. All authors contributed to the article and approved the submitted version.

FUNDING

GG was supported by grants from University of Massachusetts Medical School (an internal grant),

National Institutes of Health (R01NS076991-01, R01HL097088, 4P01HL131471-02, 1U19AI149646, and UG3 HL147367-01), and other grants from foundations and industry companies.

REFERENCES

Adachi, M., Schneck, L., Cara, J., and Volk, B. W. (1973). Spongy degeneration of the central nervous system. *Hum. Pathol.* 4, 331–347.

Adachi, M., Torii, J., Schneck, L., and Volk, B. W. (1972). Electron miscroscopic and enzyme histochemical studies of the cerebellum in spongy degeneration. *Acta Neuropathol.* 20, 22–31. doi: 10.1007/bf00687899

Ahmed, S. S., and Gao, G. (2013). Gene therapy for Canavan's disease takes a step forward. *Mol. Ther.* 21, 505–506. doi: 10.1038/mt.2013.25

Ahmed, S. S., Schattgen, S. A., Frakes, A. E., Sikoglu, E. M., Su, Q., Li, J., et al. (2016). rAAV gene therapy in a Canavan's disease mouse model reveals immune impairments and an extended pathology beyond the central nervous system. *Mol. Ther.* 24, 1030–1041. doi: 10.1038/mt.2016.68

Alisky, J. M., Hughes, S. M., and Davidson, B. L. (2003). Transduction of neurons lining the cerebral external capsules in mice with feline immunodeficiency virus based vectors. *Neurosci. Lett.* 351, 120–124. doi: 10.1016/s0304-3940(03)00833-4

Ariyannur, P. S., Arun, P., Barry, E. S., Andrews-Shigaki, B., Bosomtwi, A., Tang, H., et al. (2013). Do reductions in brain N-acetylaspartate levels contribute to the etiology of some neuropsychiatric disorders? *J. Neurosci. Res.* 91, 934–942. doi: 10.1002/jnr.23234

Ariyannur, P. S., Moffett, J. R., Manickam, P., Pattabiraman, N., Arun, P., Nitta, A., et al. (2010). Methamphetamine-induced neuronal protein NAT8L is the NAA biosynthetic enzyme: Implications for specialized acetyl coenzyme A metabolism in the CNS. *Brain Res.* 1335, 1–13. doi: 10.1016/j.brainres.2010.04.008

Arun, P., Madhavarao, C. N., Moffett, J. R., Hamilton, K., Grunberg, N. E., Ariyannur, P. S., et al. (2010). Metabolic acetate therapy improves phenotype in the tremor rat model of Canavan disease. *J. Inherit. Metab. Dis.* 33, 195–210. doi: 10.1007/s10545-010-9100-z

Ashrafi, M. R., Amanat, M., Garshasbi, M., Kameli, R., Nilipour, Y., Heidari, M., et al. (2019). An update on clinical, pathological, diagnostic, and therapeutic perspectives of childhood leukodystrophies. *Expert. Rev. Neurother.* 20, 1–20. doi: 10.1080/14737175.2020.1699060

Back, S. A., Luo, N. L., Borenstein, N. S., Volpe, J. J., and Kinney, H. C. (2002). Arrested oligodendrocyte lineage progression during human cerebral white matter development: dissociation between the timing of progenitor differentiation and myelinogenesis. *J. Neuropathol. Exp. Neurol.* 61, 197–211. doi: 10.1093/jnen/61.2.197

Barash, V., Flhor, D., Morag, B., Boneh, A., Elpeleg, O. N., and Gilon, C. (1991). A radiometric assay for aspartoacylase activity in human fibroblasts: application for the diagnosis of Canavan's disease. *Clin. Chim. Acta* 201, 175–181. doi: 10.1016/0009-8981(91)90368-m

Barkovich, A. J. (2005). Magnetic resonance techniques in the assessment of myelin and myelination. *J. Inherit. Metab. Dis.* 28, 311–343. doi: 10.1007/s10545-005-5952-z

Barres, B. A., Hart, I. K., Coles, H. S. R., Burne, J. F., Voyvodic, J. T., Richardson, W. D., et al. (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70, 31–46. doi: 10.1016/0092-8674(92)90531-g

Barres, B. A., and Raff, M. C. (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature* 361, 258–260. doi: 10.1038/361258a0

Baslow, M. H. (2002). Evidence supporting a role of NAA as a molecular water pump in myelinated neurons in the CNS an analytical review. *Neurochem. Int.* 40, 295–300. doi: 10.1016/s0197-0186(01)00095-x

Baslow, M. H., and Guilfoyle, D. N. (2009). Are astrocytes the missing link between lack of brain aspartoacylase activity and the spongiform leukodystrophy in Canavan disease? *Neurochem. Res.* 34, 1523–1534. doi: 10.1007/s11064-009-9958-z

Baslow, M. H., and Guilfoyle, D. N. (2013). Canavan disease, a rare early-onset human spongiform leukodystrophy: insights into its genesis and possible clinical interventions. *Biochimie* 95, 946–956. doi: 10.1016/j.biochi.2012.10.023

Benfenati, V., and Ferroni, S. (2010). Water transport between CNS compartments: functional and molecular interactions between aquaporins and ion channels. *Neuroscience* 168, 926–940. doi: 10.1016/j.neuroscience.2009.12.017

Benuck, M., and D'Adamo, A. F. (1968). Acetyl transport mechanisms Metabolism of N-acetyl-l-aspartic acid in the non-nervous tissues of the rat. *Biochim. Biophys. Acta* 152, 611–618. doi: 10.1016/0005-2760(68)90101-x

Best, S., Wou, K., Vora, N., Veyver, I. B. V., der, Wapner, R., and Chitty, L. S. (2018). Promises, pitfalls and practicalities of prenatal whole exome sequencing. *Prenatal. Diag.* 38, 10–19. doi: 10.1002/pd.5102

Biffi, A., Capotondo, A., Fasano, S., Carro, U., del, Marchesini, S., Azuma, H., et al. (2006). Gene therapy of metachromatic leukodystrophy reverses neurological damage and deficits in mice. *J. Clin. Invest.* 116, 3070–3082. doi: 10.1172/jci28873

Blakemore, W. F., and Crang, A. J. (1989). The relationship between type-1 astrocytes, Schwann cells and oligodendrocytes following transplantation of glial cell cultures into demyelinating lesions in the adult rat spinal cord. *J. Neurocytol.* 18, 519–528. doi: 10.1007/bf01474547

Bugiani, M., Boor, I., Powers, J. M., Scheper, G. C., and van der Knaap, M. S. (2010). Leukoencephalopathy with vanishing white matter: a review. *J. Neuropathol. Exp. Neurol.* 69, 987–996. doi: 10.1097/nen.0b013e3181f2eafa

Burger, C., Gorbatyuk, O. S., Velardo, M. J., Peden, C. S., Williams, P., Zolotukhin, S., et al. (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol. Ther.* 10, 302–317. doi: 10.1016/j.ymthe.2004.05.024

Casper, K. B., Jones, K., and McCarthy, K. D. (2007). Characterization of astrocyte-specific conditional knockouts. *Genesis* 45, 292–299. doi: 10.1002/dvg.20287

Casper, K. B., and McCarthy, K. D. (2005). GFAP Positive Progenitor Cells Produce Neurons and Oligodendrocytes Throughout the CNS. *Mol. Cell Neurosci.* 31, 676–684. doi: 10.1016/j.mcn.2005.12.006

Cearley, C. N., Vandenberghe, L. H., Parente, M. K., Carnish, E. R., Wilson, J. M., and Wolfe, J. H. (2008). Expanded repertoire of AAV vector serotypes mediate unique patterns of transduction in mouse brain. *Mol. Ther.* 16, 1710–1718. doi: 10.1038/mt.2008.166

Chahrour, M., and Zoghbi, H. Y. (2007). The story of Rett syndrome: from clinic to neurobiology. *Undefined* 56, 422–437. doi: 10.1016/j.neuron.2007.10.001

Clarner, T., Wieczorek, N., Krauspe, B., Jansen, K., Beyer, C., and Kipp, M. (2014). Astroglial redistribution of aquaporin 4 during spongy degeneration in a canavan disease mouse model. *J. Mol. Neurosci.* 53, 22–30. doi: 10.1007/s12031-013-0184-4

D'Adamo, A. F., Gidez, L. I., and Yatsu, F. M. (1968). Acetyl transport mechanisms. Involvement of N-Acetyl aspartic acid in de novo fatty acid biosynthesis in the developing rat brain. *Exp. Brain Res.* 5, 267–273. doi: 10.1007/bf00235902

Davidson, B. L., Stein, C. S., Heth, J. A., Martins, I., Kotin, R. M., Derksen, T. A., et al. (2000). Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc. Natl. Acad. Sci.U.S.A.* 97, 3428–3432. doi: 10.1073/pnas.97.7.3428

Desai, J., and Grimm, J. (2013). Communicating hydrocephalus in metachromatic leukodystrophy. *Pediatr. Neurol.* 49, 294–295. doi: 10.1016/j.pediatrneurol.2013.02.006

Eckhardt, M. (2008). The role and metabolism of sulfatide in the nervous system. *Mol. Neurobiol.* 37, 93–103. doi: 10.1007/s12035-008-8022-3

Fannon, J., Tarmier, W., and Fulton, D. (2015). Neuronal activity and AMPA-type glutamate receptor activation regulates the morphological development of oligodendrocyte precursor cells. *Glia* 63, 1021–1035. doi: 10.1002/glia.22799

Feng, L., Chao, J., Tian, E., Li, L., Ye, P., Zhang, M., et al. (2020). Cell-based therapy for canavan disease using human iPSC-derived NPCs and OPCs. *Adv. Sci.* 7:2002155. doi: 10.1002/advs.202002155

Francis, J. S., Markov, V., Wojtas, I. D., Gray, S., McCown, T., Samulski, R. J., et al. (2021). Preclinical biodistribution, tropism, and efficacy of oligotropic

AAV/Olig001 in a mouse model of congenital white matter disease. *Mol. Ther. Methods Clin. Dev.* 20, 520–534. doi: 10.1016/j.omtm.2021.01.009

Francis, J. S., Wojtas, I., Markov, V., Gray, S. J., McCown, T. J., Samulski, R. J., et al. (2016). N-acetylaspartate supports the energetic demands of developmental myelination via oligodendroglial aspartoacylase. *Neurobiol. Dis.* 96, 323–334. doi: 10.1016/j.nbd.2016.10.001

Franklin, R. J. M., Crang, A. J., and Blakemore, W. F. (1991). Transplanted type-1 astrocytes facilitate repair of demyelinating lesions by host oligodendrocytes in adult rat spinal cord. *J. Neurocytol.* 20, 420–430. doi: 10.1007/bf01355538

Gard, A. L., Burrell, M. R., Pfeiffer, S. E., Rudge, J. S., and Williams, W. C. (1995). Astroglial control of oligodendrocyte survival mediated by PDGF and leukemia inhibitory factor-like protein. *Dev. Camb. Engl.* 121, 2187–2197.

Georgiou, E., Sidiropoulou, K., Richter, J., Papaneophytou, C., Sargiannidou, I., Kagiava, A., et al. (2017). Gene therapy targeting oligodendrocytes provides therapeutic benefit in a leukodystrophy model. *Brain* 140, 599–616. doi: 10.1093/brain/aww351

Gessler, D. J., Li, D., Xu, H., Su, Q., Sanmiguel, J., Tuncer, S., et al. (2017). Redirecting N-acetylaspartate metabolism in the central nervous system normalizes myelination and rescues canavan disease. *JCI Insight* 2:e90807. doi: 10.1172/jci.insight.90807

Goursaud, S., Kozlova, E. N., Maloteaux, J., and Hermans, E. (2009). Cultured astrocytes derived from corpus callosum or cortical grey matter show distinct glutamate handling properties. *J. Neurochem.* 108, 1442–1452. doi: 10.1111/j.1471-4159.2009.05889.x

Guo, F., Bannerman, P., Ko, E. M., Miers, L., Xu, J., Burns, T., et al. (2015). Ablating N-acetylaspartate prevents leukodystrophy in a Canavan disease model. *Ann. Neurol.* 77, 884–888. doi: 10.1002/ana.24392

Haydon, P. G., and Carmignoto, G. (2006). Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol. Rev.* 86, 1009–1031. doi: 10.1152/physrev.00049.2005

Hoshino, H., and Kubota, M. (2014). Canavan disease: clinical features and recent advances in research. *Pediatr. Int.* 56, 477–483. doi: 10.1111/ped.12422

Huber, K., Hofer, D. C., Trefely, S., Pelzmann, H. J., Madreiter-Sokolowski, C., Duta-Mare, M., et al. (2018). N-acetylaspartate pathway is nutrient responsive and coordinates lipid and energy metabolism in brown adipocytes. *Biochim. Biophys. Acta* 1866, 337–348. doi: 10.1016/j.bbamcr.2018.08.017

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., and Zecevic, N. (2009). Oligodendrocyte development and the onset of myelination in the human fetal brain. *Front. Neuroanat.* 3:5. doi: 10.3389/neuro.05.005.2009

Janson, C., McPhee, S., Bilaniuk, L., Haselgrove, J., Testaiuti, M., Freese, A., et al. (2002). Gene therapy of canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. *Hum. Gene Ther.* 13, 1391–1412. doi: 10.1089/104303402760128612

Jonquieres, G., von, Fröhlich, D., Klugmann, C. B., Wen, X., Harasta, A. E., Ramkumar, R., et al. (2016). Recombinant human myelin-associated glycoprotein promoter drives selective AAV-mediated transgene expression in oligodendrocytes. *Front. Mol. Neurosci.* 9:13. doi: 10.3389/fnmol.2016.00013

Jonquieres, G., von, Mersmann, N., Klugmann, C. B., Harasta, A. E., Lutz, B., Teahan, O., et al. (2013). Glial promoter selectivity following AAV-delivery to the immature brain. *PLoS ONE* 8:e65646. doi: 10.1371/journal.pone.0065646

Jonquieres, G., von, Spencer, Z. H. T., Rowlands, B. D., Klugmann, C. B., Bongers, A., Harasta, A. E., et al. (2018). Uncoupling N-acetylaspartate from brain pathology: implications for canavan disease gene therapy. *Acta Neuropathol.* 135, 95–113. doi: 10.1007/s00401-017-1784-9

Jorge, M. S., and Bugiani, M. (2019). Astroglia in leukodystrophies. *Adv. Exp. Med. Biol.* 1175, 199–225. doi: 10.1007/978-981-13-9913-8_9

Kagiava, A., Sargiannidou, I., Bashiardes, S., Richter, J., Schiza, N., Christodoulou, C., et al. (2014). Gene delivery targeted to oligodendrocytes using a lentiviral vector. *J. Gene Med.* 16, 364–373. doi: 10.1002/jgm.2813

Kaplitt, M. G., Leone, P., Samulski, R. J., Xiao, X., Pfaff, D. W., O'Malley, K. L., et al. (1994). Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat. Genet.* 8, 148–154. doi: 10.1038/ng1094-148

Kasischke, K. A., Vishwasrao, H. D., Fisher, P. J., Zipfel, W. R., and Webb, W. W. (2004). Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science* 305, 99–103. doi: 10.1126/science.1096485

Kevelam, S., Steenweg, M., Srivastava, S., Helman, G., Naidu, S., Schiffmann, R., et al. (2016). Update on leukodystrophies: a historical perspective and adapted definition. *Neuropediatrics* 47, 349–354. doi: 10.1055/s-0036-1588020

Kile, B. T., Hentges, K. E., Clark, A. T., Nakamura, H., Salinger, A. P., Liu, B., et al. (2003). Functional genetic analysis of mouse chromosome 11. *Nature* 425, 81–86. doi: 10.1038/nature01865

Kirmani, B. F., Jacobowitz, D. M., Kallarakal, A. T., and Namboodiri, M. A. A. (2002). Aspartoacylase is restricted primarily to myelin synthesizing cells in the CNS therapeutic implications for Canavan disease. *Mol. Brain Res.* 107, 176–182. doi: 10.1016/s0169-328x(02)00490-4

Kitada, K., Akimitsu, T., Shigematsu, Y., Kondo, A., Maihara, T., Yokoi, N., et al. (2002). Accumulation of N-Acetyl-L-aspartate in the brain of the tremor rat, a mutant exhibiting absence-like seizure and spongiform degeneration in the central nervous system. *J. Neurochem.* 74, 2512–2519. doi: 10.1046/j.1471-4159.2000.0742512.x

Klugmann, M., Leichtlein, C. B., Symes, C. W., Serikawa, T., Young, D., and During, M. J. (2005). Restoration of aspartoacylase activity in CNS neurons does not ameliorate motor deficits and demyelination in a model of Canavan disease. *Mol. Ther.* 11, 745–753. doi: 10.1016/j.ymthe.2005.01.006

Knaap, M. S., van der, Schiffmann, R., Mochel, F., and Wolf, N. I. (2019). Diagnosis, prognosis, and treatment of leukodystrophies. *Lancet Neurol.* 18, 962–972. doi: 10.1016/s1474-4422(19)30143-7

Kołodziejczyk, K., Hamilton, N. B., Wade, A., Káradóttir, R., and Attwell, D. (2009). The effect of N-acetyl-aspartyl-glutamate and N-acetyl-aspartate on white matter oligodendrocytes. *Brain* 132, 1496–1508. doi: 10.1093/brain/awp087

Kordower, J. H., Emborg, M. E., Bloch, J., Ma, S. Y., Chu, Y., Leventhal, L., et al. (2000). Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 290, 767–773. doi: 10.1126/science.290.5492.767

Krivit, W., Peters, C., and Shapiro, E. G. (1999). Bone marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux-Lamy, and Sly syndromes, and Gaucher disease type III. *Curr. Opin. Neurol.* 12, 167–176. doi: 10.1097/00019052-199904000-00007

Lalo, U., Pankratov, Y., Kirchhoff, F., North, R. A., and Verkhratsky, A. (2006). NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes. *J. Neurosci.* 26, 2673–2683. doi: 10.1523/jneurosci.4689-05.2006

Lattanzi, A., Neri, M., Maderna, C., Girolamo, I., di Martino, S., Orlacchio, A., et al. (2010). Widespread enzymatic correction of CNS tissues by a single intracerebral injection of therapeutic lentiviral vector in leukodystrophy mouse models. *Hum. Mol. Genet.* 19, 2208–2227. doi: 10.1093/hmg/ddq099

Lattanzi, A., Salvagno, C., Maderna, C., Benedicenti, F., Morena, F., Kulik, W., et al. (2014). Therapeutic benefit of lentiviral-mediated neonatal intracerebral gene therapy in a mouse model of globoid cell leukodystrophy. *Hum. Mol. Genet.* 23, 3250–3268. doi: 10.1093/hmg/ddu034

Lawlor, P. A., Bland, R. J., Mouravlev, A., Young, D., and During, M. J. (2009). Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol. Ther.* 17, 1692–1702. doi: 10.1038/mt.2009.170

Leone, P., Janson, C. G., Bilaniuk, L., Wang, Z., Sorgi, F., Huang, L., et al. (2000). Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic implications for Canavan disease. *Ann. Neurol.* 48, 27–38. doi: 10.1002/1531-8249(200007)48:1<27:aid-ana6>3.0.co;2-6

Leone, P., Janson, C. G., McPhee, S. J., and During, M. J. (1999). Global CNS gene transfer for a childhood neurogenetic enzyme deficiency: Canavan disease. *Curr. Opin. Mol. Ther.* 1, 487–92.

Leone, P., Shera, D., McPhee, S. W. J., Francis, J. S., Kolodny, E. H., Bilaniuk, L. T., et al. (2012). Long-term follow-up after gene therapy for canavan disease. *Sci. Transl. Med.* 4:165ra163. doi: 10.1126/scitranslmed.3003454

Lioy, D. T., Garg, S. K., Monaghan, C. E., Raber, J., Foust, K. D., Kaspar, B. K., et al. (2011). A role for glia in the progression of Rett's syndrome. *Nature* 475, 497–500. doi: 10.1038/nature10214

Lundberg, C., Bjorklund, T., Carlsson, T., Jakobsson, J., Hantraye, P., Deglon, N., et al. (2008). Applications of lentiviral vectors for biology and gene therapy of neurological disorders. *Curr. Gene Ther.* 8, 461–473. doi: 10.2174/156652308786847996

Madhavarao, C. N., Arun, P., Moffett, J. R., Szucs, S., Surendran, S., Matalon, R., et al. (2005). Defective N-acetylaspartate catabolism reduces brain acetate levels and myelin lipid synthesis in Canavan's disease. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5221–5226. doi: 10.1073/pnas.0409184102

Maier, H., Wang-Eckhardt, L., Hartmann, D., Gieselmann, V., and Eckhardt, M. (2015). N-acetylaspartate synthase deficiency corrects the myelin phenotype in a canavan disease mouse model but does not affect survival time. *J. Neurosci.* 35, 14501–14516. doi: 10.1523/jneurosci.1056-15.2015

Marshall, M. S., Issa, Y., Jakubauskas, B., Stoskute, M., Elackattu, V., Marshall, J. N., et al. (2018). Long-term improvement of neurological signs and metabolic dysfunction in a mouse model of Krabbe's disease after global gene therapy. *Mol. Ther.* 26, 874–889. doi: 10.1016/j.ymthe.2018.01.009

Martorana, F., Brambilla, L., Valori, C. F., Bergamaschi, C., Roncoroni, C., Aronica, E., et al. (2012). The BH4 domain of Bcl-XL rescues astrocyte degeneration in amyotrophic lateral sclerosis by modulating intracellular calcium signals. *Hum. Mol. Genet.* 21, 826–40. doi: 10.1093/hmg/ddr513

Matalon, R., Michals, K., Sebesta, D., Deanching, M., Gashkoff, P., and Casanova, J. (1988). Aspartoacylase deficiency and N-acetylaspartic aciduria in patients with Canavan disease. *Am. J. Med. Genet.* 29, 463–471. doi: 10.1002/ajmg.1320290234

Matalon, R., and Michals-Matalon, K. (1999). Biochemistry and molecular biology of Canavan disease. *Neurochem. Res.* 24, 507–513. doi: 10.1023/a:1022531829100

Matalon, R., and Michals-Matalon, K. (2000). Spongy degeneration of the brain, Canavan disease: biochemical and molecular findings. *Front. Biosci.* 5, D307–D3011. doi: 10.2741/matalon

Matalon, R., Rady, P. L., Platt, K. A., Skinner, H. B., Quast, M. J., Campbell, G. A., et al. (2000). Knock-out mouse for canavan disease: a model for gene transfer to the central nervous system. *J. Gene Med.* 2, 165–175. doi: 10.1002/(SICI)1521-2254(200005/06)2:3<165::AID-JGM107>3.0.CO;2-R

Mathew, R., Arun, P., Madhavarao, C. N., Moffett, J. R., and Namboodiri, M. A. A. (2005). Progress toward acetate supplementation therapy for canavan disease: glyceryl triacetate administration increases acetate, but not N-acetylaspartate, levels in brain. *J. Pharmacol. Exp. Ther.* 315, 297–303. doi: 10.1124/jpet.105.087536

McPhee, S. W. J., Janson, C. G., Li, C., Samulski, R. J., Camp, A. S., Francis, J., et al. (2006). Immune responses to AAV in a phase I study for Canavan disease. *J. Gene Med.* 8, 577–588. doi: 10.1002/jgm.885

Mendes, M. I., Smith, D. E., Pop, A., Lennertz, P., Ojeda, M. R. F., Kanhai, W. A., et al. (2017). Clinically distinct phenotypes of Canavan disease correlate with residual aspartoacylase enzyme activity. *Hum. Mutat.* 38, 524–531. doi: 10.1002/humu.23181

Moers, A. V., Sperner, J., Michael, T. H., Scheffner, D., and Schutgens, R. H. B. (1991). Variable course of canavan disease in two boys with early infantile aspartoacylase deficiency. *Dev. Med. Child Neurol.* 33, 824–828. doi: 10.1111/j.1469-8749.1991.tb14967.x

Moffett, J. R., Arun, P., Ariyannur, P. S., and Namboodiri, A. M. A. (2013). N-Acetylaspartate reductions in brain injury: impact on post-injury neuroenergetics, lipid synthesis, and protein acetylation. *Front. Neuroenerget.* 5:11. doi: 10.3389/fnene.2013.00011

Moffett, J. R., Ross, B., Arun, P., Madhavarao, C. N., and Namboodiri, A. M. A. (2007). N-acetylaspartate in the CNS: from neurodiagnostics to neurobiology. *Progr. Neurobiol.* 81, 89–131. doi: 10.1016/j.pneurobio.2006.12.003

Muramatsu, S., Nishida, H., Nara, Y., Takino, N., Asari, S., Kodera, M., et al. (2007). AAV8 vectors transduce oligodendrocytes efficiently. *Mol. Ther.* 15:S319. doi: 10.1016/s1525-0016(16)45040-9

Naidu, S. (1999). Clinical delineation of leukodystrophies. *J. Mol. Neurosci.* 12, 185–192. doi: 10.1385/jmn:12:3:185

Namboodiri, A. M. A., Peethambaran, A., Mathew, R., Sambhu, P. A., Hershfield, J., Moffett, J. R., et al. (2006). Canavan disease and the role of N-acetylaspartate in myelin synthesis. *Mol. Cell Endocrinol.* 252, 216–223. doi: 10.1016/j.mce.2006.03.016

Nash, B., Thomson, C. E., Linington, C., Arthur, A. T., McClure, J. D., McBride, M. W., et al. (2011). Functional duality of astrocytes in myelination. *J. Neurosci.* 31, 13028–13038. doi: 10.1523/jneurosci.1449-11.2011

Neufeld, E. F. (1991). Lysosomal storage diseases. *Annu. Rev. Biochem.* 60, 257–280. doi: 10.1146/annurev.bi.60.070191.001353

Nutma, E., Gent, D., van, Amor, S., and Peferoen, L. A. N. (2020). Astrocyte and oligodendrocyte cross-talk in the central nervous system. *Cells* 9:600. doi: 10.3390/cells9030600

Pan, X., Sands, S. A., Yue, Y., Zhang, K., LeVine, S. M., and Duan, D. (2019). An engineered galactosylceramidase construct improves AAV gene therapy for krabbe disease in twitcher mice. *Hum. Gene Ther.* 30, 1039–1051. doi: 10.1089/hum.2019.008

Papadopoulos, M. C., and Verkman, A. S. (2013). Aquaporin water channels in the nervous system. *Nat. Rev. Neurosci.* 14, 265–277. doi: 10.1038/nrn3468

Patel, J. R., Williams, J. L., Muccigrosso, M. M., Liu, L., Sun, T., Rubin, J. B., et al. (2012). Astrocyte TNFR2 is required for CXCL12-mediated regulation of oligodendrocyte progenitor proliferation and differentiation within the adult CNS. *Acta Neuropathol.* 124, 847–860. doi: 10.1007/s00401-012-1034-0

Pederzolli, C. D., Mescka, C. P., Scapin, F., Rockenbach, F. J., Sgaravatti, Â. M., Sgarbi, M. B., et al. (2007). N-Acetylaspartic acid promotes oxidative stress in cerebral cortex of rats. *Int. J. Dev. Neurosci.* 25, 317–324. doi: 10.1016/j.ijdevneu.2007.04.002

Pederzolli, C. D., Rockenbach, F. J., Zanin, F. R., Henn, N. T., Romagna, E. C., Sgaravatti, Â. M., et al. (2009). Intracerebroventricular administration of N-acetylaspartic acid impairs antioxidant defenses and promotes protein oxidation in cerebral cortex of rats. *Metab. Brain Dis.* 24, 283–298. doi: 10.1007/s11011-009-9137-6

Pellerin, L., and Magistretti, P. J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc. Natl. Acad. Sci. U.S.A.* 91, 10625–10629. doi: 10.1073/pnas.91.22.10625

Pessentheiner, A., Pelzmann, H., Walenta, E., Schweiger, M., Groschner, L., Graier, W., et al. (2012). NAT8L (N-acetyltransferase 8-like) accelerates lipid turnover and increases energy expenditure in brown adipocytes. *J. Biol. Chem.* 288, 36040–36051. doi: 10.1074/jbc.m113.491324

Peters, C., and Steward, C. G. (2003). Hematopoietic cell transplantation for inherited metabolic diseases: an overview of outcomes and practice guidelines. *Bone Marrow Transpl.* 31, 229–239. doi: 10.1038/sj.bmt.1703839

Philips, T., and Rothstein, J. D. (2017). Oligodendroglia: metabolic supporters of neurons. *J. Clin. Invest.* 127, 3271–3280. doi: 10.1172/jci90610

Piguet, F., Sondhi, D., Piraud, M., Fouquet, F., Hackett, N. R., Ahouansou, O., et al. (2012). Correction of brain oligodendrocytes by AAVrh.10 intracerebral gene therapy in metachromatic leukodystrophy mice. *Hum. Gene Ther.* 23, 903–914. doi: 10.1089/hum.2012.015

Powers, J. M., and Rubio, A. (1995). Selected leukodystrophies. *Semi. Pediatr. Neurol.* 2, 200–210. doi: 10.1016/s1071-9091(05)80031-2

Prokesch, A., Pelzmann, H. J., Pessentheiner, A. R., Huber, K., Madreiter-Sokolowski, C. T., Drougard, A., et al. (2016). N-acetylaspartate catabolism determines cytosolic acetyl-CoA levels and histone acetylation in brown adipocytes. *Sci. Rep.* 6:23723. doi: 10.1038/srep23723

Rossi, D., Brambilla, L., Valori, C. F., Roncoroni, C., Crugnola, A., Yokota, T., et al. (2008). Focal degeneration of astrocytes in amyotrophic lateral sclerosis. *Cell Death Different.* 15, 1691–1700. doi: 10.1038/cdd.2008.99

Sánchez, A., García-Lareu, B., Puig, M., Prat, E., Ruberte, J., Chillón, M., et al. (2020). Cerebellar astrocyte transduction as gene therapy for megalencephalic leukoencephalopathy. *Neurotherapeutics* 17, 2041–2053. doi: 10.1007/s13311-020-00865-y

Seitelberger, F. (1984). Structural manifestations of leukodystrophies. *Neuropediatrics* 15, 53–61. doi: 10.1055/s-2008-1052382

Shaag, A., Anikster, Y., Christensen, E., Glustein, J. Z., Fois, A., Michelakakis, H., et al. (1995). The molecular basis of canavan (aspartoacylase deficiency) disease in European non-Jewish patients. *Am. J. Hum. Genet.* 57, 572–580.

Silberstein, F. C., Simone, R. D., Levi, G., and Aloisi, F. (1996). Cytokine-regulated expression of platelet-derived growth factor gene and protein in cultured human astrocytes. *J. Neurochem.* 66, 1409–1417. doi: 10.1046/j.1471-4159.1996.66041409.x

Smith, M. E. (1973). A regional survey of myelin development: some compositional and metabolic aspects. *J. Lipid Res.* 14, 541–551.

Sommer, A., and Sass, J. O. (2012). Expression of aspartoacylase (ASPA) and Canavan disease. *Gene* 505, 206–210. doi: 10.1016/j.gene.2012.06.036

Surendran, S. (2009). Upregulation of N-acetylaspartic acid alters inflammation, transcription and contractile associated protein levels in

the stomach and smooth muscle contractility. *Mol. Biol. Rep.* 36, 201–206. doi: 10.1007/s11033-007-9167-2

Surendran, S. (2010). Upregulation of N-acetylaspartic acid resulting nitric oxide toxicity induces aspartoacylase mutations and protein interaction to cause pathophysiology seen in Canavan disease. *Med. Hypoth.* 75, 533–534. doi: 10.1016/j.mehy.2010.07.017

Takahashi, H., McCaffery, J. M., Irizarry, R. A., and Boeke, J. D. (2006). Nucleocytosolic acetyl-coenzyme A synthetase is required for histone acetylation and global transcription. *Mol. Cell* 23, 207–217. doi: 10.1016/j.molcel.2006.05.040

Talbott, J. F., Loy, D. N., Liu, Y., Qiu, M. S., Bunge, M. B., Rao, M. S., et al. (2005). Endogenous Nkx2.2+/Olig2+ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes. *Exp. Neurol.* 192, 11–24. doi: 10.1016/j.expneurol.2004.05.038

Taylor, D. L., Davies, S. E. C., Obrenovitch, T. P., Doheny, M. H., Patsalos, P. N., Clark, J. B., et al. (1995). Investigation into the role of N-acetylaspartate in cerebral osmoregulation. *J. Neurochem.* 65, 275–281. doi: 10.1046/j.1471-4159.1995.65010275.x

Taymans, J.-M., Vandenberghe, L. H., Haute, C. V. D., Thiry, I., Deroose, C. M., Mortelmans, L., et al. (2007). Comparative analysis of adeno-associated viral vector serotypes 1, 2, 5, 7, and 8 in mouse brain. *Hum. Gene Ther.* 18, 195–206. doi: 10.1089/hum.2006.178

Tilborg, E., van, Heijnen, C. J., Benders, M. J., Bel, F., van, Fleiss, B., Gressens, P., et al. (2016). Impaired oligodendrocyte maturation in preterm infants: potential therapeutic targets. *Prog. Neurobiol.* 136, 28–49. doi: 10.1016/j.pneurobio.2015.11.002

Toft, P. B., GieB-Holtorff, R., Rolland, M. O., Pryds, O., Muller-Forell, W., Christensen, E., et al. (1993). Magnetic resonance imaging in juvenile Canavan disease. *Eur. J. Pediatr.* 152, 750–753. doi: 10.1007/BF01953994

Traeger, E. C., and Rapin, I. (1998). The clinical course of Canavan disease. *Pediatr. Neurol.* 18, 207–212. doi: 10.1016/s0887-8994(97)00185-9

Traka, M., Wollmann, R. L., Cerda, S. R., Dugas, J., Barres, B. A., and Popko, B. (2008). Nur7 is a nonsense mutation in the mouse aspartoacylase gene that causes spongy degeneration of the CNS. *J. Neurosci.* 28, 11537–11549. doi: 10.1523/jneurosci.1490-08.2008

Trapp, B. D., Nishiyama, A., Cheng, D., and Macklin, W. (1997). Differentiation and death of premyelinating oligodendrocytes in developing rodent brain. *J. Cell Biol.* 137, 459–468. doi: 10.1083/jcb.137.2.459

Valori, C. F., Guidotti, G., Brambilla, L., and Rossi, D. (2019). Astrocytes: emerging therapeutic targets in neurological disorders. *Trends Mol. Med.* 25, 750–759. doi: 10.1016/j.molmed.2019.04.010

van der Knaap, M. S., and Bugiani, M. (2017). Leukodystrophies: a proposed classification system based on pathological changes and pathogenic mechanism. *Acta Neuropathol.* 134, 351–382. doi: 10.1007/s00401-017-1739-1

Vázquez, E., Macaya, A., Mayolas, N., Arévalo, S., Poca, M. A., and Enríquez, G. (2008). Neonatal alexander disease: MR imaging prenatal diagnosis. *Am. J. Neuroradiol.* 29, 1973–1975. doi: 10.3174/ajnr.a1215

Wang, J., Leone, P., Wu, G., Francis, J. S., Li, H., Jain, M. R., et al. (2008). Myelin lipid abnormalities in the aspartoacylase-deficient tremor rat. *Neurochem. Res.* 34:138. doi: 10.1007/s11064-008-9726-5

Waniewski, R. A., and Martin, D. L. (1998). Preferential utilization of acetate by astrocytes is attributable to transport. *J. Neurosci.* 18, 5225–5233. doi: 10.1523/jneurosci.18-14-05225.1998

Xu, R., Janson, C., Mastakov, M., Lawlor, P., Young, D., Mouravlev, A., et al. (2001). Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. *Gene Ther.* 8, 1323–1332. doi: 10.1038/sj.gt.3301529

Yang, B., Li, S., Wang, H., Guo, Y., Gessler, D. J., Cao, C., et al. (2014). Global CNS Transduction of adult mice by intravenously delivered rAAVrh.8 and rAAVrh.10 and nonhuman primates by rAAVrh.10. *Mol. Ther.* 22, 1299–1309. doi: 10.1038/mt.2014.68

Young, K. M., Psachoulia, K., Tripathi, R. B., Dunn, S.-J., Cossell, L., Attwell, D., et al. (2013). Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling. *Neuron* 77, 873–885. doi: 10.1016/j.neuron.2013.01.006

Zamora, N. N., Cheli, V. T., González, D. A. S., Wan, R., and Paez, P. M. (2020). Deletion of voltage-gated calcium channels in astrocytes during demyelination reduces brain inflammation and promotes myelin regeneration in mice. *J. Neurosci.* 40, 3332–3347. doi: 10.1523/jneurosci.1644-19.2020

Zhang, J.-M., and An, J. (2007). Cytokines, inflammation, and pain. *Int. Anesthesiol. Clin.* 45, 27–37. doi: 10.1097/aia.0b013e318034194e

Conflict of Interest: GG is a co-founder of ASPA Therapeutics, Adrenas Therapeutics, and Voyager Therapeutics and holds equity in the companies. DG is a co-founder of ASPA Therapeutics and hold equity in the company. GG is an inventor of patents with royalties licensed to Voyager Therapeutics, ASPA Therapeutics, and other biopharmaceutical companies. DG is a member of the Clinical and Scientific Advisory Board of ASPA Therapeutics. GG and DG are inventors on a patent (PCT/US2016/058197) that is relevant to the content of this manuscript and may result in potential royalties if granted or licensed.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 22 June 2021
doi: 10.3389/fncel.2021.661857

Emerging Concepts in Vector Development for Glial Gene Therapy: Implications for Leukodystrophies

Georg von Jonquieres[1], Caroline D. Rae[2] and Gary D. Housley[1]*

[1] Translational Neuroscience Facility, Department of Physiology, School of Medical Sciences, UNSW Sydney, Sydney, NSW, Australia, [2] Neuroscience Research Australia, Randwick, NSW, Australia

Central Nervous System (CNS) homeostasis and function rely on intercellular synchronization of metabolic pathways. Developmental and neurochemical imbalances arising from mutations are frequently associated with devastating and often intractable neurological dysfunction. In the absence of pharmacological treatment options, but with knowledge of the genetic cause underlying the pathophysiology, gene therapy holds promise for disease control. Consideration of leukodystrophies provide a case in point; we review cell type – specific expression pattern of the disease – causing genes and reflect on genetic and cellular treatment approaches including *ex vivo* hematopoietic stem cell gene therapies and *in vivo* approaches using adeno-associated virus (AAV) vectors. We link recent advances in vectorology to glial targeting directed towards gene therapies for specific leukodystrophies and related developmental or neurometabolic disorders affecting the CNS white matter and frame strategies for therapy development in future.

Keywords: leukodystrophies, gene therapy, glia, adeno-associated virus, hematopoietic stem cells

OPEN ACCESS

Edited by:
Dominic J. Gessler, University of Minnesota Twin Cities, United States

Reviewed by:
Allison Bradbury, Nationwide Children's Hospital, United States
Niek Peter Van Til, Amsterdam University Medical Center, Netherlands

****Correspondence:***
Georg von Jonquieres
g.jonquieres@unsw.edu.au

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *31 January 2021*
Accepted: *12 May 2021*
Published: *22 June 2021*

Citation:
von Jonquieres G, Rae CD and Housley GD (2021) Emerging Concepts in Vector Development for Glial Gene Therapy: Implications for Leukodystrophies. Front. Cell. Neurosci. 15:661857. doi: 10.3389/fncel.2021.661857

LEUKODYSTROPHIES – A COMPLEX GROUP OF RARE DISEASES

Leukodystrophies *(leuko*, white; *dystrophy,* wasting) are a heterogeneous group of genetically determined disorders primarily affecting white matter in the central nervous system (CNS). Although each individual leukodystrophy is a rare disease, the collective overall incidence has been reported to range between 1: 5000 and 1:100.000 live births, rivaling the incidence of acquired white matter diseases including multiple sclerosis (Heim et al., 1997; Bonkowsky et al., 2010; Vanderver et al., 2012).

The term leukodystrophy was first established almost a century ago and until the mid-1980's considered to primarily affect myelin or oligodendrocytes directly (Bielschowsky and Henneberg, 1928; Morell, 1984). Advances in diagnosis through magnetic resonance imaging (MRI), whole exon and whole genome sequencing have resolved broader non-myelin components of this white matter pathophysiology. In recognition of this the term leukoencephalopathy has partially supplanted leukodystrophy, but traditionally includes acquired disorders that do not have a hereditary cause. Despite diagnostic advances, in ∼ 25 % of diagnosed leukodystrophies, the genetic cause remains enigmatic (Vanderver et al., 2016, 2020). Thus, an exhaustive definition and classification of leukodystrophies and leukoencephalopathies is ongoing. Van der Knaap et al. have recently postulated a new categorization of leukodystrophies in the clinical setting and proposed a novel, useful classification of leukodystrophies based on cellular pathology (Vanderver et al., 2015; Kevelam et al., 2016; van der Knaap and Bugiani, 2017;

van der Knaap et al., 2019). Following this classification leukodystrophies are grouped into classical *myelin disorders* that include hypomyelinating, demyelinating and vacuolizing leukodystrophies as well as *astrocytopathies*, *microgliopathies, leuko-axonopathies* and *leuko-vasculopathies* (van der Knaap and Bugiani, 2017).

While most leukodystrophies can manifest at any stage of life, there is usually an inverse correlation between the age of onset/diagnosis and disease severity. Particularly early-infantile onset leukodystrophies are typically associated with rapid progression and relentless, frequently fatal patient decline (Waldman, 2018; van der Knaap et al., 2019). However, not all leukodystrophies have a clear predictable course. Prognosis may depend on the specific mutation, age and severity at presentation and other genetic and environmental factors. More benign progression characterized by episodes of stability and even permanent improvement followed by recovery are regularly reported in some leukodystrophies (Köhler et al., 2018; van der Knaap et al., 2019).

Indisputably, advances in diagnosis and understanding of pathomechanisms have tremendous impact on management and treatment of the specific disease. Substrate reduction and pharmacological treatments generally target symptoms and have been covered in excellent reviews, but drugs that effectively treat the underlying disease and improve progression remain elusive (Adang et al., 2017; van der Knaap et al., 2019). Direct targeting of the underlying genetic basis of these diseases is therefore seen as a principal therapeutic strategy. This review will focus on the developing framework around gene therapies of these rare and debilitating genetic diseases.

CURRENT TREATMENT APPROACHES

In-depth knowledge of gene variants, associated pathomechanisms and prognosis is indispensable to make treatment decisions. The pathology of leukodystrophies range from mild to severe and often have a clear genotype – phenotype correlation. In more severe cases, the absence of pharmaceutical options for targeted treatment of CNS disorders have moved focus towards putatively curative gene and cell therapies. Due to the complexity of individual genetic causes and the limitations of different vectors, development has been lagging. It is becoming increasingly clear that there will not be a simple standardized solution. Therapy will need to be tailored and personalized.

In certain leukodystrophies, characterized by a build-up of cytotoxic metabolites in the brain, bone marrow transplant or allogenic hematopoietic stem cell transplantation (HSCT) can be very effective in attenuating disease progression. This has been the preferred treatment in pre-symptomatic X-linked Adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD) and Krabbe disease patients over the past 30 years (Escolar et al., 2005; Musolino et al., 2014; Boucher et al., 2015; Page et al., 2019). The rationale behind HSCT in slowing or arresting disease progression is that microglia, hematopoietic cells of the myelomonocytic lineage can infiltrate the CNS following myeloablation and express the missing gene necessary for degradation of the cytotoxic metabolite. In addition, intrathecal injection of cord blood-derived oligodendrocyte-like cells (DUOC-01) that have been shown to express lysosomal enzymes are currently assessed in clinical trial (NCT02254863) for their potential as an adjuvant bridging the temporal gap between allogenic HSCT transplant and engraftment of cells in the CNS thus more rapidly preventing disease progression (Kurtzberg et al., 2015; Saha, 2018). Success of HSCT largely depends on pre- or very early symptomatic treatment and the availability of a human leukocyte antigen (HLA) compatible donor, with cord blood of a non-carrier sibling being the preferred source. However even where HLA-matched donors are available, graft failure, graft versus host disease and risks of severe infections associated with prolonged immunodeficiency following myeloablation and engraftment are common complications (Boucher et al., 2015; Bonkowsky et al., 2018; Hierlmeier et al., 2018).

GENE THERAPY APPROACHES

In the following section we provide a perspective on recent progress in the field. Broadly, gene therapy approaches can be divided into *ex vivo* and *in vivo* approaches, each using either viral or non-viral vectors for delivery of coding or non-coding therapeutic nucleic acids. Despite substantive progress in the field, which have expanded gene therapy strategies personalized approaches dependent on the patient, the specific disease, and its progression at treatment are key precepts.

EX VIVO GENE THERAPY

Ex vivo gene therapy builds on HSCT and largely overlaps with cell therapy in that patients' own stem cells are extracted genetically modified via chromosomal integration of a therapeutic DNA, expanded, analyzed and reintroduced into the same patient. The advantage of this approach is that graft versus host disease and graft failure as well as other immune complications are largely eliminated (Staal et al., 2019). Pioneering this approach in a leukodystrophy, Nathalie Cartier and Patrick Aubourg's group used this autologous $CD34^+$ HSCT to successfully treat cerebral ALD in patients where no appropriate HLA – match could be obtained (Cartier et al., 2009). Following myeloablation, using a Busulfan or similar regimen, intravenous (IV) administered HSC differentiated into microglia-like cells following engraftment in the human CNS. Initially using murine leukemia virus based gammaretrovirus (γRV) vectors, followed by human immune deficiency virus-based lentivirus (LV) vectors, development of protocols and screening procedures have significantly improved the safety profile of hematopoietic stem cell gene therapy (HSC-GT) (Schmidt et al., 2007; Biffi et al., 2011). Both γRV and LV – based vectors integrate semi – randomly into the host genome producing stable replication and passage of the inserted therapeutic gene upon cell division but the corollary is the inadvertent risk of rare insertional mutagenesis – induced malignancies. While LV

preferably integrate into transcriptionally active chromatin, γRV predominantly integrate in the vicinity of gene regulatory regions (Vranckx et al., 2016). Particularly γRV show a long terminal repeat (LTR) – mediated propensity for recurrent insertion into 'hot spots' frequently near transcription start sites of proto-oncogenes explaining the heightened risk profile of early γRV vectors over LVs vectors for gene therapy applications (Cattoglio et al., 2007). Transcriptionally active LTRs are the principal determinants of this insertion mediated genotoxicity (Montini et al., 2006, 2009), and development of self – inactivating LV and γRV achieved by deletions in the 3' – LTRs that abolish intrinsic LTR promoter activity significantly enhanced the safety profile of these vectors (Zufferey et al., 1998).

Encouraging preclinical results employing self-inactivating LV vectors led to an open-label, phase II/III clinical trial involving autologous $CD34^+$ via self-inactivating lentivirus vector – mediated integration of ABC transporter D1 (*ABCD1*) cDNA in the STARBEAM study (ALD-102) in X-ALD patients (Eichler et al., 2017). Latest reports from this study suggest that disease progression stabilized in 31 out of 32 enrolled patients with all 15 patients that have completed a two year follow up remaining free of major functional disabilities (Kühl et al., 2020). Similarly, following promising clinical safety and efficacy results demonstrating improvement of Gross Motor Function Measure in 20 MLD patients for up to eight years, Libmeldy, an arylsulfatase A (*ARSA*) HSC-GT for early onset MLD has recently received a marketing authorization by the European Medicines Agency (EMA) on the basis that the benefits outweigh the risks, becoming the third approved HSC-GT after Zynteglo™ and Strimvelis™ (Biffi et al., 2013; Sessa et al., 2016; Tucci et al., 2020).

At present, *ex vivo* HSC or HSC-GT is predominantly used in leukodystrophies caused by accumulation of toxic metabolites that are degradable by a therapeutic gene product expressed in microglia-like monocyte derived macrophages after CNS infiltration, which may be supported by DUOCs in future. As for other therapeutic approaches, therapeutic benefit of HSC transplantation for leukodystrophies is inversely correlated with CNS disease progression, likely because late HSC administration alone cannot restore irreversible damage from neuron loss.

For other leukodystrophies, cell-based gene therapies may be extended to direct injection of genetically modified stem or progenitor cells in the future (Osorio and Goldman, 2016). The potential of genome editing using Zinc – finger nucleases, transcription activator like effector nucleases (TALEN) and clustered regulatory interspaced short palindromic repeats (CRISPR) – Cas9 systems offers immense opportunity for targeted correction of disease-causing mutations (Ferrari et al., 2020). Although pre-clinically gene-editing was successfully employed in HSC-GT, further safety and efficacy studies will be conducted (Schiroli et al., 2017; Pavel-Dinu et al., 2019). *Ex-vivo* gene addition and gene editing are readily achievable employing non – viral approaches including gene – electrotransfer. A notable advantage of gene editing is the preservation of normal copy numbers and gene regulation (Pinyon et al., 2019; Wagenblast et al., 2019). A recent study has modified induced neural progenitor cells (NPCs) and induced oligodendroglial progenitor cells (OPCs) from Canavan disease (CD) patients to express human aspartoacylase (*ASPA*) via lentivirus mediated gene addition and TALEN mediated gene editing. Direct injection of either of these genetically modified induced pluripotent cells (iPCs) into a CD mice resulted in pronounced histological, pathophysiology and motor behavior improvement (Feng et al., 2020). Observations from allogenic transplantation of human CNS stem cells into four Pelizaeus-Merzbacher disease patients showed engraftment and focal production of donor derived myelin in the transplanted hosts' white matter but detected an immune response and donor-specific HLA alloantibodies in half the patients (Gupta et al., 2012, 2019). Albeit holding promise, cell-based CNS gene therapies face significant hurdles to clinical translation.

IN VIVO GENE THERAPY

In vivo gene therapy relies on direct injection of the 'naked' or encapsulated therapeutic nucleic acid into the patient. While many virus derived vectors including recombinant adenovirus, LV, γRV, sindbis virus, poliovirus and herpes simplex virus have been trialed for CNS gene delivery, based on their superior safety and transduction efficiency, versatility and easy production credentials, recombinant adeno-associated virus (rAAV) has taken the center stage in the development of gene therapies for neurological disorders (Lentz et al., 2012; Ojala et al., 2015). Of the three AAV based therapeutics that have obtained regulatory approval by the European Medicines Agency (EMA) or U.S. Food and Drug Administration (FDA) since 2012, two target sensori-motor neural diseases. Luxturna® (Voretigene neparvovec) has been developed for Leber's congenital amaurosis using subretinal injection and Zolgensma® (Onasemnogene abeparvovec) is directed at spinal muscular atrophy in children under the age of two years, utilizing intravenous (IV) delivery.

In vivo gene therapy for leukodystrophies requires therapeutic gene transfer to the CNS which is compromised by the skull, the blood – brain barrier (BBB) and cerebrospinal fluid (CSF) – brain barrier (CBB). Delivery methods for rAAV to the brain include direct intraparenchymal (IP) injection, intracerebroventricular (ICV), lumbar intrathecal (IT) intra cisterna magna targeted intrathecal (ICM) and IV injections as well as nasal delivery which have been addressed in excellent recent reviews (Hocquemiller et al., 2016; Piguet et al., 2017, 2020; Hudry and Vandenberghe, 2019). In the following section, we review the advances in understanding of AAV vector targeting to provide context for the likely dominance of such gene therapy vector strategies for treating leukoencephalopathies in the future.

THE EVOLVING ADENO-ASSOCIATED VIRAL VECTOR TOOLKIT

Natural Discovery of Adeno-Associated Virus for Use as Gene Therapy Vectors

AAV is a non-enveloped, replication-deficient virus of approximately 25 nm in diameter whose icosahedral AAV

capsid harbors the single stranded (ss) DNA genome. Cell surface receptor binding, internalization, endosome escape and trafficking as well as aspects relating to immune escape and AAV production including stability and assembly are mediated through the capsid. As such the AAV capsid is the principal determinant of biodistribution, cell and tissue tropism and the site of interference with circulating neutralizing antibodies. The AAV capsid is a 60mer composed of three viral proteins VP1, VP2 and VP3 encoded in a single open reading frame, that assemble in a 1:1:10 ratio. Based on sero – reactivity, at least 13 human and non-human primate (NHP) AAV serotypes have been isolated, but seminal work from Guangping Gao and James Wilson's lab identified more than 100 variants in NHPs in the early 2000's and divided these naturally occurring AAVs into phylogenetic clades on the basis of functional and serologic similarities (Gao et al., 2004, 2005). Since then, AAVs have been isolated in different vertebrates, with many capable of cross-species transmission (Allison et al., 2013; Zinn and Vandenberghe, 2014). AAV serotypes isolated from natural sources generally have a broad tropism but often have cell type or tissue specific bias which varies depending on delivery route, target species, age of infusion and purification method (Klein et al., 2008; Zhang et al., 2011; Aschauer et al., 2013; von Jonquieres et al., 2013; Watakabe et al., 2015).

Comprehensive investigations of AAV tropism have been performed for select AAV serotypes and variants. Intraparenchymal AAV injection into the adult rodent brain using constitutive promoters to drive transgene expression suggests that most AAVs preferentially transduce neurons. Amongst others these include AAV1, AAV2, AAV5, AAV7, AAV8, AAV9, AAV.rh8, AAV.rh10, AAV.rh20, AAV.rh39 and AAV.cy5 with superior transduction efficiencies achieved by AAV1, AAV9, AAV.rh10. (Bartlett et al., 1998; Passini et al., 2003; Burger et al., 2004; Cearley and Wolfe, 2006; Lawlor et al., 2009; Castle et al., 2016). While transduction of astrocytes was observed for most serotypes, AAV8 and AAV.rh43 showed strongest astroglial tropism, with AAV8 also revealing some oligodendrocyte transduction (Davidson et al., 2000; Klein et al., 2008; Hutson et al., 2012). In the spinal cord AAV1, AAV5, AAV9 have shown strong neuronal tropism, but AAV8 was superior at targeting large diameter neurons (Jacques et al., 2012). It must be clarified, that exchanging the promoter driving transgene expression can completely change the expression profile of AAV vectors including towards oligodendrocytes, underscoring the broad tropism of AAVs in the CNS (Lawlor et al., 2009; von Jonquieres et al., 2013). Overall AAV2, AAV3 and AAV4 perform comparatively poorly in the CNS with notably reduced overall transduction efficiency and vector spread (Castle et al., 2016).

While direct IP AAV delivery is highly efficient and well tolerated, it requires complex surgeries and in diseases like leukodystrophies that affect large brain areas a trade-off between surgical risk and number of injection-sites is required. Consequently, alternative delivery routes were investigated and following ICV injection, AAV8 and AAV9 were identified as the superior serotypes crossing the CBB, while AAV4 appeared favorable when exclusively targeting the ependymal cell layer (Davidson et al., 2000; Chakrabarty et al., 2013; Bey et al., 2020). Comparison of 12 rAAV vectors for transgene expression in the CNS following IV revealed that particularly AAV7, AAV8, AAV9, AAV.rh8, AAV.rh10, AAV.rh39 and AAV.rh43 crossed the BBB (Gray et al., 2013; Samaranch et al., 2013; Yang et al., 2014). Important advances in natural discovery for CNS applications include novel clade F (AAV9 clade) AAV variants isolated from human $CD34^+$ HSC of which AAVHSC7, AAVHSC715 and AAVHSC717 effectively crossed the NHP BBB and transduce astrocytes, oligodendrocytes, neurons and cells of the choroid plexus (Smith et al., 2014; Ellsworth et al., 2019). Since AAVHSCs effectively transduce $CD34^+$ HSC they may also be employed in gene editing approaches for HSC-GT (Chatterjee et al., 2020). Using single molecule real time sequencing 81 novel capsids were identified from human tissue interestingly capsid variant AAVv66 crossed the BBB following IV administration and outperformed the prototype AAV2 in CNS biodistribution following both IP delivery (Hsu et al., 2020).

AAV Capsid Engineering

Due to its recognized potential as a gene therapy vector the crystal structure of AAV2, the most abundant naturally occurring AAV in the human population, was resolved in 2002 (Xie et al., 2002). Since then, capsid engineering has evolved rapidly to tailor rAAV vector properties to biomedical needs. **Figure 1A** depicts common strategies for identifying AAV polymorphisms to improve gene targeting and delivery. Comparison of natural AAV serotypes endemic to humans using high resolution crystallography and electron microscopy indicates that at the surface, the structural diversity of natural AAV serotypes is largely confined to nine variable regions (VR) that enable host interaction including receptor binding, endocytosis, and trafficking. These include depressions at each twofold, marked protrusions surrounding the threefold axis and pores and canyons at each fivefold (Xie et al., 2002; DiMattia et al., 2012; Gurda et al., 2013; Zinn et al., 2015). Five of these VRs are located in prominent protrusions and have been associated with serotype-specific antibody interaction and cell surface receptor binding (Tseng and Agbandje-McKenna, 2014). Transduction is thought to occur through primary proteoglycan receptors mediating attachment while secondary receptors cooperate with entry co-factors to facilitate internalization during AAV uptake. Primary receptors include heparan sulfate (AAV2, AAV3, AAV3b, AAV6), α2,3 and α 2,6N-linked sialic acid (AAV1), α2,3-O-linked sialic acid (AAV4) or terminal N-linked galactose (AAV9), while laminin receptor (AAV2, AAV3, AAV8, AAV9), fibroblast growth factor receptor, αvβ5 integrin (AAV2), and platelet derived growth factor (AAV5) have been identified as secondary receptors (Huang et al., 2014). Notably a pan AAV receptor (AAVR) that is widely distributed across tissues and cell types as well as the G-protein coupled receptor family member 108 (GPR108) have recently been recognized to be essential for efficient transduction of most known human and NHP AAVs (Mizukami et al., 1996; Pillay et al., 2016; Dudek et al., 2020).

To tailor rAAV as a gene therapy vector, important domains of AAV capsids can be harnessed in rational vector design to enhance transduction efficiencies and re-target AAV to select cell

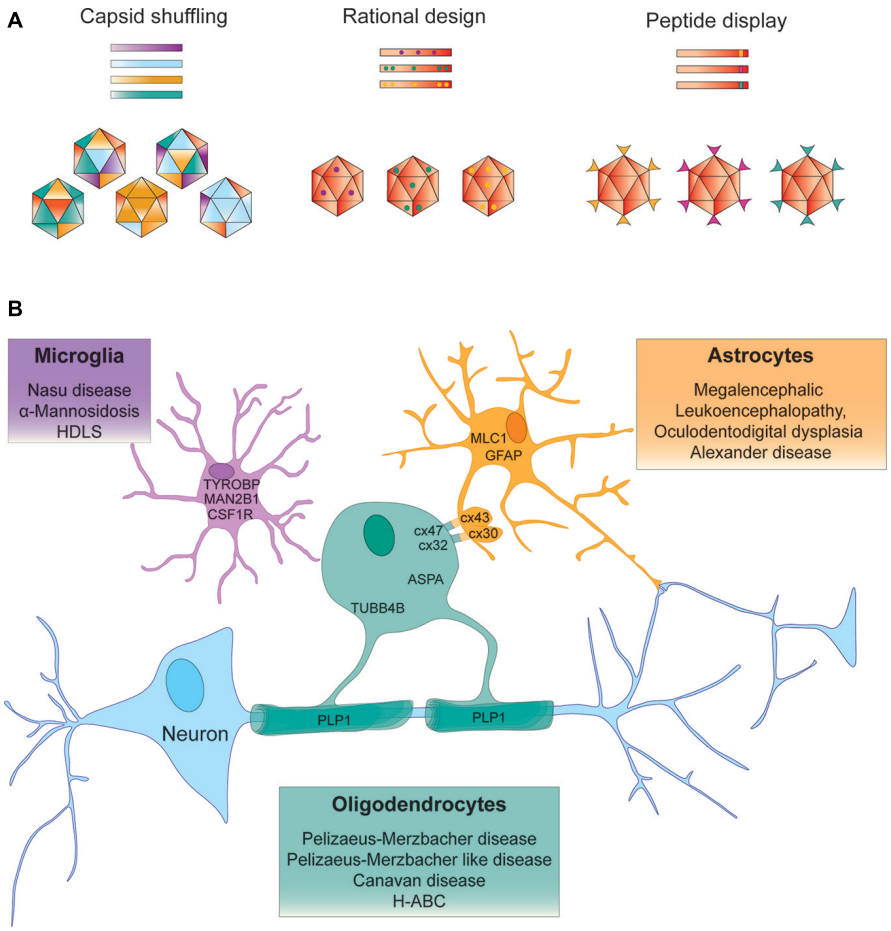


FIGURE 1 | Strategies to identify AAV vectors for AAV – mediated gene targeting to treat leukodystrophies. **(A)** Capsid shuffling uses capsid DNA from different AAV variants to generate chimeric capsid libraries to be screened for desired biological properties. Rational design relies on prior knowledge of structure – function relationships to transfer a predetermined functional aspect to another vector. Peptide display may incorporate peptides from a randomized library for screening purposes or peptides with known function into the AAV capsid. **(B)** Examples of leukodystrophy – specific gene targeting approaches that aim to deliver the therapeutic gene to the neural cell type that expresses the disease-causing gene.

types and tissues. Of the five exposed VRs located in the AAV capsid protrusions particularly VR-IV and VR-VIII have been utilized to expand tropism and re-target AAV without interfering with packaging and capsid stability. As this has been covered in reviews elsewhere (Grimm and Büning, 2017; Hüser et al., 2017; Lee et al., 2018; Büning and Srivastava, 2019; Wang et al., 2019; Li and Samulski, 2020) we only give an overview on highlights relevant to the development of CNS targeted gene therapy.

Numerous AAV capsids that have been engineered through capsid shuffling, error prone PCR or scanning mutagenesis and selected for more desirable characteristics, show the highest sequence variability in surface exposed regions (Büning et al., 2015). With potential relevance to the treatment development for some leukodystrophies, a recently identified AAV vector Olig001, generated through capsid shuffling demonstrated > 90% specificity for oligodendrocytes following direct intrastriatal injection into rats and NHPs. The most prominent differences in Olig001 from most parental capsids are located in VR-IV, VR-VII and VR-VIII. Not surprisingly the largest sequence similarity of this capsid is with AAV8, one of the stem vectors that has previously been shown to transduce oligodendrocytes (Powell et al., 2016; Mandel et al., 2017).

Microglia are the most challenging brain cell type to transduce using AAV vectors. Interestingly using targeted mutagenesis, a triple tyrosine mutant of AAV6 that effectively escaped proteasomal degradation targeted gene expression to microglia more effectively. This capsid variant may prove valuable for exploring treatments for select microgliopathies (Rosario et al., 2016; Schober et al., 2016).

More recently, the potential of *in silico* design of AAV capsids was demonstrated by ancestral capsid sequence reconstruction from 75 human and NHP capsid sequences predicting Anc80 as the ancestral node AAVs currently evaluated in clinical trials. Amino acid posterior probability calculation and alignment with AAV2 and AAV8 identified few variable residues and prompted the generation of an Anc80 capsid library, that was subsequently assessed for particle assembly and transduction efficiency *in vitro*. One variant Anc80L65 has since been praised for its outstanding

efficiency targeting sensory organs including the inner ear and retina. Anc80L65 has recently been shown to outperform AAV9 targeting the CNS, following IV injection but also revealed elevated transduction in the liver. Anc80L65 predominantly transduced astrocytes and neurons and was found to diffuse more extensively in the CNS following intraparenchymal delivery (Zinn et al., 2015; Hudry et al., 2018; Hudry and Vandenberghe, 2019). In a separate screen of a computationally designed capsid library, *in vivo* selection identified AAV.SCH9 which infected 60% of NPCs and achieved a 12-fold greater transduction volume compared to AAV9 following IP delivery (Ojala et al., 2018).

Efficient AAV uptake is key to successful AAV mediated gene delivery, but transduction efficiency of many capsids show species dependent variation, with the performance of capsids in rodent studies not always translating to larger animal models or humans. Importantly, AAV uptake itself does not guarantee transgene expression. Endosome escape, capsid degradation, intracellular transport, and nuclear translocation are significant hurdles. Only 20% of all AAV2 virions that infected fibroblasts were able to deliver their genetic payload to the nucleus and express the transgene (Hansen et al., 2000). This difference between virion uptake and 'functional transduction' resulting in transgene expression is AAV subtype, target cell and species dependent (Lisowski et al., 2014; Westhaus et al., 2020). Capsid phosphorylation at tyrosine and serine/threonine precedes ubiquitination at lysine residues and subsequent proteasomal degradation. Site specific mutagenesis of all surface exposed tyrosine serine, threonine residues and lysine residues has led to the identification of AAV2 mutants that are significantly more resistant to proteasome degradation and increase functional transduction (Zhong et al., 2008; Markusic et al., 2010; Aslanidi et al., 2013; Martino et al., 2013; Li et al., 2015).

Rational capsid design has also enabled intracellular redirection. Through integration of a mitochondrial targeting sequence, the virion directed the AAV expression cassette to mitochondria, where it facilitated transcription of the human NADH dehydrogenase 4 gene in mitochondrial genetic code (Yu et al., 2012).

Aside from optimizing capsids to navigate through the BBB and successfully transduce the target cells, CNS targeting following IV administration of therapeutic AAV requires protecting the capsid from deleterious interaction with the host immune system. Severe adverse events stemming from innate and cellular immune responses have occurred in several clinical trials associated with high-dose systemic AAV gene therapy, the exact mechanisms of which have been subject to recent review (Ronzitti et al., 2020). These include major histocompatibility complex type I (MHCI) mediated presentation of proteolytically processed capsids and the resulting cytotoxic T-cell activation as well as innate immune responses through pattern recognition receptors and the complement system. Due to the large prevalence throughout the population and broad cross-reactivity between serotypes, neutralizing antibodies can significantly counteract gene therapy and consequently, participants in many AAV clinical trials are now routinely screened to rule out pre-existing humoral immunity. More immune-evasive capsid variants including AAV-DJ that retain potent transduction efficiency have been generated through capsid shuffling (Grimm et al., 2008). Similarly, mapping of specific antibody epitopes on the capsids, which also appear enriched in variable regions and particularly in conserved residues along the three-fold axis are subjected to rational design of virions able to evade specific antibodies (Giles et al., 2018). Aside from genetic approaches a number of chemical avenues have been assessed to shield the AAV capsid from the host immune response and other undesired interactions (Kaygisiz and Synatschke, 2020).

AAV Peptide Display

A different approach to amend AAV tropism is the insertion of peptides into the AAV capsid. Peptides can be inserted into all AAV VPs since encoded in the same open reading frame, targeting VP3 will also alter VP1 and VP2 leading to peptide display on all 60 capsid subunits. Peptide insertions into the AAV2-I587 and I588 sites in VR-VIII destroy the HSPG binding motif and are commonly used in peptide display libraries and screens for cell surface receptor re-targeting (Büning and Srivastava, 2019). Corresponding sites have been used to alter vector tropism in various AAV serotypes and *in vivo* screening of barcoded capsid libraries is rapidly developing. To date only relatively few mutants with select cell type – specific tropism have been published. Notably, AAV9P1 has performed exceptionally well on cultured astrocytes (Kunze et al., 2018; Börner et al., 2020) and AAV.r3.45 with a seven amino acid peptide insertion selected from $> 3x10^6$ capsid variants through direct evolution, transduced rat NPCs with great efficiency *in vitro* (Jang et al., 2011). AAV vectors with enhanced retrograde transport efficiencies or select dopaminergic neuron tropism have also been identified, but peptide displaying capsids that selectively transduce oligodendrocytes or microglia remain to be identified (Davidsson et al., 2019).

Significant advances in CNS targeting following IV delivery have been achieved and these are important to reduce the extraordinarily large vector doses needed for IV AAV administration (Yang et al., 2014; Mendell et al., 2017). Due to its ability to cross the BBB, effectively transduce neural cells and its comparatively low prevalence in the human population, AAV9 has emerged as one of the most promising AAV vectors for CNS gene therapy. In a tour de force Adachi et al. used genome barcoding and alanine-scanning mutagenesis combined with biodistribution assays to link AAV9 capsid characteristics including the neutralizing antibody binding epitope and the galactosyl-proteoglycan-binding site to specific amino acids. Subsequent transfer of 10 key amino acids harboring the galactosyl-proteoglycan-binding site into heparin binding deficient AAV2 transferred this tropism characteristic to the resulting AAV vector (Adachi et al., 2014). Separately, identification of the BBB traversing footprint of AAV.rh10 and subsequent grafting of eight amino acids onto AAV1 converted AAV1RX into a BBB traversing vector with reduced transduction in liver and vasculature following IV delivery (Albright et al., 2018). Incorporation of 'functional transduction' parameters in the screening of an AAV9 capsid library that contained a randomized seven amino acid peptide in VP3 and coupled the

Cap gene with a Cre invertible switch, identified AAV.PhP.B, that crosses the murine BBB ∼ 40-fold more efficiently. Thus biodistribution of AAV.PhP.B was effectively shifted towards the CNS (Deverman et al., 2016). Expansion of the technology has since identified capsid variants that predominantly transduce CNS neurons (AAV.PhP.eB), peripheral neurons (AAV.PhP.S), equally transduce excitatory and inhibitory neurons (AAV.PhP.N) or vascular endothelial cells and astrocytes (AAV.PhP.V1) (Chan et al., 2017; Ravindra Kumar et al., 2020). Functionally, the enhanced BBB traversing efficiency of AAV PHP.B and PHP.eB was linked to glycosylphosphatidylinositol (GPI)-anchored lymphocyte antigen 6 complex (Ly6a) expressed on brain endothelial cells (Hordeaux et al., 2019). Although the improved ability of the above variants to cross the BBB is mouse strain dependent and did not translate to the marmoset, multiplexed Cre-recombination-based AAV targeted evolution (CREATE) identified capsid variant AAV.PhB.C1-3 that demonstrate enhanced efficiency traversing the BBB across all mouse strains and exhibit strong bias towards astrocytes (Hordeaux et al., 2018; Matsuzaki et al., 2018; Ravindra Kumar et al., 2020).

An exciting advance was the targeted insertion of nonviral moieties including designed ankyrin repeat proteins (DARPins) into the AAV capsid to elicit a desired receptor binding function (Büning and Srivastava, 2019). Development of an GluA4 DARPin expressing AAV2 that has a > 90% specificity for interneurons, highlights the potential of this technology (Hartmann et al., 2019). Similarly, AAV re-targeting was achieved via incorporation of unnatural amino acids that enabled peptide or aptamer conjugation to the capsid surface using click chemistry (Kelemen et al., 2016; Katrekar et al., 2018). Notably, switchable AAV2, that was de-targeted from HSPG binding through mutation in the above-mentioned protrusion can bind re-targeting DARPins after vector production (Münch et al., 2015). These studies are framed by the caveat that the human brain is likely to challenge these pre-clinical proof of concept studies, but none-the-less, validate the strategy and translational potential of hybrid AAV vector technologies for leukoencephalopathy-associated gene targeting.

The Recombinant AAV Genome

The key component of a gene therapy is the genetic payload and the regulation of its expression. The wildtype (wt) ssDNA genome of naturally occurring AAVs spans approximately 4.7 kilobases (kb) and, restricted by virion size, packaging capacity is capped at ∼5 kb or 2.3 kb in self-complementary (sc) genomes (Dong et al., 1996). scAAV genomes are palindromic and form a double stranded DNA in the transduced cell to facilitate rapid transgene expression without prior DNA replication (McCarty, 2008). With the exception of the terminal inverted repeats (ITRs), the only *cis*-acting element (CRE) required for rAAV vector production, the entire wt genome is replaced by the recombinant expression cassette consisting of promoter, genetic payload and regulatory elements. The ITRs harbor the essential replication and packaging signals and are vital for episome formation and protection from cellular nucleases. Most AAV vectors are produced via cross-packaging where ITRs and Rep genes from one serotype are packaged into the capsid from another. The most commonly used ITRs stem from AAV2 (Wilmott et al., 2019).

Ideally a gene therapy should aim to restore as close to natural gene expression profile as possible to reset the proteomic and metabolomic micro-environment. Genetic payloads and mechanisms to control transgene expression have become increasingly versatile and can include complete coding sequences for proteins, untranslated regions (UTRs) and regulatory elements beyond the polyadenylation signals including post transcriptional regulatory elements, ribozymes, aptamers, regulatory RNAs like short hairpin RNAs (shRNAs), microRNAs (miRNAs), other non-coding RNAs and guide RNAs for gene editing (Domenger and Grimm, 2019; Wang et al., 2020). These combined aspects of the AAV toolkit need to be considered in a gene therapy strategy for gene replacement, gene editing, gene addition and to modulate or abrogate target gene expression. Ubiquitous unregulated expression of a genetic payload can have detrimental side effects. Consequently, implementation of mechanisms that restrict and control transgene expression are rapidly gaining attention in the field.

The first level of gene regulation is governed by the promoter that recruits transcription factors (TFs) in a sequence specific manner to initiate transcription through RNA polymerases. The proximal core promoter is generally located within the first 1 kb of the transcription start site, but additional *cis*-regulatory elements (CREs) carrying clusters of binding sites for silencers and enhancers may be located up to 1 megabase pair up or downstream in intergenic or intronic regions. rAAV – mediated transgene expression has predominantly employed strong constitutive eukaryotic, viral or hybrid promoters that are thought to drive expression in all cell types. Prominent examples include the cytomegalovirus (CMV) promoter, the phosphoglycerate kinase (PGK), elongation factor 1 α (EF1α), ubiquitin C (UbiC) and chicken β-actin promoter and various hybrids thereof. The 1.6kb CAG promoter comprises the CMV immediate early enhancer and the chicken β-actin promoter fused to the first exon and intron and the splice acceptor of the rabbit β-globin gene. In light of the limited packaging capacity of AAV vectors, size reduction of the of CAG promoter hybrids to ∼ 800 bp by replacing the 5'-UTR of CBA with either a truncated simian virus 40 intron or minute virus of mice VP intron were welcomed achievements (Wang et al., 2003; Gray et al., 2011). CBA hybrids with the CMV immediate early enhancer (CAGGS) a truncation thereof (CBh) enable long term transgene expression in the CNS. CMV and CBA promoter hybrids are now employed in FDA approved AAV drugs (Niwa et al., 1991; Klein et al., 2002; Gaudet et al., 2013; Mendell et al., 2015, 2017; Armbruster et al., 2016; Weleber et al., 2016; Pennesi et al., 2018; Al-Zaidy et al., 2019; Gray et al., 2019). Direct intraparenchymal injections of different AAV serotypes into the mammalian CNS employing these constitutive promoters yielded strong, predominantly neuronal transgene expression, thus giving AAV the reputation to be a largely neurotropic vector, but most AAV serotypes and variants have since been shown to have a relatively broad tropism.

While capsid evolution and rationally designed AAV variants may home in on specific cell types, employing cell type – specific promoters can restrict transgene expression to select cell types (Klein et al., 2008; Lawlor et al., 2009; von Jonquieres et al., 2013; Gessler et al., 2019). Among the first, the relatively large 1.8 kb human neuron specific enolase (NSE) promoter, the 1.5 kb human platelet derived growth factor (PDGF) promoter and the 1.3 kb Ca^{2+}/calmodulin kinase II (CamKII) promoter successfully to drive AAV – mediated transgene expression in neurons. Shorter neuron specific promoters including the 0.47 kb human synapsin (hSYN1) promoter, a compacted 0.4 kb truncated neuronal-specific CamKII promoter and the 0.23 kb methyl CpG binding protein (MeCP2) promoter have been generated since (Peel et al., 1997; Kügler et al., 2001; Shevtsova et al., 2005; Nathanson et al., 2009; Gray et al., 2011; Chandler et al., 2017).

With regards to AAV gene therapies targeting some leukodystrophies, promoter mediated restriction of transgene expression to glial cells is desirable. The 2.2 kb human glial acidic glycoprotein (GFAP) promoter was the first to show clear astrocyte specificity in the AAV setting (Brenner et al., 1994). Following identification of a *cis*-regulatory element (CRE) required for silencing activity in neurons, this promoter has since been compacted to a twofold more active 681 bp gfaABC$_1$D promoter with comparable astrocyte specificity (Lee et al., 2008) and a gfa2(B$_3$) variant targeting GFAP positive astrocytes in mouse basal ganglia. More recently, following the identification of aldehyde dehydrogenase 1 like protein 1 (ALDH1L1) as a pan-astrocytic marker, the 1.3 kb human ALDH1L1 promoter has restricted AAV – mediated transgene expression to astrocytes in certain brain regions in mice (Cahoy et al., 2008; Koh et al., 2017).

Oligodendrocyte specific expression in the context of AAV was first achieved with the 1.9 kb mouse myelin basic protein promoter (Mbp) promoter, but heavily depends on the timepoint of AAV infusion after onset of myelination in mice (Chen et al., 1999; Lawlor et al., 2009; von Jonquieres et al., 2013). This promoter has since been used to target oligodendrocytes in preclinical gene therapies for Pelizaeus – Merzbacher like disease and for CD (Georgiou et al., 2017; von Jonquieres et al., 2018). Following identification of the myelin gene regulatory factor (MRF) as a critical transcriptions factor regulating myelination in the CNS and characterization of its consensus binding motif (Emery et al., 2009; Koenning et al., 2012), screening for this motif in evolutionary conserved regions in close proximity to the transcription start sites of known myelin associated genes, identified the 0.3 kb human myelin acidic glycoprotein (MAG) which was able to restrict AAV – mediated expression to oligodendrocytes *in vivo* (von Jonquieres et al., 2016). More recently oligodendrocyte specific expression of a synthetic miRNA was achieved using the 1.8 kb human 2',3'-cyclic nucleotide 3- phosphodiesterase (CNP) proximal promoter (Li et al., 2019).

In the context of targeting microglia, several promoters including CD68 and CD11b have been assessed with the F4/80 promoter being the most promising to date (Rosario et al., 2016). However, CD68 is upregulated in activated phagocytosing microglia and consequently the CD68 promoter may prove more effective and valuable in a disease setting (Zotova et al., 2013).

Founded on the growing availability of genomic data new bioinformatic strategies for rational and synthetic promoter design are evolving. Transcription is governed through large and convoluted deoxyribonucleoprotein complexes that can often harbor both repressor and activator potential. Promoter activity depends on the tissue, cell type and environment specific nucleoproteome, chromatin condensation and epigenetic footprint within the given cell. Higher throughput bioinformatic directed parallel design for example through the Pleiades Promoter Project has identified cell type and tissue specific core promoter regions and CREs. Subsequent compaction of the core promoter and multiple conserved computationally – predicted CREs to AAV compatible 'MiniPromoters' has led to the identification of four glial specific promoters stemming from S100B (Ple266), UTG8 (Ple267) and Olig1 (Ple304, Ple305) (Portales-Casamar et al., 2010; de Leeuw et al., 2016). Many CREs have been shown to retain their activity when used as building blocks in modular synthetic or compacted promoters (Tornøe et al., 2002; Lee et al., 2008; Domenger and Grimm, 2019). Screening of a barcoded library containing 230 synthetic promoters incorporating similar bioinformatic designs found 11% of promoters drive expression in specific cell types in the CNS. Notable examples are ProC17 which efficiently restricts AAV PhP.B mediated transgene expression following IV injection to parvalbumin positive neurons in the CNS, while ProB12 generated by ordered assembly of evolutionary conserved CREs directed transgene expression to a subpopulation of protoplasmic astrocytes (Jüttner et al., 2019). With regards to promoter development in the AAV context, it is important to note, that the ITRs themselves have weak inherent promoter activity and CRE have been identified in AAV2 ITRs that may affect the transgene expression (Flotte et al., 1992, 1993; Haberman et al., 2000; Logan et al., 2017). In addition, it has recently been observed, that promoter – capsid interactions cause a shift in the AAV9 gene expression profile from neurons to oligodendrocytes demonstrating that interactions with the capsid and the recombinant genome may affect functional transduction in a cell type – specific manner *in vivo* (Powell et al., 2020). This fundamental discovery emphasizes the importance of screening capsid and expression cassette elements for functional transduction in conjunction (Bohlen et al., 2020; Powell et al., 2020). Together these findings underscore the importance of integrating screening for functional transduction in humanized animal models replicating target cell and disease state as closely as possible.

In the case of liver directed gene therapies, this was achieved by repopulating the liver of *Fah*$^{-/-}$; *Rag2*$^{-/-}$; *Il2rg*$^{-/-}$ (FRG) mice with primary human hepatocytes where screening for functional transduction revealed that bioengineered capsids outperform natural serotypes (Lisowski et al., 2014; Westhaus et al., 2020). While to our knowledge this has not yet been done in the brain, appropriate models are available. For example, the CNS of the *Mbp*$^{-/-}$ *Rag2*$^{-/-}$ shiverer mouse has successfully been repopulated with human glial progenitors and human iPSC (Wang et al., 2013; Windrem et al., 2014;

Osorio and Goldman, 2016). Similarly, the recent advances in preclinical cell therapy for CD using human iPSCs may lend itself to similar screens in the CNS (Feng et al., 2020). In fact, AAV – mediated expression of Oct4, Klf, Sox2, and Myc has also been used to induce pluripotency (Senís et al., 2018).

Ideally a therapeutic transgene should self – regulate its own expression tailored to the requirements of the disease state. This might also be achieved by CREs in the promoter responding to metabolic or physiological changes associated with specific disease states in future. An exemplar is the incorporation of hypoxia response elements into the astrocyte specific, compacted $GfaABC_1D$ variant generating a hypoxia – inducible GFAP promoter (Prentice et al., 2011). Similarly, introduction of a ARF5/AuxRE transcription factor/response element enabled cleaved caspase 3 dependent transcriptional activation in cells undergoing apoptosis (Vagner et al., 2015). It remains to be determined, if similar system enable autoregulation of therapeutic gene expression controlled by the disease state in leukodystrophies.

A different advance was the incorporation of riboswitches that facilitate inducible inhibition of a highly active synthetic ribozyme that reliably cleaves the transgenic mRNA unless inhibited with a specific antisense oligonucleotide or small molecules, thereby effectively allowing control of transgene expression (Strobel et al., 2020; Zhong et al., 2020). Another key gene therapy safety element that is under investigation is the control of AAV – mediated transgene expression in off-target tissue where transgene expression may be undesirable or induce an immune response. Because these strategies have been recently reviewed, we will only highlight a notable advance with direct relevance to CNS targeted gene therapy (Domenger and Grimm, 2019; Ingusci et al., 2019).

RNA interference, through incorporation of miRNAs can either be driven by cell type – specific RNA polymerase lI promoters and/or incorporated in the 3'UTR of the transgenic mRNAs. The latter is possible because the active secondary structure of many miRNAs is determined by specific RNA binding proteins and the alternative splicing machinery differs among cell types. Notably, a successful IV AAV9-*ASPA* gene replacement therapy in a CD mouse model, incorporated a miRNA with muscle specific miRNA-1 and liver specific miRNA-122 sites ablating transgenic *ASPA* expression in these organs, but not in the CNS (Ahmed et al., 2013).

Particularly in the case of gene replacement in which the host's immune system is naïve to the transgene, transduction of antigen-presenting dendritic cells, macrophages or B-lymphocytes can lead to transgene – derived peptide presentation on MHCI molecules and trigger $CD8^+$ cytotoxic T-cell activation. Incorporation of the hematopoietic lineage specific synthetic miRNA-142 targeting the transgene in the 3'UTR of the same AAV vector was sufficient to suppress this immunotoxicity to the point that allowed re-administration of the same transgene albeit with a different AAV serotype (Xiao et al., 2019). This strategy may also mitigate immune reactions targeting the bacterial Cas9 transgene in AAV – mediated CRISPR gene editing *in vivo*. Strategies and advances in this field can be pursued via a recent review (Wang et al., 2020).

CONSIDERATIONS FOR THE NEXT GENERATION OF GENE THERAPIES FOR LEUKODYSTROPHIES

Although currently assessed in clinical trials, rAAV vector spread may be a limiting factor in intracerebral treatment of neurological diseases affecting large areas of the CNS (Hudry and Vandenberghe, 2019). In fact, it has been estimated, that over 100 needle tracts would be required to achieve adequate coverage of the entire human brain (Hinderer et al., 2018a). Since the discovery of BBB and CBB traversing AAV vectors including AAV9 and growing confidence in their excellent safety profile, the prospect of achieving widespread gene transfer to the CNS via non-invasive vector delivery attracted great attention. Following a breadth of supportive clinical trials this advanced to regulatory approval of Zolgensma®. However, the recent tragic deaths following severe hepatobiliary disease, bacterial infection and sepsis associated with high dose (3×10^{14} vg/kg) IV delivery of AAV vector (AAV8-MTM1) in the ASPIRO phase I/II clinical trial targeting X-linked myotubular myopathy (NCT03199469), is a reminder that despite comprehensive pre-clinical safety and efficacy studies and the excellent safety profile of AAV even at high doses, outcomes in first human trials may result in the severest adverse events (Philippidis, 2020; Wilson and Flotte, 2020). Growing safety concerns regarding liver toxicity in nonhuman primates and pigs subjected to systemic high dose (AAV9-SMN, 1.2×10^{14} vg/kg) and genotoxic integration potentially increasing the risk of liver cancer founded on clonal hepatocyte expansion observed in dogs (AAV9-cFVIII, 2.5×10^{13} vg/kg) emphasize the importance of continued pursuit of the safest and most efficient AAV vectors, particularly for the treatment of leukodystrophies in which the entire CNS needs to be targeted, which inherently requires a high vector dose (Hinderer et al., 2018b; Nguyen et al., 2021). This entails AAV vector development with enhanced ability to traverse the BBB and/or CBB. In addition, de-targeting the therapeutic rAAV capsid from off-target organs and improving transduction of a selected cell types and tailored therapeutic gene expression to these cells will reduce the vector dose required to achieve therapeutic benefit. At present, regardless of the disease, experience in clinical trials with the current gold standard AAV9 indicates effective CNS targeting via IV, IT or ICM delivery requires vector doses above 1×10^{14} vg/kg. However, as discussed above promising progress is made in vector development and up to 40-fold dose reductions are already achievable in mice (Deverman et al., 2016, 2018). In addition, it is increasingly evident that gene therapy for many genetic diseases would benefit from a cell type targeted and inducible approach, that restricts transduction and/or expression of the genetic payload to a specific target cell type. **Table 1** gives an overview of naturally – occurring and engineered AAV capsids and promoters observed when expressing green fluorescent protein and similar fluorescent markers, that may be considered when targeting neural cell types. Ongoing development in this area of the next generation gene therapies will be relevant to the treatment of select leukodystrophies

TABLE 1 | Summery of AAV capsids and cell type targeted promoters that achieve AAV mediated transgene expression in neural cells.

	*Capsid**	*Promoter*
Neurons	**AAV1, AAV9, AAV.rh10, AAV.v66, Anc80L65, PhP.B, PhP.eB,** AAV2, AAV5, AAV7, AAV8, AAV.rh8, AAV.rh20, AAV.rh39, AAV.cy5 AAVHSC7,15,17, AAV.PhB.C1-3 6	*NSE PDGF CamKII hSYN1 MeCP2 ProC17 Ple155*
Astrocytes	**AAV8, AAV.rh43, Anc80L65, AAV.PhB.C1-3, AAVHSC.7, AAVHSC.15, AAVHSC.17,** AAV1, AAV2, AAV5, AAV7, AAV8, AAV9, AAV.rh8, AAV.rh10, AAV.rh20, AAV.rh39, AAV.cy5, AAV.v66	*GFAP gfaABC1D gfa2 (B_3) ProB12 Ple266 Ple267*
Oligodendrocytes	**AAV8, Olig001, AAV.rh10 AAV9**, **AAV.rh20, AAV.rh39, AAV.cy5,** PhP.B, AAV.v66, AAVHSC7,15,17, AAV1/2	*Mbp MAG CNP Ple304 Ple305*
Microglia	**rAAV6, AAV.v66,** AAV5, AAV7, AAV8, AAV9, AAV.rh10	*F4/80 CD68 CD11b*
Ependymal cells & vascular endothelia	**PhP.V1**, AAV1, AAV4, AAV5, AAV7, AAV8, AAV9	*Ple261*
Neural progenitor cells	**AAV.SCH9, AAV.r3.45**	to be identified

**Bold font indicates superior transduction was reported for the AAV capsid.*

and leukoencephalopathies. Gene therapy should first and foremost be safe, halt disease progression, improve the quality of life by resolving the underlying pathophysiological cause and ultimately aim to restore as close to natural gene expression profile in the CNS while minimizing potential off target effects associated with the treatment. With this focus in mind, **Table 2** summarizes the publicly available cellular expression profile of known leukodystrophy associated genes, founded on RNA sequencing of neural cells isolated from the murine cerebral cortex (Zhang et al., 2014). Inclusion of leukodystrophies in **Table 2** is founded on a classification according to pathogenic mechanisms and pathological changes (van der Knaap and Bugiani, 2017). The leukodystrophies selected are exemplary and include leukodystrophies from each category with a clear CNS white matter phenotype, however this selection is not meant to be exhaustive. **Figure 1** provides a graphical overview of targeting strategies for leukodystrophy gene therapy development when aiming to restore the normal, natural cell type specificity of gene expression.

LEUKODYSTROPHIES THAT MAY BENEFIT FROM OLIGODENDROCYTE TARGETING

Oligodendrocytes are the myelin forming cell in the CNS, where they provide axon insulation and enable saltatory impulse propagation which is critical to ordered connectivity between brain regions and with the peripheral nervous system (PNS), where this role is taken by Schwann cells (Kuhn et al., 2019). Particularly during active myelination oligodendrocytes have among the highest metabolic rates in any cell type and require high levels of iron, an essential cofactor in the mitochondrial respiratory chain. In combination with their high turnover, the elevated production of reactive oxygen species and low level of glutathione make oligodendrocytes particularly vulnerable to oxidative stress and free radical formation culminating in lipid peroxidation and cell death (Bradl and Lassmann, 2010). This explains why white matter and oligodendrocyte pathology is prevalent in many leukodystrophies where the mutated gene is not primarily expressed in oligodendrocytes. Nevertheless, RNA sequencing data summarized in **Table 2** indicates that in many hypomyelinating and some vacuolating leukodystrophies the mutated gene is almost exclusively expressed in oligodendrocytes, while in many demyelinating leukodystrophies the expression pattern is often broader.

Pelizaeus-Merzbacher Disease

Pelizaeus-Merzbacher disease (PMD) is a X-linked, recessive dysmyelinating leukodystrophy with the most common and severe forms caused by proteolipid protein 1 (*PLP1*) gene duplication (Inoue et al., 1996; Hudson, 2003; Osório and Goldman, 2018). Resulting PLP1 overexpression triggers oligodendrocyte dysfunction and prevents proper myelin formation leading to classic PMD. This presents with nystagmus, head tremor, systemic hypotonia and hypomyelination usually in the first year of life, followed by spasticity and progressive motor and cognitive decline (Osório and Goldman, 2018). Notably, without addressing the clinical cause, a pre-clinical trial found feeding PMD mice a high cholesterol diet delayed PMD pathology and preserved myelin (Saher et al., 2012). Whether the high dose cholesterol diet is beneficial or sustainable in humans remains to be determined.

Addressing the causative overexpression of *PLP1* was recently achieved using oligodendrocyte specific RNA interference in PMD mice. To restore as close to natural PLP1 protein levels as possible, careful choice of the miRNA backbone, targeting sequence and promoter is needed to avoid off-target effects and RNA interference (RNAi) mediated toxicity. Direct intraparenchymal injection of scAAV1/2 mediating oligodendrocyte restricted expression of a synthetic miR-155 harboring a PLP1 directed short hairpin prevented oligodendrocyte demise, restored myelin, and improved neurological phenotypes and survival (Li et al., 2019). These findings indicate that AAV – mediated oligodendrocyte targeted RNAi for PMD holds promise when the disease is caused by gene duplication.

In contrast to CNS myelin formed by oligodendrocytes, peripheral myelin generated by Schwann cells can tolerate gene duplication but is more vulnerable to nonsense mutations in the PLP1 gene (Shy et al., 2003). Thus, when targeting gene duplication derived PMD, ICM AAV delivery (found to be the superior CSF delivery route in non-human primates) may be the most promising route of administration, mitigating

TABLE 2 | Gene expression of leukodystrophy associated genes across neural cell types.

Disease	Gene	Genebank ID	Astrocyte	Neuron	OPCs	Oligo-dendrocyte	Microglia	Vascular endothelial cells
Hypomyelinating								
Pelizaeus-Merzbacher like disease	*Gjc2*	*118454*	-	-	-	+++	-	-
Pelizaeus-Merzbacher disease	*Plp1*	*18823*	-	-	-	+++	-	-
Hypomyelination with atrophy of the Basal Ganglia and cerebellum (H-ABC)	*Tubb4a*	*22153*	-	-	-	+++	-	-
Sox10 associated PCWH syndrome	*Sox10*	*20665*	-	-	++	++	-	-
Vacuolating								
Canavan disease	*Aspa*	*11484*	-	-	-	+++	-	-
Cx32- related Charcot-Marie-Tooth disease	*Gjb1*	*14618*	-	-	-	+++	-	-
Demyelinating								
Krabbe disease	*GalC*	*14420*	+	-	++	++	-	+
Metachromatic leukodystrophy	*Arsa*	*11883*	+	+	+	+	+	+
X-linked adrenoleukodystrophy,	*Abcd1*	*11666*	-	-	-	+	++	-
Multiple sulfatase deficiency	*Sumf1*	*58911*	+	+	+	+	++	+
Astrocytopathies								
Megalencephalic leukodystrophy with subcortical cysts	*Mlc1*	*170790*	+++	-	-	-	-	-
Oculodentodigital Dysplasia with cerebral white matter abnormalities	*Gja1*	*14609*	+++	-	-	-	-	+
Alexander disease	*Gfap*	*14580*	+++	-	-	-	-	-
ClC2 related leukoencephalopathy	*Clcn2*	*12724*	++	+	+	++	-	-
Vanishing White Matter Disease	*eif2b1 – 5**	*209354, 217715, 108067, 13667, 224045*	+	+	+	+	+	+
Aicardi Goutieres Syndrome	*Rnaseh2a, b, c*	*69724, 67153, 68209*	+/+/+	+/+/+	+/++/+	+/+/+	+/+/++	+/+/++
Microgliopathies								
Hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS)	*Csf1R*	*12978*	-	-	-	-	+++	-
Nasu disease	*Tyrobp*	*22177*	-	-	-	-	+++	-
α-Mannosidosis	*Man2b1*	*17159*	-	-	-	-	+++	-
Disease	**Gene**	**Genebank ID**	**Astrocyte**	**Neuron**	**OPCs**	**Oligo-dendrocyte**	**Microglia**	**Vascular endothelial cells**
Leuko-axonopathies								
Developmental and epileptic encephalopathy 29	*Aars1*	*234734*	+	+	+	+	+	-
Leukoencephalopathy, progressive with ovarian failure	*Aars2*	*224805*	+	+	+	+	+	+
Hypomyelination with brainstem spinal cord involvement & leg spasticity	*Dars1*	*226414*	+	+	+	+	-	+
Leukoencephalopathy w brainstem, spinal cord involvement, elevated lactate	*Dars2*	*226539*	+	+	+	+	+	+
Combined oxidative phosphorylation deficiency 12	*Ears2*	*67417*	+	+	+	+	+	-
Leukodystrophy, hypomyelinating 9	*Rars1*	*104458*	+	+	+	+	+	-
AIMP1 related disease	*Aimp1*	*13722*	-	+	+	+	-	+
H4 leukodystrophy	*Polr3a, b**	*218832, 70428*	+	+	+	+	+	+
GM1 Gangliosidosis	*Glb1*	*12091*	+	+	+	+	+	-
Tay-Sachs GM2 Gangliosidosis	*Hexa, Hexb**	*15211*	-	-	-	-	+++	-
Sanfilippo syndrome (MPS IIIA)	*Naglu/Sgsh*	*27419, 27029*	- / +	- / -	- / +	- / +	++	- / +
Leuko-vasculopathies								
Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)	*Notch3*	*18131*	+++	-	-	-	-	+
Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL)	*Htra1*	*56213*	++	-	+	-	-	+

**Expression pattern of genes in this family are similar.*

the risk of complex surgery associated with direct multisite intraparenchymal injection and of immune – response and high off target transduction from intravenous delivery (Hinderer et al., 2014). This relies on a capsid that efficiently crosses the CBB via transcytosis of the ependymal layer and readily transduces oligodendroglial cells in the brain. This is currently achieved with AAV9 (Hinderer et al., 2018a). However, more efficient capsid variants are likely to become available enabling dose reduction.

PLP1 is the prototype of a dosage sensitive gene with missense mutations leading to absence of PLP1 causing much milder disease while PLP1 nonsense mutations generating misfolded or incomplete PLP1 frequently promote dysmyelination and early oligodendrocyte death. In these cases, gene replacement in combination with RNAi is unlikely to achieve the exact non-toxic gene dose required during oligodendrocyte differentiation, myelination, and maintenance. Therefore, a gene editing approach may be a better gene therapy option, but immunological complications associated with bacterial Cas9 expressions will still need to be resolved. Antisense oligonucleotides (ASOs), particularly when packaged into oligodendrogliotrophic exosomes that effectively cross the BBB are another developing technology that may hold promise in future. Indeed, therapeutic promise has recently been demonstrated in the 'jimpy' mouse model of PMD where single administration of a *Plp1* – targeting ASO restored oligodendrocyte numbers, increased myelination, improved motor performance and extended lifespan (Elitt et al., 2020).

Pelizaeus-Merzbacher Like Disease

Pelizaeus-Merzbacher like disease (PMLD) is a hypomyelinating leukodystrophy with very similar clinical manifestation to PMD but caused by autosomal recessive loss-of-function mutations in the gap junction protein connexin 47 (*GJC2*) gene (Orthmann-Murphy et al., 2007). High levels of GJC2 are found exclusively in the CNS where it is selectively expressed by oligodendrocytes (**Table 2**). AAV1/2 – mediated *Mbp* promoter – driven *GJC2* gene replacement by IP injection re-established oligodendrocyte gap junction connectivity and rescued the severe demyelination in a mouse model of PMLD. This underscores the therapeutic potential of an oligodendrocyte targeted AAV – mediated gene therapy (Georgiou et al., 2017). Given the high *GJC2* expression throughout the CNS white matter including in the spinal cord, broad CNS targeting is required and likely sufficient and achievable through ICM delivery into the CSF which can reduce the vector doses and potential immunogenicity, toxicity, and off target risks of systemic IV administration (Nualart-Marti et al., 2013; Hinderer et al., 2014, 2018a,b).

Hypomyelination With Atrophy of the Basal Ganglia and Cerebellum

Typically, H-ABC presents in early childhood with varied rate of progression and severity involving complex pathology including spasticity, dystonia, dyskinesia ataxia and tremor. Motor function is commonly more severely affected than cognition. H-ABC has been associated with toxic 'gain of function' mutations in the TUBB4a gene encoding the microtubule associated protein tubulin β-4a (Nahhas et al., 1993; Hamilton et al., 2014; Curiel et al., 2017). Resolution of the cell type – specific expression in the mouse brain reveals that TUBB4a is almost exclusively expressed in the oligodendroglial lineage in the cortex, while in humans low expression in astrocytes and neurons was detected (Zhang et al., 2014, 2016). Recent development of an accurate mouse model of H-ABC now opens up pre-clinical investigation (Sase et al., 2020). Based on the gain of function hypothesis, a potential therapy would be largely oligodendrocyte focused, directed to reduce mutated tubulin β-4a protein while restoring its wt form. This may be achieved via AAV – mediated expression of a synthetic *TUBB4a* miRNA. To prevent potential undesired effects associated with loss TUBB4A, targeting the miRNA to the untranslated region and simultaneous expression of transgenic wt *TUBB4a* both driven by a predominantly oligodendrocyte – specific promoter may be viable. The AAV serotype chosen, and associated delivery route, would need to efficiently transduce oligodendrocytes. Similar to the above, AAV – CRISPR mediated gene correction and potentially ASO may become additional treatment options in future.

SOX10-Associated PCWH

SOX10-associated peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease (PCWH) presents with a complex central and peripheral pathology (Sánchez-Mejías et al., 2010). Because Sox10 is a transcription factor known to drive oligodendrocyte differentiation and myelination, Sox10-associated PCWH would rely on *SOX10* gene replacement in oligodendroglial progenitor cells (OPC). The infantile forms may be particularly amendable to AAV – mediated gene replacement, but suitable capsids enabling selective OPC transduction following efficient delivery to the CNS across the CBB or BBB and appropriate promoters remain to be identified. Potentially AAV variants SCH9 or AAV.r3.45 that have been shown to effectively transduce NPCs are promising candidates.

Canavan Disease

Canavan disease is a spongiform leukodystrophy caused by loss-of function mutations in the oligodendroglia aspartoacetylase (*ASPA*) encoding gene. Its substrate *N*-acetylaspartate (NAA) is the second most abundant brain metabolite, being predominantly synthesized by aspartate-*N*-acetyltransferase NAT8L in neuronal mitochondria. Although the exact function of NAA remains enigmatic, lack of NAA hydrolysis by ASPA which is exclusively expressed in oligodendrocytes causes its toxic accumulation and severe white matter vacuolization. Age of onset generally correlates with disease severity and in the most common infantile form children present with hypotonia, macrocephaly and fail to reach developmental milestones in months after birth before progressively losing motor function, seizure development and premature demise (Hoshino and Kubota, 2014; Gessler and Gao, 2016). CD was the first CNS disease to ever be treated in a gene therapy in humans more than 20 years ago, utilizing a lipid-entrapped, polycation-condensed delivery system (LPD) with an *ASPA* encoding plasmid DNA (Leone et al., 2000).

Later, pre-clinical AAV – mediated *ASPA* gene replacement benefit was observed following intraparenchymal injection of a first generation rAAV2-CBA-*ASPA* vector into CD mice and rats (Matalon et al., 2003; McPhee et al., 2005). Since that time due to the availability of accurate rodent models (Matalon et al., 2000; Traka et al., 2008; Ahmed et al., 2013; von Jonquieres et al., 2014), several pre-clinical and clinical studies have achieved promising outcomes using AAV – mediated gene replacement, RNA interference and recently cell therapy advancing understanding and prospect of the field. In 2013 the Gao lab was first to employ IV rAAV-CAG-*ASPA* delivery using AAV9, AAV.rh8 and AAV.rh10 vectors and included a liver, heart and skeletal muscle de-targeting miRNA. In a CD mouse model, injections up to postnatal day (P)20 targeted predominantly neurons and achieved long-lasting therapeutic benefit by lowering NAA levels, preventing spongiform white matter vacuolization and markedly improved motor performance (Ahmed et al., 2013). This outcome was further improved in the next generation vectors targeting astroglia with codon optimized *ASPA*. Intravenous AAV9-phGFAP-*ASPA* at P1 achieved widespread ASPA expression throughout the CNS and completely reversed the CD phenotype to the point that at the highest dose treated animals performed better than their untreated WT littermates in some behavioral tests (Gessler et al., 2017). In a separate study, targeting oligodendrocytes via direct intraparenchymal injection of AAV.cy5-Mbp-*ASPA* at P30 following disease onset, achieved near complete correction of the CD phenotype including NAA levels vacuolization and motor performance (von Jonquieres et al., 2018). Following the realization that NAA is largely dispensable, at least for myelination and motor function in mice (Guo et al., 2015; Maier et al., 2015; von Jonquieres et al., 2018), an shRNA mediated *Nat8l* knockdown strategy using P1 ICV and ICM delivery of AAV8-U6-*Nat8l*-shRNA achieved comparable therapeutic outcome indicating that in the absence of oligodendroglial ASPA reduction of NAA levels provides key therapeutic benefit (Bannerman et al., 2018). Phenotypic improvement was achieved regardless of the target cell, strategy or delivery route as long as widespread ASPA expression was achieved throughout the CNS. This is underscored by the observation that induced pluripotent stem cells directly injected into the CD mouse CNS differentiated into astrocytes, oligodendrocytes and neurons providing therapeutic benefit comparable to gene therapy approaches (Feng et al., 2020).

Pioneering AAV – mediated gene therapy for leukodystrophies in humans, direct intraparenchymal injection of AAV2-*ASPA* into six sites was performed in phase I clinical trials. Importantly, emphasizing safety and underscoring the immune privilege of the CNS, no neutralizing antibodies and only mild immune response was detected (McPhee et al., 2006). In a 10-year follow up study modest improvement of NAA levels and seizure frequency were accompanied by slowed atrophy and stabilization of clinical status (Leone et al., 2012). Looking forward, glial cell targeted *ASPA* replacement employing novel capsids in combination with peripheral organ de-targeting should enable dose reduction for CSF or IV administration and holds significant promise for CD gene therapy. In addition, NAT8L targeted ASOs were able to promote rapid NAA reduction and reduce vacuolization following postsymptomatic intracisternal administration (Hull et al., 2020). It will be exciting to validate the benefit of presymptomatic and/or repeated ASO administration particularly in the early stage of the disease stage when combined with *ASPA* gene replacement, as *Nat8l* targeting ASOs rapidly decrease NAA load bridging valuable time until transgenic ASPA expression is fully established. Based on the natural ASPA expression pattern, identification of capsid and promoter combinations that efficiently target AAV mediated ASPA expression to human glia will likely hold clinical promise. Parental capsids included in functional transduction screens for efficient human oligodendrocyte specific vectors that cross the BBB and CBB should include Olig001, that has been reported to selectively transduce oligodendrocytes in mice (Powell et al., 2016, 2020).

Krabbe Disease (Globoid Cell Leukodystrophy)

Krabbe disease is a demyelinating leukodystrophy caused by autosomal recessive mutations of the galactosylceramidase (*GALC*) gene rendering the lysosomal hydrolytic enzyme that is required for myelin turnover inactive. This leads to toxic accumulation of psychosine, a byproduct of galactosylceramide synthesis and GALC substrate (Orsini et al., 1993; Weinstock et al., 2020). In the frequent severe form, children present with hypersensitivity, spasticity, arrested psychomotor development often rapidly deteriorating with seizures leading to death before 3 years of age (Duffner et al., 2011). Currently, pre-symptomatic bone marrow transplant (BMT) and HSCT aiming to restore some level of GALC in the CNS through microglia, are the only treatment options but even these only slow disease progressions.

Based on the severity of the disease, therapeutic approaches spanning from substrate reduction and enzyme replacement to cell- and AAV- mediated gene therapies using a number of different capsids (AAV1, 2, 5, 9, rh.10 and Olig001) and delivery routes (IP, IV, IT, ICM, ICV) have been trialed in mouse models of the disease, many lagging behind expectations. The severity of the disease and extent of gene replacement required to treat Krabbe disease is underscored by the fact that even in combination with bone marrow transplant it required neonatal IV, IT and intraparenchymal AAV9-CAGGS-*GALC* to achieve extension of lifespan to 9 months with long-term improvement of neurological and physiological signs (Marshall et al., 2018). Considering these data, recent findings that ICM administration of high dose AAV9-CAGGS-GALC ($1x10^{14}$ vg) prior to disease onset in a naturally occurring canine model for Krabbe disease decreased psychosine concentration and inflammation, improved myelination, nerve conduction and extended lifespan from 16 weeks to at least 2.5 years are very promising (Bradbury et al., 2020). In contrast, combined IV and ICV AAV.rh10-CAG-*GALC* at a lower dose ($3.8x10^{13}$ vg) while ameliorating CNS and PNS disease only doubled the lifespan (Bradbury et al., 2018). Recent promising results were observed upon combination of high dose IV of AAVrh10 – mGALC (1.6×10^{14} vg/kg) with bone marrow transplant between

P10 – P11 in twitcher mice extended median survival over 500 days (Karumuthil-Melethil et al., 2016; Ricca and Gritti, 2016; Pan et al., 2019; Rafi et al., 2020). Although the rapid disease progression and limited peripheral involvement warrant an AAV mediated approach, therapeutic outcome is likely to be further improved by combination with next generation HSC-GTs that have addressed the pitfall of selective GALC toxicity in the hematopoietic compartment by introducing a mir126 target sequence in the LV GALC expression vector, without affecting HSC engraftment and monocyte derived macrophage mediated GALC expression in the CNS (Ungari et al., 2015). In a separate murine study, better GALC secretion and BBB transfer was achieved by attaching the iduronate-2-sulfatase signaling peptide and the apolipoprotein B low-density lipoprotein receptor to the GALC coding sequence improving myelination, motor function and life span compared to previous studies (Pan et al., 2019).

Based on studies in mice the vulnerability to *GalC* ablation appears to be highest between P4 and P6 before onset of myelination, and *GalC* expression is highest in OPCs, newly formed oligodendrocytes and astrocytes, but drops in mature oligodendrocytes (Weinstock et al., 2020). This indicates that ensuring widespread transduction and gene expression in OPCs, neural crest cells as well as early myelinating oligodendrocytes and Schwann cells which are most in need of GALC may be important and should be addressed during design of the next generation of Krabbe disease targeting gene therapies. Notably, aside from the *MAG*, the *CNP* and the *Mbp* promoter, in context of AAV9 the CBh promoter showed strong functional transduction in oligodendrocytes but it remains to be determined if this includes OPCs and early myelinating oligodendrocytes (Powell et al., 2020). The same holds true for the oligodendrocyte targeting capsid variant Olig001 and the neural stem cell targeting AAV.SCH9.

Krabbe disease pathology demands rapid onset and strong GALC expression and thus scAAV genomes may be best suited and the synthetic Olig2 derived promoters Ple266 and Ple267, or the 0.3 kb *MAG* promoter may be candidates; bearing in mind that some astroglial expression could prove beneficial. Lastly, even when combined with HSC-GT, an AAV vector capable of highly efficient transcytosis through the CBB or BBB with reduced off target transduction would be pivotal.

LEUKODYSTROPHIES THAT REQUIRE ASTROCYTE TARGETING

Astrocytes are the most abundant cell type in the human brain where they control perivascular homeostasis, integrity of the blood brain barrier and provide trophic support of neurons and oligodendrocytes. In addition, astrocytes are key to neurotransmitter recycling and directly modulate synaptic activity (Santello et al., 2019; Sweeney et al., 2019; Cohen-Salmon et al., 2020; Nutma et al., 2020). In white matter tracts, astrocytes and oligodendrocytes are directly connected through gap junctions formed by connexin hemichannels, generating a panglial syncytium with ependymal cell that enables free flow of water and small molecules, including lactate, amino acids, gliotransmitters and ions. Astrocytes are thought to prevent intramyelinic oedema by diverting excessive osmotic water arising in paranodal myelin from action potential associated ion fluxes. Astrogliopathies caused by mutations in genes coding for astrocytic connexin hemichannels including Cx43 and Cx30 required for gap junction formation with oligodendrocytes and in genes encoding proteins associated with ion-water homeostasis, frequently present as vacuolating leukodystrophies (Lutz et al., 2009; Tress et al., 2012; van der Knaap and Bugiani, 2017). In addition, iron supply provided by astrocytes is essential for oligodendrocyte metabolism and myelination (Nutma et al., 2020). Reactive astrogliosis in the brain induced by transient stressors or progressive neurodegeneration generally contributes to restoration of metabolic homeostasis and overall neuroprotection and repair, but may be detrimental if the driver persits (Garcia et al., 2020). In some leukodystrophies the underlying genetic cause is originates from mutations of astrocyte specific genes and their monogenic cause, disease severity and absence of alternate treatments puts these leukodystrophies in scope for gene therapy development.

Alexander Disease

Alexander disease (AxD) is caused by autosomal dominant *gain of function* mutations in the gene encoding the intermediate filament glial acidic fibrillary protein (*GFAP*). In its severe infantile presentation AxD is characterized by megalocephaly, hypomyelination, developmental delay, psychomotor decline and seizures culminating in premature demise (Prust et al., 2011; Köhler et al., 2018). The brain pathophysiology involves a toxic accumulation of the mutated GFAP protein in fibrous multiprotein complexes with ubiquitin, vimentin, heat shock protein HSP27, plectin and αβ-crystallin in eosinophile complexes termed Rosenthal fibers. AxD astrocytes are characterized by a bushy, activated morphology, with decreased glutamate buffering activity and an inhibitory effect on oligodendrogenesis and myelination (Brenner et al., 2001; Lanciotti et al., 2013; Brignone et al., 2015; Olabarria and Goldman, 2017; Li et al., 2018).

Since the disease is caused by toxic accumulation of a mutant misfolded protein and has predominantly neurological manifestation, gene therapy should be CNS targeted and aim to reduce the expression of mutant GFAP in astrocytes. This may be achieved through AAV – mediated expression of a GFAP targeting synthetic miRNA, preferentially incorporated into an astrocyte enriched primary miRNA backbone (Jovièiæ and Gitler, 2017) and driven by an astrocyte specific promoter. If the miRNAs' complementary target sequence is located in the 5'-UTR or 3'-UTR of the *GFAP* mRNA, simultaneous expression of transgenic wt *GFAP* is feasible. In a different approach, a single intrathecal injection of a GFAP mRNA targeted ASO with enhanced nuclease resistance, binding affinity and lower toxicity has achieved remarkable efficacy providing elimination of GFAP throughout the CNS, reversal of Rosenthal fibers and rescue of hippocampal neurogenesis in an AxD mouse model (Hagemann et al., 2018).

Van der Knaap Disease

Autosomal recessive mutations in Megalencephalic Leukoencephalopathy with subcortical cysts (*MLC)1*, or dominant heterozygous mutations in *GLIALCAM* disrupt membrane localization of MLC1 to astrocyte endfeet and astrocyte junctions, where it stabilizes water channels including aquaporin-4 and the inwardly rectifying potassium channel Kir4.1 (Noell et al., 2011; Brignone et al., 2015). Disruption of the ion-water homeostasis and cell volume regulation in van der Knaap disease is thought to be the key pathophysiological hallmark leading to this spongiform leukodystrophy that morphologically manifests with megalocephaly and intramyelinic vacuolization. The more common infantile form is characterized by megalencephaly caused by chronic white matter oedema. In the classical progressive form of the disease, loss of motor functions, epilepsy, and mild mental decline follow, and patients are often wheelchair – bound by their teenage years. Interestingly, dominant mutations in *GLIALCAM* have been shown to cause a remitting form of Van der Knaap disease.

MLC1 is exclusively expressed in gray and white matter astrocytes including cerebellar Bergmann glia in the CNS. In a recently published murine MLC1 knockout model that mirrors the spongiform phenotype of the human disease, a single astrocyte targeted AAV.rh10-*GFAP-MLC1* gene replacement prevented vacuolization when administered before disease onset at 5 months, and even at 15 months provided near complete remission of vacuolization (Sánchez et al., 2020). While likely due to the advanced age at infusion, the authors observed relatively poor transduction in the CNS following IT or ICV delivery. Interestingly, AAV delivery into the intracerebellar subarachnoid space provided more efficient and widespread transduction throughout the cerebellum compared with deeper injections into the white matter or molecular layer. Because this delivery route does not require physical penetration deep into the CNS it is less invasive and appears worth further investigation as an alternative or additional AAV delivery route for many leukodystrophies and leukoencephalopathies.

Two further albeit later onset and usually milder leukodystrophies may benefit from astroglial gene transfer include chloride voltage gated channel 2 (*CLCN2*) related vacuolating leukoencephalopathy and the Oculodentodigital dysplasia with cerebral white matter abnormalities (ODDD), a hypomyelinating leukodystrophy caused by an autosomal dominant mutation in the Gap junction α 1 (Connexin-43) encoding *GJA1* gene (Abrams and Scherer, 2012). Both genes are predominantly expressed in astrocytes in mice and humans. Given that using current vectors the efficiency of ICV, ICM and IV gene delivery rapidly decreases with age, development of better BBB and CBB penetrating capsids is required.

MICROGLIA AS A TARGET FOR LEUKODYSTROPHY TREATMENTS

Microglia are the only hematopoietic stem cell derived cell type in the healthy CNS, where they are considered to be the resident macrophages. They are ontogenically distinct from other mononuclear phagocytes, including dendritic cells, monocytes and macrophages. They originate from the yolk sac and infiltrate the developing CNS where they compose ∼10% of all glial cells and self – maintain. Microglia are highly dynamic and constantly survey their microenvironment by active projection and retraction and are now known to perform much more than immune – related functions in the CNS (Wu et al., 2015). Over the past decade microglia have been shown to actively participate in synapse remodeling and stripping, as well as to actively maintain or compromise the integrity of the BBB (Wake et al., 2009; Akiyoshi et al., 2018; Haruwaka et al., 2019). Nevertheless, in their crucial role as immune cells, upon insult, infection or neural cell degeneration microglial rapidly change from a highly ramified to a more amoeba-like shape, proliferate and express a range of pro-inflammatory cytokines. With regards to leukodystrophies, microglia have become both a target in case of certain microgliopathies and a therapeutic tool as they are critical to clearance of myelin debris, a prerequisite for remyelination.

In a few leukodystrophies classified as microgliopathies, mutations in microglia specific genes are the root cause of the disease. These include colony stimulating factor 1 receptor (CSF1R) associated "hereditary diffuse neuroaxonal leukoencephalopathy with axonal spheroids' and the 'pigmentary orthochromatic leukodystrophy' (Rademakers et al., 2011; Nicholson et al., 2013) as well as Nasu – Hakola disease caused by *DAP12* gene mutations encoding the TYRO protein tyrosine kinase binding protein (Sasaki, 2017). These microgliopathies are usually adult onset leukodystrophies exhibiting neurological phenotypes as well as peripheral manifestations. Although microglia self-renew locally, under certain disease conditions or following myeloablation, circulating monocytic precursor cells can infiltrate the CNS and differentiate along the macrophage pathway into microglia-like cells and perform microglial functions (Greter et al., 2015; Beins et al., 2016). This mechanism in combination with the close ontogenic relationship indicates that some microgliopathies may be at least partially amendable by *ex vivo* HSC-GT.

Interestingly, RNAseq data assessing cell type – specific gene expression profiles in mice (**Table 2**) demonstrates that many LSDs associated genes are expressed at comparably high levels in microglia and microglial damage has been shown to precede myelin damage in MLD and X-ALD (Bergner et al., 2019). Engraftment of autologous bone marrow or cord blood derived genetically 'corrected' HSCs expressing a functioning copy of the mutated gene are becoming a viable treatment option for some leukodystrophies. Following myeloablation between 5 and 20% of microglia are physically and functionally replaced with transgenic, HSC – derived microglia-like macrophages capable of cross-correction through lysosomal enzyme secretion or intracellular processing of substrate that would otherwise accumulate to toxic levels.

Ex vivo HSC-GT has shown immense promise halting disease progression when performed in pre-symptomatic or early symptomatic patients and is currently in various stages of clinical trial for lysosomal and peroxisomal storage disorders including

MLD and X-ALD disease, (Dahl et al., 2015; Nagree et al., 2019). The first HSC-GT, for a CNS disease 'Libmeldy', has just been approved by the European Commission for use in children with late-infantile onset MLD. MLD is an autosomal recessive demyelinating leukodystrophy caused by of arylsulfatase A (*ARSA*) deficiencies triggering an accumulation of the myelin lipid sulfatide in oligodendrocytes and Schwann cells, as well as several peripheral organs, because the enzyme can no longer support its degradation and recycling. In this most common and severe late-infantile form of the disease, children lose the ability to walk and talk within months, and following rapid progression of motor symptoms, commonly die within seven years of diagnosis making the success of 'Libmeldy' a truly remarkable testament to the field. Pioneering the technology, HSC-GT successfully halts disease progression in X-ALD, even though in this case the peroxisomal very long chain fatty acid transporter ABCD1 is not secreted. In the STARBEAM study, HSC-GT for X-ALD is now also in late-stage Phase II/III clinical trial (NCT01896102, NCT02698579, NCT02559830). Recent success in early symptomatic treatment of adult-onset X-ALD using HSCT underscores the potential feasibility of this approach for adult-onset LSDs and the above mentioned microgliopathies (Matsukawa et al., 2020).

Other notable LSDs that manifest with neurological symptoms, frequently present with white matter abnormalities, and may benefit from HSC-GT include Tay-Sachs disease from monosialic ganglioside accumulation following mutation in the hexosaminidase A (*HEXA*) gene, Sandhoff disease associated with HEXB mutations and Sanfilippo syndrome, a mucopolysaccharidosis (MPSIII) stemming from deficiencies in α-N-acetylglucosaminidase (*NAGLU*) or N-sulfoglucosamine sulfohydrolase (*SGSH*) gene. Recent development of a bicistronic lentivirus *HEXA* and *HEXB* encoding vector promoting stoichiometric synthesis of both subunits of the enzyme is capable of targeting both Tay-Sachs and Sandhoff disease in HSC-GT (Ornaghi et al., 2020). Interestingly, α – mannosidase encoding MAN2B, mutations in which cause α – mannosidosis, is also predominantly expressed in microglia, indicating that this lysosomal storage disease may also benefit from HSC-GT, and potentially add to recent achievement in AAV mediated preclinical success in a feline model (Zhang et al., 2014; Yoon et al., 2020).

A pitfall of *ex vivo* gene therapy for leukodystrophies and leukoencephalopathies is the crucial importance for early treatment; arrest of disease progression in X-ALD took 12-18 months in which demyelinating lesions continued to expand (Cartier et al., 2014). Although myeloablation efficiently creates a niche and HSC engraftment occurs within hours to days, functional microglia – like differentiation increases over months after treatment with superior efficacy of ICV over IV HSC administration recently reported (Capotondo et al., 2017). Aside from engraftment and differentiation additional factors including transduction efficiency and transgene expression may contribute to the observed delay in halting often irreversible disease progression. A recent notable advance that may drastically improve the outcome of HSC-GT demonstrated that targeted inhibition of the endogenous microglial proliferation through inhibition of CSF1R effectively before myeloablation enabled near complete engraftment of (>92%) transgenic microglial like cells (Xu et al., 2020).

Separately the pitfall of a delayed effectiveness of HSC-GT for treatment of some neurometabolic disorders may be overcome by gene replacement through rAAV because it yields expression of a missing gene within days in case of scAAV, or weeks for ssAAV counterparts. Indeed, clinical trials employing broad spectrum AAV vectors with transgene expression driven by ubiquitous promoters are currently underway for MLD, Tay – Sachs disease and Sandhoff disease, as well as Sanfilippo syndrome in which both direct intraparenchymal delivery of ssAAVs (NCT03612869) and systemic delivery of scAAV9 (NCT02716246) are investigated. A recently initiated phase I dose escalation study for Tay – Sachs and Sandhoff disease (NCT04669535) combines dual ICM and IT administration of AAV.rh8-HexA and AAV.rh8-HexB at a 1:1 with bilateral intrathalamic delivery.

Looking forward, bicistronic HexA and HexB which can circumvent dual AAV delivery have shown promise in murine models of Sandhoff disease (Lahey et al., 2020). To date, the AAV – mediated gene therapies selectively targeting microglia have been hampered by poor transduction efficiencies (<20 %) *in vivo*. However, the success of HSC-GT in combination with the observation that microglial damage precedes myelin damage indicate that development of therapeutic AAV vectors with better microglial tropism may promote therapeutic outcomes even for leukodystrophies like MLD that may require a broader cell type overlapping approach.

LEUKODYSTROPHIES REQUIRING TARGETING OF MULTIPLE CNS CELL TYPES

Based on the relatively broad expression and the ubiquitous requirement of the gene product, a number of leukodystrophies caused by 'loss of function' that do not result in the accumulation of a toxic metabolite are likely to require broad cell type overlapping gene replacement. Their gene products are frequently part of multiprotein complexes, disease severity and onset are variable and additional pathologies in the PNS and peripheral organs are often involved. Many of these enzymes are ubiquitously expressed, and absence or complete loss of function are usually embryonically lethal. Examples include autosomal recessive aminoacyl transfer RNA (tRNA) related leukodystrophies, 'hypomyelination with brainstem and spinal cord involvement and leg spasticity' (HBSL) caused by mutations in the cytosolic aspartyl-tRNA synthetase 1 (*DARS1*) (Taft et al., 2013; Fröhlich et al., 2017, 2018), 'leukoencephalopathy with brain stem spinal cord involvement ad lactate elevation' (LBSL) associated with mitochondrial *DARS2* mutations (van der Knaap et al., 1995; Scheper et al., 2007), the cytosolic and mitochondrial alanyl-tRNA synthetase (*AARS1, AARS2*) underlie progressive 'leukoencephalopathy with ovarian failure and epileptic encephalopathy 29' (Dallabona et al., 2014; Simons et al., 2015), mutations in' glutamyl-tRNA synthetase

(*EARS1*) cause 'leukoencephalopathy with thalamus and brainstem involvement and high lactate' (LTBL) (Steenweg et al., 2012), arginyl-tRNA synthetase (*RARS1*) cause 'hypomyelinating Leukodystrophy 9' (Mendes et al., 2020) and the aminoacyl-tRNA synthase complex-interacting multifunctional protein 1 (AIMP1) (Feinstein et al., 2010). Similarly, '4H-leukodystrophy' originates from mutations in RNA polymerase 3 (*POLR3A* and *POLR3B*) encoding genes and is typically characterized by hypomyelination, hypodontia, and hypogonadotropic hypogonadism. Age of onset varies but averages four years of age and *POL3A* mutations are associated with more severe disease. POLR3A and POLR3B are the main building blocks of this RNA polymerase that transcribes tRNAs and ribosomal RNA (rRNA) (Wolf et al., 2014; Thiffault et al., 2015; van der Knaap and Bugiani, 2017). To date discovery of adequate animal models that enable preclinical testing of therapeutic strategies is ongoing, however gene therapies will likely need to reflect the broad expression profile of these genes.

Aicardi-Goutieres Syndrome (AGS) is caused by mutations in a number of genes involved in nucleotide metabolism. These include *RNASEH2A, RNASEH2B RNASEH2C.* AGS manifests as microcephaly with progressive spasticity, psychomotor retardation. Neonatal onset has been associated with an approximately 35% fatality in early childhood. RNASEH2 is a DNA repair enzyme and its absence leads to DNA damage response and accumulation of cytosolic DNA aggregates triggering inflammation. While expressed across neural cell types, astrocytes rather than infiltrating leukocytes were found responsible for high cytokine and particularly interferon-α production in post-mortem brains indicating that gene replacement therapies, while targeting all cell types, should first and foremost ensure good astroglial coverage, but not prevent expression in other cells. Either way, a gene therapy for RNASEH2 related leukodystrophies must be carefully designed and tested because the enzyme consists of three albeit small subunits and to provide constraints around stoichiometry will be challenging. In addition, it should be noted that *RNASEH2C* is considered a metastasis susceptibility gene and interfering with the expression of cell cycle regulated genes must be very carefully considered as it may increase downstream risks of malignancies (Crow et al., 2006; Orcesi et al., 2009; Crow and Manel, 2015; Deasy et al., 2019).

Lysosomes play essential roles in the turnover of structurally diverse compounds including lipids, proteins, nucleic acids and oligosaccharides and thus control homeostasis through the autophagy – lysosomal pathway. Mutations in many genes associated with these pathways cause lysosomal storage diseases (LSD) characterized by the accumulation of intermediate metabolites that are frequently toxic at higher concentrations and prevent homeostasis, eventually triggering cell death. Many LSDs have significant neurological and CNS involvement. While cross-correction by externally delivered lysosomal enzymes can mitigate peripheral aspects of the pathology, in the CNS uptake is largely prevented by the BBB, promoting the development of gene therapies that have the potential to overcome limitations of traditional enzyme replacement therapies. We apologize to our colleagues in this field whose valued contributions could not be included in this manuscript due to space constraints. Recent reviews have provided comprehensive overviews on clinical progress of gene therapies (Hocquemiller et al., 2016; Nagree et al., 2019; Parenti et al., 2021). Aside from the above mentioned MLD, Krabbe disease, Tay-Sachs and Sandhoff disease, GM1 gangliosidosis is an autosomal recessive LSD associated with progressive neuronal cell death in the brain and spinal cord caused by mutations in the β-galactosidase encoding gene *GLB1* that is expressed by all cell types in the CNS (Brunetti-Pierri and Scaglia, 2008). Reflecting the rapid neurological decline associated with neuron loss in GM1 gangliosidosis, early, ideally presymptomatic treatment is essential. Likely due to insufficient enzyme expression in the CNS a trial of HSCT could not prevent neurological disease progression (Shield et al., 2005). In contrast, following promising results in preclinical research in murine and feline animal models employing AAV in which GLB1 expression was driven by strong constitutive promoters (Broekman et al., 2007; Weismann et al., 2015; Gray-Edwards et al., 2017; Hinderer et al., 2020), intravenous AAV9 (NCT03952637), ICM administration of AAVhu.68 (NCT04713475) and AAVrh.10 (NCT04273269) have advanced into clinical trial. Further, multiple sulfatase deficiency (MSD), a similar lysosomal storage – related leukodystrophy with significant CNS involvement arising from autosomal recessive mutation in the sulfatase-modifying factor 1 (SUMF1) encoding gene, causes profound reduction in downstream sulfatase activities, including the sulfatases associated with other LSDs like MLD and various mucopolysaccharidosis (MPS) (Fraldi et al., 2007). MSD patients display combined clinical symptoms of these sulfatase deficiencies, with severity depending on the specific mutation (Schlotawa et al., 2011). In LSDs disease onset is inversely with correlated neurological decline and severity. To prevent neurological deterioration rapid and widespread *SUMF1* expression and activity is paramount. Combined ICV and IV infusion of AAV9 -CMV-SUMF1 was able to activate sulfatases, clear accumulated glycosaminoglycans, decrease inflammation and improve motor and memory performance (Spampanato et al., 2011). In future, scAAV vectors promoting quicker onset of lysosomal enzyme expression following IP, ICM or systemic IV delivery in LSDs are likely to emerge.

Vanishing White Matter Disease

VWM disease, also known as 'childhood ataxia with central nervous system hypomyelination' is a prominent leukodystrophy that is caused by loss of function in eukaryotic translation initiation factor 2B subunit 1 to 5 (*EIF2B1, EIF2B2, EIF2B3, EIF2B4, EIF2B5*) encoding genes, that show a clear genotype – phenotype correlation. The nucleotide exchange factor eIF2B is required for eIF2 delivery of initiator Met-tRNA to the ribosome and initiation of translation (Leegwater et al., 2001). Classical VWM disease is associated with progressive neurological deterioration, mild spasticity, and cognitive impairment. Stress causes episodes of major deterioration that may be fatal. Indeed, eI2FB is the critical regulator of the integrated

stress response during protein translation and is activated by oxidative stress or starvation (Carter, 2007). Based on this crucial role, EIF2B appears fairly uniformly expressed across CNS cell types (**Table 2**) but the most striking histopathological changes in VWM are observed in astrocytes and oligodendrocytes.

Recent evidence from a glial progenitor cell therapy study suggested that phenotypic improvement is correlated with increased astroglial differentiation underscoring the hypothesis that astrocytes are central to the VWM pathology (Dooves et al., 2019). In addition, co – cultures of mutated astrocytes with wildtype oligodendrocytes impaired maturation of the oligodendrocytes, while mutated oligodendrocytes mature normally in the presence of wildtype astrocytes (Dooves et al., 2016). These findings indicate that primary gene replacement must also first and foremost target astrocytes. However, the broad expression of EIF2B1 – 5 across different cell types in the CNS indicates, broad cell type targeting within the CNS will be required. All *EIF2B* genes are expressed at high levels throughout peripheral organs and peripheral phenotypes such as endocrine effects have been observed and may also need to be addressed. Nevertheless, the pre-clinical benefits achieved through glial progenitor cell therapy are encouraging. Since most AAV vectors that penetrate the BBB or CBB transduce astrocytes with high efficacy following IV or ICM administration, AAV gene replacement with constitutively active promoters appears to be a viable treatment option for severe infantile VWM disease. For treatment development, capsid and promoter selection would certainly benefit from a screening for 'functional transduction' in a humanized animal model.

CONCLUSION AND FUTURE DIRECTIONS

Nucleic acid-based treatments of neurological disorders have rapidly expanded in the last decade and the long – recognized potential for gene therapy is now becoming a reality for patients and families suffering from a multitude of devastating genetic diseases. The first hematopoietic stem cell-based *ex vivo* gene therapies as well as AAV – mediated *in vivo* gene therapies have been approved by the FDA. In addition, the first ASOs have also successfully advanced to this stage. In addition, most big pharma companies are supporting development and increasingly managing to address issues associated with large scale production. Nevertheless, despite the immense potential and undoubtedly growing importance of these evolving genetic drugs in modern medicine, recent clinical and pre-clinical studies also expose risks and pitfalls underscoring the importance of a cautious and considered advance. In the case of *ex vivo* HSC-GT, this has ensured development of self-inactivating lentiviral vectors and rigorous mapping of integration sites. With regards to AAV – mediated *in vivo* gene therapy programs, which are establishing excellent safety and efficiency profiles, the recent tragic deaths of three patients in the high dose cohort of the ASPIRO trial targeting myotubular myopathy as well as preclinical evidence of toxicity and severe immune response associated with high vector doses following systemic AAV delivery are a reminder of the risks and imperative for refinement, particularly around vector dose. This is of particularly relevant to treatments for leukodystrophies and leukoencephalopathies that rely on relatively high vector doses associated with targeting the entire CNS. Since direct intraparenchymal injections are associated with risk of hemorrhage and infection and it has been estimated that about 100 injection sites may be required to achieve full CNS coverage, the discovery of BBB and CBB traversing AAV serotypes has moved systemic IV and CSF vector administration into focus. Importantly, comparison of delivery routes and modes of rAAV vector administration is ongoing, but recent studies indicate when administered into the CSF a substantially better vector spread throughout the CNS and spinal cord is achieved by injection into the cisterna magna over other cerebroventricular and intrathecal approaches. A key to dose reduction is to increase this ability in the next generation of AAV vectors while ensuring immune – evasion and substantial reduction in off-target tissue transduction. Among others, notable preclinical progress has been made in this field through discovery of novel naturally – occurring AAV variants, alongside rational capsid design, capsid shuffling peptide insertion combined with high throughput screening and targeted selection to extend the repertoire of vectors and associated cell and tissue targeting profiles.

By reviewing the natural expression profile of genes associated with leukodystrophies across neural cell types, we hope to provide a better understanding of which cells may naturally be important targets. Highlighting the importance of the choice of promoters and *cis*-regulatory elements to achieve gene expression in the target cell we compiled current capsid and promoters that may be considered as a starting point with regards to development of improved targeting strategies and potentially toggleable expression control. Notably, recent findings that direct capsid – promoter interactions determine the cell type – selective gene expression in the CNS highlight the importance of screening these aspects in conjunction. Because beyond uptake, capsid components influence intracellular transport, stability and gene expression, and cross species differences are evident even when targeting the same cell type, it will be increasingly important to confirm functional transduction in humanized CNS models. AAV capsid / promoter combinations identified via screens resulting from selective pressure for functional transduction of the target cell in humanized models may be a promising strategy to achieve the needed vector dose reduction required for treatment of CNS disorders. Beyond vector engineering and choice, other patient specific, parameters in addition to body weight may need to be considered when choosing vector dose. These include developmental stage, disease progression, immunity, and metabolic profile. As gene therapies for leukodystrophies and leukoencephalopathies advance, the programs will need to broaden to assess and mitigate risk. In many, if not most cases, a single shot 'Magic Bullet' curative treatment may be elusive, but such challenges are driving the

rapid advance in sophistication of the vectors and therapeutic payloads to yield genetic medicines to treat these devastating neurological diseases.

AUTHOR CONTRIBUTIONS

GJ led the project and wrote the draft manuscript. GJ, CR, and GH contributed to the manuscript preparation. All authors read and approved the final manuscript.

FUNDING

This work was supported by the NHMRC (Ideas Grant APP1189113) to GH and GJ.

ACKNOWLEDGMENTS

We acknowledge the work of all colleagues that due to constraints could not be included in this manuscript.

REFERENCES

Abrams, C. K., and Scherer, S. S. (2012). Gap junctions in inherited human disorders of the central nervous system. *Biochim. Biophys. Acta* 1818, 2030–2047. doi: 10.1016/j.bbamem.2011.08.015

Adachi, K., Enoki, T., Kawano, Y., Veraz, M., and Nakai, H. (2014). Drawing a high-resolution functional map of adeno-associated virus capsid by massively parallel sequencing. *Nat. Commun.* 5:3075. doi: 10.1038/ncomms4075

Adang, L. A., Sherbini, O., Ball, L., Bloom, M., Darbari, A., Amartino, H., et al. (2017). Revised consensus statement on the preventive and symptomatic care of patients with leukodystrophies. *Mol. Genet. Metab.* 122, 18–32. doi: 10.1016/j.ymgme.2017.08.006

Ahmed, S. S., Li, H., Cao, C., Sikoglu, E. M., Denninger, A. R., Su, Q., et al. (2013). A single intravenous rAAV injection as late as P20 achieves efficacious and sustained CNS Gene therapy in Canavan mice. *Mol. Ther.* 21, 2136–2147. doi: 10.1038/mt.2013.138

Akiyoshi, R., Wake, H., Kato, D., Horiuchi, H., Ono, R., Ikegami, A., et al. (2018). Microglia enhance synapse activity to promote local network synchronization. *eNeuro* 5:ENEURO.0088-18.2018. doi: 10.1523/eneuro.0088-18.2018

Albright, B. H., Storey, C. M., Murlidharan, G., Castellanos Rivera, R. M., Berry, G. E., Madigan, V. J., et al. (2018). Mapping the structural determinants required for AAVrh.10 transport across the blood-brain barrier. *Mol. Ther.* 26, 510–523. doi: 10.1016/j.ymthe.2017.10.017

Allison, A. B., Kohler, D. J., Fox, K. A., Brown, J. D., Gerhold, R. W., Shearn-Bochsler, V. I., et al. (2013). Frequent cross-species transmission of parvoviruses among diverse carnivore hosts. *J. Virol.* 87, 2342–2347. doi: 10.1128/jvi.02428-12

Al-Zaidy, S., Pickard, A. S., Kotha, K., Alfano, L. N., Lowes, L., and Paul, G. (2019). Health outcomes in spinal muscular atrophy type 1 following AVXS-101 gene replacement therapy. *Pediatr. Pulmonol.* 54, 179–185. doi: 10.1002/ppul.24203

Armbruster, N., Lattanzi, A., Jeavons, M., Van Wittenberghe, L., Gjata, B., Marais, T., et al. (2016). Efficacy and biodistribution analysis of intracerebroventricular administration of an optimized scAAV9-SMN1 vector in a mouse model of spinal muscular atrophy. *Mol. Ther. Methods Clin. Dev.* 3:16060. doi: 10.1038/mtm.2016.60

Aschauer, D. F., Kreuz, S., and Rumpel, S. (2013). Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PLoS One* 8:e76310. doi: 10.1371/journal.pone.0076310

Aslanidi, G. V., Rivers, A. E., Ortiz, L., Song, L., Ling, C., Govindasamy, L., et al. (2013). Optimization of the capsid of recombinant adeno-associated virus 2 (AAV2) vectors: the final threshold? *PLoS One* 8:e59142. doi: 10.1371/journal.pone.0059142

Bannerman, P., Guo, F., Chechneva, O., Burns, T., Zhu, X., Wang, Y., et al. (2018). Brain Nat8l knockdown suppresses spongiform leukodystrophy in an aspartoacylase-deficient canavan disease mouse model. *Mol. Ther.* 26, 793–800. doi: 10.1016/j.ymthe.2018.01.002

Bartlett, J. S., Samulski, R. J., and McCown, T. J. (1998). Selective and rapid uptake of adeno-associated virus type 2 in brain. *Hum. Gene Ther.* 9, 1181–1186. doi: 10.1089/hum.1998.9.8-1181

Beins, E., Ulas, T., Ternes, S., Neumann, H., Schultze, J. L., and Zimmer, A. (2016). Characterization of inflammatory markers and transcriptome profiles of differentially activated embryonic stem cell-derived microglia. *Glia* 64, 1007–1020. doi: 10.1002/glia.22979

Bergner, C. G., van der Meer, F., Winkler, A., Wrzos, C., Türkmen, M., Valizada, E., et al. (2019). Microglia damage precedes major myelin breakdown in X-linked adrenoleukodystrophy and metachromatic leukodystrophy. *Glia* 67, 1196–1209. doi: 10.1002/glia.23598

Bey, K., Deniaud, J., Dubreil, L., Joussemet, B., Cristini, J., Ciron, C., et al. (2020). Intra-CSF AAV9 and AAVrh10 Administration in nonhuman primates: promising routes and vectors for which neurological diseases? *Mol. Ther. Methods Clin. Dev.* 17, 771–784. doi: 10.1016/j.omtm.2020.04.001

Bielschowsky, M. H., and Henneberg, R. (1928). Über familiäre diffuse Sklerose (Leukodystrophia cerebri progressiva hereditaria). *J. Psychol. Neurol.* 36, 131–181.

Biffi, A., Aubourg, P., and Cartier, N. (2011). Gene therapy for leukodystrophies. *Hum. Mol. Genet.* 20, R42–R53. doi: 10.1093/hmg/ddr142

Biffi, A., Montini, E., Lorioli, L., Cesani, M., Fumagalli, F., Plati, T., et al. (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 341:1233158. doi: 10.1126/science.1233158

Bohlen, M. O., McCown, T. J., Powell, S. K., El-Nahal, H. G., Daw, T., and Basso, M. A. (2020). Adeno-associated virus capsid-promoter interactions in the brain translate from rat to the nonhuman primate. *Hum. Gene Ther.* 31, 1155–1168. doi: 10.1089/hum.2020.196

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, 718–725. doi: 10.1212/WNL.0b013e3181eee46b

Bonkowsky, J. L., Wilkes, J., and Shyr, D. C. (2018). Scope and burden of non-standard of care hematopoietic stem cell transplantation in pediatric leukodystrophy patients. *J. Child Neurol.* 33, 882–887. doi: 10.1177/0883073818798090

Börner, K., Kienle, E., Huang, L.-Y., Weinmann, J., Sacher, A., Bayer, P., et al. (2020). Pre-arrayed Pan-AAV peptide display libraries for rapid single-round screening. *Mol. Ther.* 28, 1016–1032. doi: 10.1016/j.ymthe.2020.02.009

Boucher, A. A., Miller, W., Shanley, R., Ziegler, R., Lund, T., Raymond, G., et al. (2015). Long-term outcomes after allogeneic hematopoietic stem cell transplantation for metachromatic leukodystrophy: the largest single-institution cohort report. *Orphanet J. Rare Dis.* 10:94. doi: 10.1186/s13023-015-0313-y

Bradbury, A. M., Bagel, J. H., Nguyen, D., Lykken, E. A., Pesayco Salvador, J., Jiang, X., et al. (2020). Krabbe disease successfully treated via monotherapy of intrathecal gene therapy. *J. Clin. Invest.* 130, 4906–4920. doi: 10.1172/jci133953

Bradbury, A. M., Rafi, M. A., Bagel, J. H., Brisson, B. K., Marshall, M. S., Pesayco Salvador, J., et al. (2018). AAVrh10 gene therapy ameliorates central and peripheral nervous system disease in canine globoid cell leukodystrophy (Krabbe Disease). *Hum. Gene Ther.* 29, 785–801. doi: 10.1089/hum.2017.151

Bradl, M., and Lassmann, H. (2010). Oligodendrocytes: biology and pathology. *Acta Neuropathol.* 119, 37–53. doi: 10.1007/s00401-009-0601-5

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, 117–120. doi: 10.1038/83679

Brenner, M., Kisseberth, W. C., Su, Y., Besnard, F., and Messing, A. (1994). GFAP promoter directs astrocyte-specific expression in transgenic mice. *J. Neurosci.* 14(3 Pt 1), 1030–1037. doi: 10.1523/jneurosci.14-03-01030.1994

Brignone, M. S., Lanciotti, A., Camerini, S., De Nuccio, C., Petrucci, T. C., Visentin, S., et al. (2015). MLC1 protein: a likely link between leukodystrophies and brain channelopathies. *Front. Cell Neurosci.* 9:66. doi: 10.3389/fncel.2015.00106

Broekman, M. L., Baek, R. C., Comer, L. A., Fernandez, J. L., Seyfried, T. N., and Sena-Esteves, M. (2007). Complete correction of enzymatic deficiency and neurochemistry in the GM1-gangliosidosis mouse brain by neonatal adeno-associated virus-mediated gene delivery. *Mol. Ther.* 15, 30–37. doi: 10.1038/sj.mt.6300004

Brunetti-Pierri, N., and Scaglia, F. (2008). GM1 gangliosidosis: review of clinical, molecular, and therapeutic aspects. *Mol. Genet. Metab.* 94, 391–396. doi: 10.1016/j.ymgme.2008.04.012

Büning, H., Huber, A., Zhang, L., Meumann, N., and Hacker, U. (2015). Engineering the AAV capsid to optimize vector-host-interactions. *Curr. Opin. Pharmacol.* 24, 94–104. doi: 10.1016/j.coph.2015.08.002

Büning, H., and Srivastava, A. (2019). Capsid modifications for targeting and improving the efficacy of AAV vectors. *Mol. Ther. Methods Clin. Dev.* 12, 248–265. doi: 10.1016/j.omtm.2019.01.008

Burger, C., Gorbatyuk, O. S., Velardo, M. J., Peden, C. S., Williams, P., Zolotukhin, S., et al. (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol. Ther.* 10, 302–317. doi: 10.1016/j.ymthe.2004.05.024

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., et al. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 28, 264–278. doi: 10.1523/jneurosci.4178-07.2008

Capotondo, A., Milazzo, R., Garcia-Manteiga, J. M., Cavalca, E., Montepeloso, A., Garrison, B. S., et al. (2017). Intracerebroventricular delivery of hematopoietic progenitors results in rapid and robust engraftment of microglia-like cells. *Sci. Adv.* 3:e1701211. doi: 10.1126/sciadv.1701211

Carter, C. J. (2007). eIF2B and oligodendrocyte survival: where nature and nurture meet in bipolar disorder and schizophrenia? *Schizophr. Bull.* 33, 1343–1353. doi: 10.1093/schbul/sbm007

Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutschera, I., et al. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 326, 818–823. doi: 10.1126/science.1171242

Cartier, N., Lewis, C. A., Zhang, R., and Rossi, F. M. (2014). The role of microglia in human disease: therapeutic tool or target? *Acta Neuropathol.* 128, 363–380. doi: 10.1007/s00401-014-1330-y

Castle, M. J., Turunen, H. T., Vandenberghe, L. H., and Wolfe, J. H. (2016). Controlling AAV tropism in the nervous system with natural and engineered capsids. *Methods Mol. Biol.* 1382, 133–149. doi: 10.1007/978-1-4939-3271-9_10

Cattoglio, C., Facchini, G., Sartori, D., Antonelli, A., Miccio, A., Cassani, B., et al. (2007). Hot spots of retroviral integration in human CD34+ hematopoietic cells. *Blood* 110, 1770–1778. doi: 10.1182/blood-2007-01-068759

Cearley, C. N., and Wolfe, J. H. (2006). Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol. Ther.* 13, 528–537. doi: 10.1016/j.ymthe.2005.11.015

Chakrabarty, P., Rosario, A., Cruz, P., Siemienski, Z., Ceballos-Diaz, C., Crosby, K., et al. (2013). Capsid serotype and timing of injection determines AAV transduction in the neonatal mice brain. *PLoS One* 8:e67680. doi: 10.1371/journal.pone.0067680

Chan, K. Y., Jang, M. J., Yoo, B. B., Greenbaum, A., Ravi, N., Wu, W. L., et al. (2017). Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. *Nat. Neurosci.* 20, 1172–1179. doi: 10.1038/nn.4593

Chandler, R. J., Williams, I. M., Gibson, A. L., Davidson, C. D., Incao, A. A., Hubbard, B. T., et al. (2017). Systemic AAV9 gene therapy improves the lifespan of mice with Niemann-Pick disease, type C1. *Hum. Mol. Genet.* 26, 52–64. doi: 10.1093/hmg/ddw367

Chatterjee, S., Sivanandam, V., and Wong, K. K. Jr. (2020). Adeno-associated virus and hematopoietic stem cells: the potential of adeno-associated virus hematopoietic stem cells in genetic medicines. *Hum. Gene Ther.* 31, 542–552. doi: 10.1089/hum.2020.049

Chen, H., McCarty, D. M., Bruce, A. T., and Suzuki, K. (1999). Oligodendrocyte-specific gene expression in mouse brain: use of a myelin-forming cell type-specific promoter in an adeno-associated virus. *J. Neurosci. Res.* 55, 504–513. doi: 10.1002/(sici)1097-4547(19990215)55:4<504::Aid-jnr10<3.0.Co;2-0

Cohen-Salmon, M., Slaoui, L., Mazaré, N., Gilbert, A., Oudart, M., Alvear-Perez, R., et al. (2020). Astrocytes in the regulation of cerebrovascular functions. *Glia* 69, 817–841. doi: 10.1002/glia.23924

Crow, Y. J., Leitch, A., Hayward, B. E., Garner, A., Parmar, R., Griffith, E., et al. (2006). Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutières syndrome and mimic congenital viral brain infection. *Nat. Genet.* 38, 910–916. doi: 10.1038/ng1842

Crow, Y. J., and Manel, N. (2015). Aicardi-Goutières syndrome and the type I interferonopathies. *Nat. Rev. Immunol.* 15, 429–440. doi: 10.1038/nri3850

Curiel, J., Rodríguez Bey, G., Takanohashi, A., Bugiani, M., Fu, X., Wolf, N. I., et al. (2017). TUBB4A mutations result in specific neuronal and oligodendrocytic defects that closely match clinically distinct phenotypes. *Hum. Mol. Genet.* 26, 4506–4518. doi: 10.1093/hmg/ddx338

Dahl, M., Doyle, A., Olsson, K., Månsson, J. E., Marques, A. R. A., Mirzaian, M., et al. (2015). Lentiviral gene therapy using cellular promoters cures type 1 Gaucher disease in mice. *Mol. Ther.* 23, 835–844. doi: 10.1038/mt.2015.16

Dallabona, C., Diodato, D., Kevelam, S. H., Haack, T. B., Wong, L. J., Salomons, G. S., et al. (2014). Novel (ovario) leukodystrophy related to AARS2 mutations. *Neurology* 82, 2063–2071. doi: 10.1212/wnl.0000000000000497

Davidson, B. L., Stein, C. S., Heth, J. A., Martins, I., Kotin, R. M., Derksen, T. A., et al. (2000). Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3428–3432. doi: 10.1073/pnas.050581197

Davidsson, M., Wang, G., Aldrin-Kirk, P., Cardoso, T., Nolbrant, S., Hartnor, M., et al. (2019). A systematic capsid evolution approach performed *in vivo* for the design of AAV vectors with tailored properties and tropism. *Proc. Natl. Acad. Sci. U.S.A.* 116, 27053–27062. doi: 10.1073/pnas.1910061116

de Leeuw, C. N., Korecki, A. J., Berry, G. E., Hickmott, J. W., Lam, S. L., Lengyell, T. C., et al. (2016). rAAV-compatible MiniPromoters for restricted expression in the brain and eye. *Mol. Brain* 9:52. doi: 10.1186/s13041-016-0232-4

Deasy, S. K., Uehara, R., Vodnala, S. K., Yang, H. H., Dass, R. A., Hu, Y., et al. (2019). Aicardi-Goutières syndrome gene Rnaseh2c is a metastasis susceptibility gene in breast cancer. *PLoS Genet.* 15:e1008020. doi: 10.1371/journal.pgen.1008020

Deverman, B. E., Pravdo, P. L., Simpson, B. P., Kumar, S. R., Chan, K. Y., Banerjee, A., et al. (2016). Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nat. Biotechnol.* 34, 204–209. doi: 10.1038/nbt.3440

Deverman, B. E., Ravina, B. M., Bankiewicz, K. S., Paul, S. M., and Sah, D. W. Y. (2018). Gene therapy for neurological disorders: progress and prospects. *Nat. Rev. Drug Discov.* 17, 641–659. doi: 10.1038/nrd.2018.110

DiMattia, M. A., Nam, H. J., Van Vliet, K., Mitchell, M., Bennett, A., Gurda, B. L., et al. (2012). Structural insight into the unique properties of adeno-associated virus serotype 9. *J. Virol.* 86, 6947–6958. doi: 10.1128/jvi.07232-11

Domenger, C., and Grimm, D. (2019). Next-generation AAV vectors-do not judge a virus (only) by its cover. *Hum. Mol. Genet.* 28, R3–R14. doi: 10.1093/hmg/ddz148

Dong, J. Y., Fan, P. D., and Frizzell, R. A. (1996). Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum. Gene Ther.* 7, 2101–2112. doi: 10.1089/hum.1996.7.17-2101

Dooves, S., Bugiani, M., Postma, N. L., Polder, E., Land, N., and Horan, S. T. (2016). Astrocytes are central in the pathomechanisms of vanishing white matter. *J. Clin. Invest.* 126, 1512–1524. doi: 10.1172/jci83908

Dooves, S., Leferink, P. S., Krabbenborg, S., Breeuwsma, N., Bots, S., Hillen, A. E. J., et al. (2019). Cell replacement therapy improves pathological hallmarks in a mouse model of leukodystrophy vanishing white matter. *Stem Cell Rep.* 12, 441–450. doi: 10.1016/j.stemcr.2019.01.018

Dudek, A. M., Zabaleta, N., Zinn, E., Pillay, S., Zengel, J., Porter, C., et al. (2020). GPR108 is a highly conserved AAV entry factor. *Mol. Ther.* 28, 367–381. doi: 10.1016/j.ymthe.2019.11.005

Duffner, P. K., Barczykowski, A., Jalal, K., Yan, L., Kay, D. M., and Carter, R. L. (2011). Early infantile Krabbe disease: results of the world-wide krabbe registry. *Pediatr. Neurol.* 45, 141–148. doi: 10.1016/j.pediatrneurol.2011.05.007

Eichler, F., Duncan, C., Musolino, P. L., Orchard, P. J., De Oliveira, S., Thrasher, A. J., et al. (2017). Hematopoietic stem-cell gene therapy for cerebral adrenoleukodystrophy. *N. Engl. J. Med.* 377, 1630–1638. doi: 10.1056/NEJMoa1700554

Elitt, M. S., Barbar, L., Shick, H. E., Powers, B. E., Maeno-Hikichi, Y., Madhavan, M., et al. (2020). Suppression of proteolipid protein rescues Pelizaeus–Merzbacher disease. *Nature* 585, 397–403. doi: 10.1038/s41586-020-2494-3

Ellsworth, J. L., Gingras, J., Smith, L. J., Rubin, H., Seabrook, T. A., Patel, K., et al. (2019). Clade F AAVHSCs cross the blood brain barrier and transduce the central nervous system in addition to peripheral tissues following intravenous administration in nonhuman primates. *PLoS One* 14:e0225582. doi: 10.1371/journal.pone.0225582

Emery, B., Agalliu, D., Cahoy, J. D., Watkins, T. A., Dugas, J. C., Mulinyawe, S. B., et al. (2009). Myelin gene regulatory factor is a critical transcriptional regulator required for CNS myelination. *Cell* 138, 172–185. doi: 10.1016/j.cell.2009.04.031

Escolar, M. L., Poe, M. D., Provenzale, J. M., Richards, K. C., Allison, J., Wood, S., et al. (2005). Transplantation of umbilical-cord blood in babies with infantile Krabbe's disease. *N. Engl. J. Med.* 352, 2069–2081. doi: 10.1056/NEJMoa042604

Feinstein, M., Markus, B., Noyman, I., Shalev, H., Flusser, H., Shelef, I., et al. (2010). Pelizaeus-Merzbacher-like disease caused by AIMP1/p43 homozygous mutation. *Am. J. Hum. Genet.* 87, 820–828. doi: 10.1016/j.ajhg.2010.10.016

Feng, L., Chao, J., Tian, E., Li, L., Ye, P., Zhang, M., et al. (2020). Cell-based therapy for canavan disease using human iPSC-Derived NPCs and OPCs. *Adv. Sci.* 7:2002155. doi: 10.1002/advs.202002155

Ferrari, G., Thrasher, A. J., and Aiuti, A. (2020). Gene therapy using haematopoietic stem and progenitor cells. *Nat. Rev. Genet.* 22, 216–234. doi: 10.1038/s41576-020-00298-5

Flotte, T. R., Afione, S. A., Solow, R., Drumm, M. L., Markakis, D., Guggino, W. B., et al. (1993). Expression of the cystic fibrosis transmembrane conductance regulator from a novel adeno-associated virus promoter. *J. Biol. Chem.* 268, 3781–3790.

Flotte, T. R., Solow, R., Owens, R. A., Afione, S., Zeitlin, P. L., and Carter, B. J. (1992). Gene expression from adeno-associated virus vectors in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 7, 349–356. doi: 10.1165/ajrcmb/7.3.349

Fraldi, A., Biffi, A., Lombardi, A., Visigalli, I., Pepe, S., Settembre, C., et al. (2007). SUMF1 enhances sulfatase activities *in vivo* in five sulfatase deficiencies. *Biochem. J.* 403, 305–312. doi: 10.1042/bj20061783

Fröhlich, D., Suchowerska, A. K., Spencer, Z. H., von Jonquieres, G., Klugmann, C. B., Bongers, A., et al. (2017). *in vivo* characterization of the aspartyl-tRNA synthetase DARS: homing in on the leukodystrophy HBSL. *Neurobiol. Dis.* 97(Pt A), 24–35. doi: 10.1016/j.nbd.2016.10.008

Fröhlich, D., Suchowerska, A. K., Voss, C., He, R., Wolvetang, E., von Jonquieres, G., et al. (2018). Expression pattern of the Aspartyl-tRNA Synthetase DARS in the human brain. *Front. Mol. Neurosci.* 11:81. doi: 10.3389/fnmol.2018.00081

Gao, G., Vandenberghe, L. H., Alvira, M. R., Lu, Y., Calcedo, R., Zhou, X., et al. (2004). Clades of Adeno-associated viruses are widely disseminated in human tissues. *J. Virol.* 78, 6381–6388. doi: 10.1128/jvi.78.12.6381-6388.2004

Gao, G., Vandenberghe, L. H., and Wilson, J. M. (2005). New recombinant serotypes of AAV vectors. *Curr. Gene Ther.* 5, 285–297. doi: 10.2174/1566523054065057

Garcia, L. M., Hacker, J. L., Sase, S., Adang, L., and Almad, A. (2020). Glial cells in the driver seat of leukodystrophy pathogenesis. *Neurobiol. Dis.* 146:105087. doi: 10.1016/j.nbd.2020.105087

Gaudet, D., Méthot, J., Déry, S., Brisson, D., Essiembre, C., Tremblay, G., et al. (2013). Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene Ther.* 20, 361–369. doi: 10.1038/gt.2012.43

Georgiou, E., Sidiropoulou, K., Richter, J., Papaneophytou, C., Sargiannidou, I., Kagiava, A., et al. (2017). Gene therapy targeting oligodendrocytes provides therapeutic benefit in a leukodystrophy model. *Brain* 140, 599–616. doi: 10.1093/brain/aww351

Gessler, D. J., and Gao, G. (2016). Gene therapy for the treatment of neurological disorders: metabolic disorders. *Methods Mol. Biol.* 1382, 429–465. doi: 10.1007/978-1-4939-3271-9_30

Gessler, D. J., Li, D., Xu, H., Su, Q., Sanmiguel, J., Tuncer, S., et al. (2017). Redirecting N-acetylaspartate metabolism in the central nervous system normalizes myelination and rescues Canavan disease. *JCI Insight* 2:e90807. doi: 10.1172/jci.insight.90807

Gessler, D. J., Tai, P. W. L., Li, J., and Gao, G. (2019). Intravenous Infusion of AAV for widespread gene delivery to the nervous system. *Methods Mol. Biol.* 1950, 143–163. doi: 10.1007/978-1-4939-9139-6_8

Giles, A. R., Govindasamy, L., Somanathan, S., and Wilson, J. M. (2018). Mapping an adeno-associated virus 9-Specific neutralizing epitope to develop next-generation gene delivery vectors. *J. Virol.* 92:e01011-18. doi: 10.1128/jvi.01011-18

Gray, A. L., O'Leary, C., Liao, A., Agúndez, L., Youshani, A. S., Gleitz, H. F., et al. (2019). An improved adeno-associated virus vector for neurological correction of the mouse model of mucopolysaccharidosis IIIA. *Hum. Gene Ther.* 30, 1052–1066. doi: 10.1089/hum.2018.189

Gray, S. J., Foti, S. B., Schwartz, J. W., Bachaboina, L., Taylor-Blake, B., Coleman, J., et al. (2011). Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors. *Hum. Gene Ther.* 22, 1143–1153. doi: 10.1089/hum.2010.245

Gray, S. J., Nagabhushan Kalburgi, S., McCown, T. J., and Jude Samulski, R. (2013). Global CNS gene delivery and evasion of anti-AAV-neutralizing antibodies by intrathecal AAV administration in non-human primates. *Gene Ther.* 20, 450–459. doi: 10.1038/gt.2012.101

Gray-Edwards, H. L., Jiang, X., Randle, A. N., Taylor, A. R., Voss, T. L., Johnson, A. K., et al. (2017). Lipidomic evaluation of feline neurologic disease after AAV Gene therapy. *Mol. Ther. Methods Clin. Dev.* 6, 135–142. doi: 10.1016/j.omtm.2017.07.005

Greter, M., Lelios, I., and Croxford, A. L. (2015). Microglia versus myeloid cell nomenclature during brain inflammation. *Front. Immunol.* 6:249. doi: 10.3389/fimmu.2015.00249

Grimm, D., and Büning, H. (2017). Small but increasingly mighty: latest advances in AAV vector research. design, and evolution. *Hum. Gene Ther.* 28, 1075–1086. doi: 10.1089/hum.2017.172

Grimm, D., Lee, J. S., Wang, L., Desai, T., Akache, B., Storm, T. A., et al. (2008). *In vitro* and *in vivo* gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. *J. Virol.* 82, 5887–5911. doi: 10.1128/jvi.00254-08

Guo, F., Bannerman, P., Mills Ko, E., Miers, L., Xu, J., Burns, T., et al. (2015). Ablating N-acetylaspartate prevents leukodystrophy in a Canavan disease model. *Ann. Neurol.* 77, 884–888. doi: 10.1002/ana.24392

Gupta, N., Henry, R. G., Kang, S.-M., Strober, J., Lim, D. A., Ryan, T., et al. (2019). Long-Term safety, immunologic response, and imaging outcomes following neural stem cell transplantation for pelizaeus-Merzbacher Disease. *Stem Cell Rep.* 13, 254–261. doi: 10.1016/j.stemcr.2019.07.002

Gupta, N., Henry, R. G., Strober, J., Kang, S.-M., Lim, D. A., Bucci, M., et al. (2012). Neural stem cell engraftment and myelination in the human brain. *Sci. Transl. Med.* 4:155ra137. doi: 10.1126/scitranslmed.3004373

Gurda, B. L., DiMattia, M. A., Miller, E. B., Bennett, A., McKenna, R., Weichert, W. S., et al. (2013). Capsid antibodies to different adeno-associated virus serotypes bind common regions. *J. Virol.* 87, 9111–9124. doi: 10.1128/jvi.00622-13

Haberman, R. P., McCown, T. J., and Samulski, R. J. (2000). Novel transcriptional regulatory signals in the adeno-associated virus terminal repeat A/D junction element. *J. Virol.* 74, 8732–8739. doi: 10.1128/jvi.74.18.8732-8739.2000

Hagemann, T. L., Powers, B., Mazur, C., Kim, A., Wheeler, S., Hung, G., et al. (2018). Antisense suppression of glial fibrillary acidic protein as a treatment for Alexander disease. *Ann. Neurol.* 83, 27–39. doi: 10.1002/ana.25118

Hamilton, E. M., Polder, E., Vanderver, A., Naidu, S., Schiffmann, R., Fisher, K., et al. (2014). Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation. *Brain* 137(Pt 7), 1921–1930. doi: 10.1093/brain/awu110

Hansen, J., Qing, K., Kwon, H. J., Mah, C., and Srivastava, A. (2000). Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. *J. Virol.* 74, 992–996. doi: 10.1128/jvi.74.2.992-996.2000

Hartmann, J., Thalheimer, F. B., Höpfner, F., Kerzel, T., Khodosevich, K., García-González, D., et al. (2019). GluA4-Targeted AAV vectors deliver genes selectively to interneurons while relying on the AAV receptor for entry. *Mol. Ther. Methods Clin. Dev.* 14, 252–260. doi: 10.1016/j.omtm.2019.07.004

Haruwaka, K., Ikegami, A., Tachibana, Y., Ohno, N., Konishi, H., Hashimoto, A., et al. (2019). Dual microglia effects on blood brain barrier permeability induced by systemic inflammation. *Nat. Commun.* 10:5816. doi: 10.1038/s41467-019-13812-z

Heim, P., Claussen, M., Hoffmann, B., Conzelmann, E., Gärtner, J., Harzer, K., et al. (1997). Leukodystrophy incidence in Germany. *Am. J. Med. Genet.* 71, 475–478.

Hierlmeier, S., Eyrich, M., Wölfl, M., Schlegel, P. G., and Wiegering, V. (2018). Early and late complications following hematopoietic stem cell transplantation in pediatric patients - A retrospective analysis over 11 years. *PLoS One* 13:e0204914. doi: 10.1371/journal.pone.0204914

Hinderer, C., Bell, P., Katz, N., Vite, C. H., Louboutin, J. P., Bote, E., et al. (2018a). Evaluation of intrathecal routes of administration for adeno-associated viral vectors in large animals. *Hum. Gene Ther.* 29, 15–24. doi: 10.1089/hum.2017.026

Hinderer, C., Katz, N., Buza, E. L., Dyer, C., Goode, T., Bell, P., et al. (2018b). Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. *Hum. Gene Ther.* 29, 285–298. doi: 10.1089/hum.2018.015

Hinderer, C., Bell, P., Vite, C. H., Louboutin, J. P., Grant, R., Bote, E., et al. (2014). Widespread gene transfer in the central nervous system of cynomolgus macaques following delivery of AAV9 into the cisterna magna. *Mol. Ther. Methods Clin. Dev.* 1:14051. doi: 10.1038/mtm.2014.51

Hinderer, C., Nosratbakhsh, B., Katz, N., and Wilson, J. M. (2020). A single injection of an optimized adeno-associated viral vector into cerebrospinal fluid corrects neurological disease in a murine model of GM1 gangliosidosis. *Hum. Gene Ther.* 31, 1169–1177. doi: 10.1089/hum.2018.206

Hocquemiller, M., Giersch, L., Audrain, M., Parker, S., and Cartier, N. (2016). Adeno-associated virus-based gene therapy for CNS diseases. *Hum. Gene Ther.* 27, 478–496. doi: 10.1089/hum.2016.087

Hordeaux, J., Wang, Q., Katz, N., Buza, E. L., Bell, P., and Wilson, J. M. (2018). The Neurotropic properties of AAV-PHP.B Are limited to C57BL/6J Mice. *Mol. Ther.* 26, 664–668. doi: 10.1016/j.ymthe.2018.01.018

Hordeaux, J., Yuan, Y., Clark, P. M., Wang, Q., Martino, R. A., Sims, J. J., et al. (2019). The GPI-Linked protein LY6A Drives AAV-PHP.B transport across the blood-brain barrier. *Mol. Ther.* 27, 912–921. doi: 10.1016/j.ymthe.2019.02.013

Hoshino, H., and Kubota, M. (2014). Canavan disease: clinical features and recent advances in research. *Pediatr. Int.* 56, 477–483. doi: 10.1111/ped.12422

Hsu, H. L., Brown, A., Loveland, A. B., Lotun, A., Xu, M., Luo, L., et al. (2020). Structural characterization of a novel human adeno-associated virus capsid with neurotropic properties. *Nat. Commun.* 11:3279. doi: 10.1038/s41467-020-17047-1

Huang, L. Y., Halder, S., and Agbandje-McKenna, M. (2014). Parvovirus glycan interactions. *Curr. Opin. Virol.* 7, 108–118. doi: 10.1016/j.coviro.2014.05.007

Hudry, E., Andres-Mateos, E., Lerner, E. P., Volak, A., Cohen, O., Hyman, B. T., et al. (2018). Efficient gene transfer to the central nervous system by single-stranded Anc80L65. *Mol. Ther. Methods Clin. Dev.* 10, 197–209. doi: 10.1016/j.omtm.2018.07.006

Hudry, E., and Vandenberghe, L. H. (2019). Therapeutic AAV gene transfer to the nervous system: a clinical reality. *Neuron* 101, 839–862. doi: 10.1016/j.neuron.2019.02.017

Hudson, L. D. (2003). Pelizaeus-Merzbacher disease and spastic paraplegia type 2: two faces of myelin loss from mutations in the same gene. *J. Child Neurol.* 18, 616–624. doi: 10.1177/08830738030180090801

Hull, V., Wang, Y., Burns, T., Zhang, S., Sternbach, S., McDonough, J., et al. (2020). Antisense oligonucleotide reverses leukodystrophy in canavan disease mice. *Ann. Neurol.* 87, 480–485. doi: 10.1002/ana.25674

Hüser, D., Khalid, D., Lutter, T., Hammer, E. M., Weger, S., Heßler, M., et al. (2017). High prevalence of infectious adeno-associated virus (AAV) in human peripheral blood mononuclear cells indicative of T lymphocytes as sites of AAV persistence. *J. Virol.* 91:e02137-16. doi: 10.1128/jvi.02137-16

Hutson, T. H., Verhaagen, J., Yáñez-Muñoz, R. J., and Moon, L. D. (2012). Corticospinal tract transduction: a comparison of seven adeno-associated viral vector serotypes and a non-integrating lentiviral vector. *Gene Ther.* 19, 49–60. doi: 10.1038/gt.2011.71

Ingusci, S., Verlengia, G., Soukupova, M., Zucchini, S., and Simonato, M. (2019). Gene therapy tools for brain diseases. *Front. Pharmacol.* 10:724. doi: 10.3389/fphar.2019.00724

Inoue, K., Osaka, H., Sugiyama, N., Kawanishi, C., Onishi, H., Nezu, A., et al. (1996). A duplicated PLP gene causing pelizaeus-merzbacher disease detected by comparative multiplex PCR. *Am. J. Hum. Genet.* 59, 32–39.

Jacques, S. J., Ahmed, Z., Forbes, A., Douglas, M. R., Vigenswara, V., Berry, M., et al. (2012). AAV8(gfp) preferentially targets large diameter dorsal root ganglion neurones after both intra-dorsal root ganglion and intrathecal injection. *Mol. Cell Neurosci.* 49, 464–474. doi: 10.1016/j.mcn.2012.03.002

Jang, J. H., Koerber, J. T., Kim, J. S., Asuri, P., Vazin, T., Bartel, M., et al. (2011). An evolved adeno-associated viral variant enhances gene delivery and gene targeting in neural stem cells. *Mol. Ther.* 19, 667–675. doi: 10.1038/mt.2010.287

Jovièiæ, A., and Gitler, A. D. (2017). Distinct repertoires of microRNAs present in mouse astrocytes compared to astrocyte-secreted exosomes. *PLoS One* 12:e0171418. doi: 10.1371/journal.pone.0171418

Jüttner, J., Szabo, A., Gross-Scherf, B., Morikawa, R. K., Rompani, S. B., Hantz, P., et al. (2019). Targeting neuronal and glial cell types with synthetic promoter AAVs in mice, non-human primates and humans. *Nat. Neurosci.* 22, 1345–1356. doi: 10.1038/s41593-019-0431-2

Karumuthil-Melethil, S., Marshall, M. S., Heindel, C., Jakubauskas, B., Bongarzone, E. R., and Gray, S. J. (2016). Intrathecal administration of AAV/GALC vectors in 10-11-day-old twitcher mice improves survival and is enhanced by bone marrow transplant. *J. Neurosci. Res.* 94, 1138–1151. doi: 10.1002/jnr.23882

Katrekar, D., Moreno, A. M., Chen, G., Worlikar, A., and Mali, P. (2018). Oligonucleotide conjugated multi-functional adeno-associated viruses. *Sci. Rep.* 8:3589. doi: 10.1038/s41598-018-21742-x

Kaygisiz, K., and Synatschke, C. V. (2020). Materials promoting viral gene delivery. *Biomater. Sci.* 8, 6113–6156. doi: 10.1039/d0bm01367f

Kelemen, R. E., Mukherjee, R., Cao, X., Erickson, S. B., Zheng, Y., and Chatterjee, A. (2016). A precise chemical strategy to alter the receptor specificity of the adeno-associated virus. *Angew. Chem. Int. Ed. Engl.* 55, 10645–10649. doi: 10.1002/anie.201604067

Kevelam, S. H., Steenweg, M. E., Srivastava, S., Helman, G., Naidu, S., Schiffmann, R., et al. (2016). Update on leukodystrophies: a historical perspective and adapted definition. *Neuropediatrics* 47, 349–354. doi: 10.1055/s-0036-1588020

Klein, R. L., Dayton, R. D., Tatom, J. B., Henderson, K. M., and Henning, P. P. (2008). AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. *Mol. Ther.* 16, 89–96. doi: 10.1038/sj.mt.6300331

Klein, R. L., Hamby, M. E., Gong, Y., Hirko, A. C., Wang, S., Hughes, J. A., et al. (2002). Dose and promoter effects of adeno-associated viral vector for green fluorescent protein expression in the rat brain. *Exp. Neurol.* 176, 66–74. doi: 10.1006/exnr.2002.7942

Koenning, M., Jackson, S., Hay, C. M., Faux, C., Kilpatrick, T. J., Willingham, M., et al. (2012). Myelin gene regulatory factor is required for maintenance of myelin and mature oligodendrocyte identity in the adult CNS. *J. Neurosci.* 32, 12528–12542. doi: 10.1523/jneurosci.1069-12.2012

Koh, W., Park, Y. M., Lee, S. E., and Lee, C. J. (2017). AAV-Mediated astrocyte-specific gene expression under human ALDH1L1 promoter in mouse thalamus. *Exp. Neurobiol.* 26, 350–361. doi: 10.5607/en.2017.26.6.350

Köhler, W., Curiel, J., and Vanderver, A. (2018). Adulthood leukodystrophies. *Nat. Rev. Neurol.* 14, 94–105. doi: 10.1038/nrneurol.2017.175

Kügler, S., Meyn, L., Holzmüller, H., Gerhardt, E., Isenmann, S., Schulz, J. B., et al. (2001). Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinant adenoviral vectors. *Mol. Cell Neurosci.* 17, 78–96. doi: 10.1006/mcne.2000.0929

Kühl, J. S., Eichler, F., Duncan, C., Orchard, P. J., De Oliveira, S., Thrasher, A. J., Lund, T., et al. (2020). The 46th annual meeting of the European society for blood and marrow transplantation: physicians oral session (0010-0173). *Bone Marrow Transpl.* 55, 22–174. doi: 10.1038/s41409-020-01119-3

Kuhn, S., Gritti, L., Crooks, D., and Dombrowski, Y. (2019). Oligodendrocytes in development, myelin generation and beyond. *Cells* 8:1424. doi: 10.3390/cells8111424

Kunze, C., Börner, K., Kienle, E., Orschmann, T., Rusha, E., Schneider, M., et al. (2018). Synthetic AAV/CRISPR vectors for blocking HIV-1 expression in persistently infected astrocytes. *Glia* 66, 413–427. doi: 10.1002/glia.23254

Kurtzberg, J., Buntz, S., Gentry, T., Noeldner, P., Ozamiz, A., Rusche, B., et al. (2015). Preclinical characterization of DUOC-01, a cell therapy product derived from banked umbilical cord blood for use as an adjuvant to umbilical cord blood

transplantation for treatment of inherited metabolic diseases. *Cytotherapy* 17, 803–815. doi: 10.1016/j.jcyt.2015.02.006

Lahey, H. G., Webber, C. J., Golebiowski, D., Izzo, C. M., Horn, E., Taghian, T., et al. (2020). Pronounced therapeutic benefit of a single bidirectional AAV vector administered systemically in sandhoff mice. *Mol. Ther.* 28, 2150–2160. doi: 10.1016/j.ymthe.2020.06.021

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. doi: 10.2478/s13380-013-0118-1

Lawlor, P. A., Bland, R. J., Mouravlev, A., Young, D., and During, M. J. (2009). Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol. Ther.* 17, 1692–1702. doi: 10.1038/mt.2009.170

Lee, E. J., Guenther, C. M., and Suh, J. (2018). Adeno-associated virus (AAV) vectors: rational design strategies for capsid engineering. *Curr. Opin. Biomed. Eng.* 7, 58–63. doi: 10.1016/j.cobme.2018.09.004

Lee, Y., Messing, A., Su, M., and Brenner, M. (2008). GFAP promoter elements required for region-specific and astrocyte-specific expression. *Glia* 56, 481–493. doi: 10.1002/glia.20622

Leegwater, P. A., Vermeulen, G., Könst, A. A., Naidu, S., Mulders, J., Visser, A., et al. (2001). Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. *Nat. Genet.* 29, 383–388. doi: 10.1038/ng764

Lentz, T. B., Gray, S. J., and Samulski, R. J. (2012). Viral vectors for gene delivery to the central nervous system. *Neurobiol. Dis.* 48, 179–188. doi: 10.1016/j.nbd.2011.09.014

Leone, P., Janson, C. G., Bilaniuk, L., Wang, Z., Sorgi, F., Huang, L., et al. (2000). Aspartoacylase gene transfer to the mammalian central nervous system with therapeutic implications for Canavan disease. *Ann. Neurol.* 48, 27–38. doi: 10.1002/1531-8249(200007)48:1<27::aid-ana6<3.0.co;2-6

Leone, P., Shera, D., McPhee, S. W., Francis, J. S., Kolodny, E. H., Bilaniuk, L. T., et al. (2012). Long-term follow-up after gene therapy for canavan disease. *Sci. Transl. Med.* 4:165ra163. doi: 10.1126/scitranslmed.3003454

Li, B., Ma, W., Ling, C., Van Vliet, K., Huang, L. Y., Agbandje-McKenna, M., et al. (2015). Site-directed mutagenesis of surface-exposed lysine residues leads to improved transduction by AAV2, But Not AAV8, vectors in murine hepatocytes *in vivo*. *Hum. Gene Ther. Methods* 26, 211–220. doi: 10.1089/hgtb.2015.115

Li, C., and Samulski, R. J. (2020). Engineering adeno-associated virus vectors for gene therapy. *Nat. Rev. Genet.* 21, 255–272. doi: 10.1038/s41576-019-0205-4

Li, H., Okada, H., Suzuki, S., Sakai, K., Izumi, H., Matsushima, Y., et al. (2019). Gene suppressing therapy for pelizaeus-merzbacher disease using artificial microRNA. *JCI Insight* 4:e125052. doi: 10.1172/jci.insight.125052

Li, L., Tian, E., Chen, X., Chao, J., Klein, J., Qu, Q., et al. (2018). GFAP mutations in astrocytes impair oligodendrocyte progenitor proliferation and myelination in an hiPSC model of alexander disease. *Cell Stem Cell* 23, 239.e6–251.e6. doi: 10.1016/j.stem.2018.07.009

Lisowski, L., Dane, A. P., Chu, K., Zhang, Y., Cunningham, S. C., Wilson, E. M., et al. (2014). Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature* 506, 382–386. doi: 10.1038/nature12875

Logan, G. J., Dane, A. P., Hallwirth, C. V., Smyth, C. M., Wilkie, E. E., Amaya, A. K., et al. (2017). Identification of liver-specific enhancer-promoter activity in the 3' untranslated region of the wild-type AAV2 genome. *Nat. Genet.* 49, 1267–1273. doi: 10.1038/ng.3893

Lutz, S. E., Zhao, Y., Gulinello, M., Lee, S. C., Raine, C. S., and Brosnan, C. F. (2009). Deletion of astrocyte connexins 43 and 30 leads to a dysmyelinating phenotype and hippocampal CA1 vacuolation. *J. Neurosci.* 29, 7743–7752. doi: 10.1523/jneurosci.0341-09.2009

Maier, H., Wang-Eckhardt, L., Hartmann, D., Gieselmann, V., and Eckhardt, M. (2015). N-Acetylaspartate synthase deficiency corrects the myelin phenotype in a canavan disease mouse model but does not affect survival time. *J. Neurosci.* 35, 14501–14516. doi: 10.1523/jneurosci.1056-15.2015

Mandel, R. J., Marmion, D. J., Kirik, D., Chu, Y., Heindel, C., McCown, T., et al. (2017). Novel oligodendroglial alpha synuclein viral vector models of multiple system atrophy: studies in rodents and nonhuman primates. *Acta Neuropathol. Commun.* 5:47. doi: 10.1186/s40478-017-0451-7

Markusic, D. M., Herzog, R. W., Aslanidi, G. V., Hoffman, B. E., Li, B., Li, M., et al. (2010). High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. *Mol. Ther.* 18, 2048–2056. doi: 10.1038/mt.2010.172

Marshall, M. S., Issa, Y., Jakubauskas, B., Stoskute, M., Elackattu, V., Marshall, J. N., et al. (2018). Long-term improvement of neurological signs and metabolic dysfunction in a mouse model of krabbe's disease after global gene therapy. *Mol. Ther.* 26, 874–889. doi: 10.1016/j.ymthe.2018.01.009

Martino, A. T., Basner-Tschakarjan, E., Markusic, D. M., Finn, J. D., Hinderer, C., Zhou, S., et al. (2013). Engineered AAV vector minimizes *in vivo* targeting of transduced hepatocytes by capsid-specific CD8+ T cells. *Blood* 121, 2224–2233. doi: 10.1182/blood-2012-10-460733

Matalon, R., Rady, P. L., Platt, K. A., Skinner, H. B., Quast, M. J., Campbell, G. A., et al. (2000). Knock-out mouse for Canavan disease: a model for gene transfer to the central nervous system. *J. Gene Med.* 2, 165–175. doi: 10.1002/(sici)1521-2254(200005/06)2:3<165::Aid-jgm107<3.0.Co;2-r

Matalon, R., Surendran, S., Rady, P. L., Quast, M. J., Campbell, G. A., Matalon, K. M., et al. (2003). Adeno-associated virus-mediated aspartoacylase gene transfer to the brain of knockout mouse for canavan disease. *Mol. Ther.* 7(5 Pt 1), 580–587. doi: 10.1016/s1525-0016(03)00066-2

Matsukawa, T., Yamamoto, T., Honda, A., Toya, T., Ishiura, H., Mitsui, J., et al. (2020). Clinical efficacy of haematopoietic stem cell transplantation for adult adrenoleukodystrophy. *Brain Commun.* 2:fcz048. doi: 10.1093/braincomms/fcz048

Matsuzaki, Y., Konno, A., Mochizuki, R., Shinohara, Y., Nitta, K., Okada, Y., et al. (2018). Intravenous administration of the adeno-associated virus-PHP.B capsid fails to upregulate transduction efficiency in the marmoset brain. *Neurosci. Lett.* 665, 182–188. doi: 10.1016/j.neulet.2017.11.049

McCarty, D. M. (2008). Self-complementary AAV vectors; advances and applications. *Mol. Ther.* 16, 1648–1656. doi: 10.1038/mt.2008.171

McPhee, S. W., Francis, J., Janson, C. G., Serikawa, T., Hyland, K., Ong, E. O., et al. (2005). Effects of AAV-2-mediated aspartoacylase gene transfer in the tremor rat model of Canavan disease. *Brain Res. Mol. Brain Res.* 135, 112–121. doi: 10.1016/j.molbrainres.2004.12.007

McPhee, S. W., Janson, C. G., Li, C., Samulski, R. J., Camp, A. S., Francis, J., et al. (2006). Immune responses to AAV in a phase I study for Canavan disease. *J. Gene Med.* 8, 577–588. doi: 10.1002/jgm.885

Mendell, J. R., Al-Zaidy, S., Shell, R., Arnold, W. D., Rodino-Klapac, L. R., Prior, T. W., et al. (2017). Single-dose gene-replacement therapy for spinal muscular atrophy. *N. Engl. J. Med.* 377, 1713–1722. doi: 10.1056/NEJMoa1706198

Mendell, J. R., Sahenk, Z., Malik, V., Gomez, A. M., Flanigan, K. M., Lowes, L. P., et al. (2015). A phase 1/2a follistatin gene therapy trial for becker muscular dystrophy. *Mol. Ther.* 23, 192–201. doi: 10.1038/mt.2014.200

Mendes, M. I., Green, L. M. C., Bertini, E., Tonduti, D., Aiello, C., Smith, D., et al. (2020). RARS1-related hypomyelinating leukodystrophy: expanding the spectrum. *Ann. Clin. Transl. Neurol.* 7, 83–93. doi: 10.1002/acn3.50960

Mizukami, H., Young, N. S., and Brown, K. E. (1996). Adeno-associated virus type 2 binds to a 150-kilodalton cell membrane glycoprotein. *Virology* 217, 124–130. doi: 10.1006/viro.1996.0099

Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Bartholomae, C. C., Ranzani, M., et al. (2009). The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J. Clin. Invest.* 119, 964–975. doi: 10.1172/jci37630

Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Ponzoni, M., Bartholomae, C., et al. (2006). Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat. Biotechnol.* 24, 687–696. doi: 10.1038/nbt1216

Morell, P. (1984). A correlative synopsis of the leukodystrophies. *Neuropediatrics* 15(Suppl.), 62–65. doi: 10.1055/s-2008-1052383

Münch, R. C., Muth, A., Muik, A., Friedel, T., Schmatz, J., Dreier, B., et al. (2015). Off-target-free gene delivery by affinity-purified receptor-targeted viral vectors. *Nat. Commun.* 6:6246. doi: 10.1038/ncomms7246

Musolino, P. L., Lund, T. C., Pan, J., Escolar, M. L., Paker, A. M., Duncan, C. N., et al. (2014). Hematopoietic stem cell transplantation in the leukodystrophies: a systematic review of the literature. *Neuropediatrics* 45, 169–174. doi: 10.1055/s-0033-1364179

Nagree, M. S., Scalia, S., McKillop, W. M., and Medin, J. A. (2019). An update on gene therapy for lysosomal storage disorders. *Expert Opin. Biol. Ther.* 19, 655–670. doi: 10.1080/14712598.2019.1607837

Nahhas, N., Conant, A., Hamilton, E., Curiel, J., Simons, C., van der Knaap, M., et al. (1993). "TUBB4A-related leukodystrophy," in *GeneReviews(®)*, eds M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, et al. (Seattle, WA: University of Washington, Seattle).

Nathanson, J. L., Yanagawa, Y., Obata, K., and Callaway, E. M. (2009). Preferential labeling of inhibitory and excitatory cortical neurons by endogenous tropism of adeno-associated virus and lentivirus vectors. *Neuroscience* 161, 441–450. doi: 10.1016/j.neuroscience.2009.03.032

Nguyen, G. N., Everett, J. K., Kafle, S., Roche, A. M., Raymond, H. E., Leiby, J., et al. (2021). A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat. Biotechnol.* 39, 47–55. doi: 10.1038/s41587-020-0741-7

Nicholson, A. M., Baker, M. C., Finch, N. A., Rutherford, N. J., Wider, C., Graff-Radford, N. R., et al. (2013). CSF1R mutations link POLD and HDLS as a single disease entity. *Neurology* 80, 1033–1040. doi: 10.1212/WNL.0b013e31828726a7

Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108, 193–199. doi: 10.1016/0378-1119(91)90434-d

Noell, S., Wolburg-Buchholz, K., Mack, A. F., Beedle, A. M., Satz, J. S., Campbell, K. P., et al. (2011). Evidence for a role of dystroglycan regulating the membrane architecture of astroglial endfeet. *Eur. J. Neurosci.* 33, 2179–2186. doi: 10.1111/j.1460-9568.2011.07688.x

Nualart-Marti, A., Solsona, C., and Fields, R. D. (2013). Gap junction communication in myelinating glia. *Biochim. Biophys. Acta* 1828, 69–78. doi: 10.1016/j.bbamem.2012.01.024

Nutma, E., van Gent, D., Amor, S., and Peferoen, L. A. N. (2020). Astrocyte and oligodendrocyte cross-talk in the central nervous system. *Cells* 9:600. doi: 10.3390/cells9030600

Ojala, D. S., Amara, D. P., and Schaffer, D. V. (2015). Adeno-associated virus vectors and neurological gene therapy. *Neuroscientist* 21, 84–98. doi: 10.1177/1073858414521870

Ojala, D. S., Sun, S., Santiago-Ortiz, J. L., Shapiro, M. G., Romero, P. A., and Schaffer, D. V. (2018). *in vivo* selection of a computationally designed SCHEMA AAV library yields a novel variant for infection of adult neural stem cells in the SVZ. *Mol. Ther.* 26, 304–319. doi: 10.1016/j.ymthe.2017.09.006

Olabarria, M., and Goldman, J. E. (2017). Disorders of astrocytes: alexander disease as a model. *Annu. Rev. Pathol.* 12, 131–152. doi: 10.1146/annurev-pathol-052016-100218

Orcesi, S., La Piana, R., and Fazzi, E. (2009). Aicardi–Goutières syndrome. *Br. Med. Bull.* 89, 183–201. doi: 10.1093/bmb/ldn049

Ornaghi, F., Sala, D., Tedeschi, F., Maffia, M. C., Bazzucchi, M., Morena, F., et al. (2020). Novel bicistronic lentiviral vectors correct β-Hexosaminidase deficiency in neural and hematopoietic stem cells and progeny: implications for *in vivo* and *ex vivo* gene therapy of GM2 gangliosidosis. *Neurobiol. Dis.* 134:104667. doi: 10.1016/j.nbd.2019.104667

Orsini, J. J., Escolar, M. L., Wasserstein, M. P., and Caggana, M. (1993). "Krabbe disease," in *GeneReviews(®)*, eds M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, et al. (Seattle, WA: University of Washington, Seattle).

Orthmann-Murphy, J. L., Freidin, M., Fischer, E., Scherer, S. S., and Abrams, C. K. (2007). Two distinct heterotypic channels mediate gap junction coupling between astrocyte and oligodendrocyte connexins. *J. Neurosci.* 27, 13949–13957. doi: 10.1523/jneurosci.3395-07.2007

Osorio, M. J., and Goldman, S. A. (2016). Glial progenitor cell-based treatment of the childhood leukodystrophies. *Exp. Neurol.* 283(Pt B), 476–488. doi: 10.1016/j.expneurol.2016.05.010

Osório, M. J., and Goldman, S. A. (2018). Neurogenetics of pelizaeus-merzbacher disease. *Handb. Clin. Neurol.* 148, 701–722. doi: 10.1016/b978-0-444-64076-5.00045-4

Page, K. M., Stenger, E. O., Connelly, J. A., Shyr, D., West, T., Wood, S., et al. (2019). Hematopoietic stem cell transplantation to treat leukodystrophies: clinical practice guidelines from the hunter's hope leukodystrophy care network. *Biol. Blood Marrow Transplant* 25, e363–e374. doi: 10.1016/j.bbmt.2019.09.003

Pan, X., Sands, S. A., Yue, Y., Zhang, K., LeVine, S. M., and Duan, D. (2019). An engineered galactosylceramidase construct improves AAV gene therapy for krabbe disease in twitcher mice. *Hum. Gene Ther.* 30, 1039–1051. doi: 10.1089/hum.2019.008

Parenti, G., Medina, D. L., and Ballabio, A. (2021). The rapidly evolving view of lysosomal storage diseases. *EMBO Mol. Med.* 13:e12836. doi: 10.15252/emmm.202012836

Passini, M. A., Watson, D. J., Vite, C. H., Landsburg, D. J., Feigenbaum, A. L., and Wolfe, J. H. (2003). Intraventricular brain injection of adeno-associated virus type 1 (AAV1) in neonatal mice results in complementary patterns of neuronal transduction to AAV2 and total long-term correction of storage lesions in the brains of beta-glucuronidase-deficient mice. *J. Virol.* 77, 7034–7040. doi: 10.1128/jvi.77.12.7034-7040.2003

Pavel-Dinu, M., Wiebking, V., Dejene, B. T., Srifa, W., Mantri, S., Nicolas, C. E., et al. (2019). Gene correction for SCID-X1 in long-term hematopoietic stem cells. *Nat. Commun.* 10:1634. doi: 10.1038/s41467-019-09614-y

Peel, A. L., Zolotukhin, S., Schrimsher, G. W., Muzyczka, N., and Reier, P. J. (1997). Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type-specific promoters. *Gene Ther.* 4, 16–24. doi: 10.1038/sj.gt.3300358

Pennesi, M. E., Weleber, R. G., Yang, P., Whitebirch, C., Thean, B., Flotte, T. R., et al. (2018). Results at 5 Years after gene therapy for RPE65-Deficient retinal dystrophy. *Hum. Gene Ther.* 29, 1428–1437. doi: 10.1089/hum.2018.014

Philippidis, A. (2020). After third death, Audentes' AT132 remains on clinical hold. *Hum. Gene Ther.* 31, 908–910. doi: 10.1089/hum.2020.29133.bfs

Piguet, F., Alves, S., and Cartier, N. (2017). Clinical gene therapy for neurodegenerative diseases: past, present, and future. *Hum. Gene Ther.* 28, 988–1003. doi: 10.1089/hum.2017.160

Piguet, F., de Saint Denis, T., Audouard, E., Beccaria, K., Arthur, A., Wurtz, G., et al. (2020). The challenge of gene therapy for neurological diseases: strategies and tools to achieve efficient delivery to the central nervous system. *Hum. Gene Ther.* 32, 349–374. doi: 10.1089/hum.2020.105

Pillay, S., Meyer, N. L., Puschnik, A. S., Davulcu, O., Diep, J., Ishikawa, Y., et al. (2016). An essential receptor for adeno-associated virus infection. *Nature* 530, 108–112. doi: 10.1038/nature16465

Pinyon, J. L., von Jonquieres, G., Crawford, E. N., Duxbury, M., Al Abed, A., Lovell, N. H., et al. (2019). Neurotrophin gene augmentation by electrotransfer to improve cochlear implant hearing outcomes. *Hear. Res.* 380, 137–149. doi: 10.1016/j.heares.2019.06.002

Portales-Casamar, E., Swanson, D. J., Liu, L., de Leeuw, C. N., Banks, K. G., Ho Sui, S. J., et al. (2010). A regulatory toolbox of MiniPromoters to drive selective expression in the brain. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16589–16594. doi: 10.1073/pnas.1009158107

Powell, S. K., Khan, N., Parker, C. L., Samulski, R. J., Matsushima, G., Gray, S. J., et al. (2016). Characterization of a novel adeno-associated viral vector with preferential oligodendrocyte tropism. *Gene Ther.* 23, 807–814. doi: 10.1038/gt.2016.62

Powell, S. K., Samulski, R. J., and McCown, T. J. (2020). AAV capsid-promoter interactions determine CNS Cell-selective gene expression *in vivo*. *Mol. Ther.* 28, 1373–1380. doi: 10.1016/j.ymthe.2020.03.007

Prentice, H. M., Biswal, M. R., Dorey, C. K., and Blanks, J. C. (2011). Hypoxia-regulated retinal glial cell-specific promoter for potential gene therapy in disease. *Invest. Ophthalmol. Vis. Sci.* 52, 8562–8570. doi: 10.1167/iovs.10-6835

Prust, M., Wang, J., Morizono, H., Messing, A., Brenner, M., Gordon, E., et al. (2011). GFAP mutations, age at onset, and clinical subtypes in Alexander disease. *Neurology* 77, 1287–1294. doi: 10.1212/WNL.0b013e3182309f72

Rademakers, R., Baker, M., Nicholson, A. M., Rutherford, N. J., Finch, N., Soto-Ortolaza, A., et al. (2011). Mutations in the colony stimulating factor 1 receptor (CSF1R) gene cause hereditary diffuse leukoencephalopathy with spheroids. *Nat. Genet.* 44, 200–205. doi: 10.1038/ng.1027

Rafi, M. A., Luzi, P., and Wenger, D. A. (2020). Conditions for combining gene therapy with bone marrow transplantation in murine Krabbe disease. *Bioimpacts* 10, 105–115. doi: 10.34172/bi.2020.13

Ravindra Kumar, S., Miles, T. F., Chen, X., Brown, D., Dobreva, T., Huang, Q., et al. (2020). Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types. *Nat. Methods* 17, 541–550. doi: 10.1038/s41592-020-0799-7

Ricca, A., and Gritti, A. (2016). Perspective on innovative therapies for globoid cell leukodystrophy. *J. Neurosci. Res.* 94, 1304–1317. doi: 10.1002/jnr.23752

Ronzitti, G., Gross, D. A., and Mingozzi, F. (2020). Human immune responses to adeno-associated virus (AAV) Vectors. *Front. Immunol.* 11:670. doi: 10.3389/fimmu.2020.00670

Rosario, A. M., Cruz, P. E., Ceballos-Diaz, C., Strickland, M. R., Siemienski, Z., Pardo, M., et al. (2016). Microglia-specific targeting by novel capsid-modified AAV6 vectors. *Mol. Ther. Methods Clin. Dev.* 3:16026. doi: 10.1038/mtm.2016.26

Saha, A. (2018). DUOC-01, a cell therapy product derived from human cord blood, accelerates remyelination. *Stem Cells Transl. Med.* 7(Suppl. 1), S5–S5. doi: 10.1002/sctm.12356

Saher, G., Rudolphi, F., Corthals, K., Ruhwedel, T., Schmidt, K. F., Löwel, S., et al. (2012). Therapy of Pelizaeus-Merzbacher disease in mice by feeding a cholesterol-enriched diet. *Nat. Med.* 18, 1130–1135. doi: 10.1038/nm.2833

Samaranch, L., Salegio, E. A., San Sebastian, W., Kells, A. P., Bringas, J. R., Forsayeth, J., et al. (2013). Strong cortical and spinal cord transduction after AAV7 and AAV9 delivery into the cerebrospinal fluid of nonhuman primates. *Hum. Gene Ther.* 24, 526–532. doi: 10.1089/hum.2013.005

Sánchez, A., García-Lareu, B., Puig, M., Prat, E., Ruberte, J., Chillón, M., et al. (2020). Cerebellar astrocyte transduction as gene therapy for megalencephalic leukoencephalopathy. *Neurotherapeutics* 17, 2041–2053. doi: 10.1007/s13311-020-00865-y

Sánchez-Mejías, A., Watanabe, Y., M Fernández, R., López-Alonso, M., Antiñolo, G., Bondurand, N., et al. (2010). Involvement of SOX10 in the pathogenesis of Hirschsprung disease: report of a truncating mutation in an isolated patient. *J. Mol. Med.* 88, 507–514. doi: 10.1007/s00109-010-0592-7

Santello, M., Toni, N., and Volterra, A. (2019). Astrocyte function from information processing to cognition and cognitive impairment. *Nat. Neurosci.* 22, 154–166. doi: 10.1038/s41593-018-0325-8

Sasaki, A. (2017). Microglia and brain macrophages: an update. *Neuropathology* 37, 452–464. doi: 10.1111/neup.12354

Sase, S., Almad, A. A., Boecker, C. A., Guedes-Dias, P., Li, J. J., Takanohashi, A., et al. (2020). TUBB4A mutations result in both glial and neuronal degeneration in an H-ABC leukodystrophy mouse model. *eLife* 9:e52986. doi: 10.7554/eLife.52986

Scheper, G. C., van der Klok, T., van Andel, R. J., van Berkel, C. G., Sissler, M., Smet, J., et al. (2007). Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. *Nat. Genet.* 39, 534–539. doi: 10.1038/ng2013

Schiroli, G., Ferrari, S., Conway, A., Jacob, A., Capo, V., Albano, L., et al. (2017). Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Sci. Transl. Med.* 9:eaan0820. doi: 10.1126/scitranslmed.aan0820

Schlotawa, L., Ennemann, E. C., Radhakrishnan, K., Schmidt, B., Chakrapani, A., Christen, H.-J., et al. (2011). SUMF1 mutations affecting stability and activity of formylglycine generating enzyme predict clinical outcome in multiple sulfatase deficiency. *Eur. J. Hum. Genet.* 19, 253–261. doi: 10.1038/ejhg.2010.219

Schmidt, M., Schwarzwaelder, K., Bartholomae, C., Zaoui, K., Ball, C., Pilz, I., et al. (2007). High-resolution insertion-site analysis by linear amplification-mediated PCR (LAM-PCR). *Nat. Methods* 4, 1051–1057. doi: 10.1038/nmeth1103

Schober, A. L., Gagarkin, D. A., Chen, Y., Gao, G., Jacobson, L., and Mongin, A. A. (2016). Recombinant adeno-associated virus serotype 6 (rAAV6) potently and preferentially transduces rat astrocytes *in vitro* and *in vivo*. *Front. Cell Neurosci.* 10:262. doi: 10.3389/fncel.2016.00262

Senís, E., Mosteiro, L., Wilkening, S., Wiedtke, E., Nowrouzi, A., Afzal, S., et al. (2018). AAVvector-mediated *in vivo* reprogramming into pluripotency. *Nat. Commun.* 9:2651. doi: 10.1038/s41467-018-05059-x

Sessa, M., Lorioli, L., Fumagalli, F., Acquati, S., Redaelli, D., Baldoli, C., et al. (2016). Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial. *Lancet* 388, 476–487. doi: 10.1016/s0140-6736(16)30374-9

Shevtsova, Z., Malik, J. M., Michel, U., Bähr, M., and Kügler, S. (2005). Promoters and serotypes: targeting of adeno-associated virus vectors for gene transfer in the rat central nervous system *in vitro* and *in vivo*. *Exp. Physiol.* 90, 53–59. doi: 10.1113/expphysiol.2004.028159

Shield, J. P., Stone, J., and Steward, C. G. (2005). Bone marrow transplantation correcting beta-galactosidase activity does not influence neurological outcome in juvenile GM1-gangliosidosis. *J. Inherit Metab. Dis.* 28, 797–798. doi: 10.1007/s10545-005-0089-7

Shy, M. E., Hobson, G., Jain, M., Boespflug-Tanguy, O., Garbern, J., Sperle, K., et al. (2003). Schwann cell expression of PLP1 but not DM20 is necessary to prevent neuropathy. *Ann. Neurol.* 53, 354–365. doi: 10.1002/ana.10466

Simons, C., Griffin, L. B., Helman, G., Golas, G., Pizzino, A., Bloom, M., et al. (2015). Loss-of-function alanyl-tRNA synthetase mutations cause an autosomal-recessive early-onset epileptic encephalopathy with persistent myelination defect. *Am. J. Hum. Genet.* 96, 675–681. doi: 10.1016/j.ajhg.2015.02.012

Smith, L. J., Ul-Hasan, T., Carvaines, S. K., Van Vliet, K., Yang, E., Wong, K. K. Jr., et al. (2014). Gene transfer properties and structural modeling of human stem cell-derived AAV. *Mol. Ther.* 22, 1625–1634. doi: 10.1038/mt.2014.107

Spampanato, C., De Leonibus, E., Dama, P., Gargiulo, A., Fraldi, A., Sorrentino, N. C., et al. (2011). Efficacy of a combined intracerebral and systemic gene delivery approach for the treatment of a severe lysosomal storage disorder. *Mol. Ther.* 19, 860–869. doi: 10.1038/mt.2010.299

Staal, F. J. T., Aiuti, A., and Cavazzana, M. (2019). Autologous stem-cell-based gene therapy for inherited disorders: state of the art and perspectives. *Front. Pediatr.* 7:443. doi: 10.3389/fped.2019.00443

Steenweg, M. E., Ghezzi, D., Haack, T., Abbink, T. E., Martinelli, D., van Berkel, C. G., et al. (2012). Leukoencephalopathy with thalamus and brainstem involvement and high lactate 'LTBL' caused by EARS2 mutations. *Brain* 135(Pt 5), 1387–1394. doi: 10.1093/brain/aws070

Strobel, B., Düchs, M. J., Blazevic, D., Rechtsteiner, P., Braun, C., Baum-Kroker, K. S., et al. (2020). A small-molecule-responsive riboswitch enables conditional induction of viral vector-mediated gene expression in mice. *ACS Synth. Biol.* 9, 1292–1305. doi: 10.1021/acssynbio.9b00410

Sweeney, M. D., Zhao, Z., Montagne, A., Nelson, A. R., and Zlokovic, B. V. (2019). Blood-brain barrier: from physiology to disease and back. *Physiol. Rev.* 99, 21–78. doi: 10.1152/physrev.00050.2017

Taft, R. J., Vanderver, A., Leventer, R. J., Damiani, S. A., Simons, C., Grimmond, S. M., et al. (2013). Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. *Am. J. Hum. Genet.* 92, 774–780. doi: 10.1016/j.ajhg.2013.04.006

Thiffault, I., Wolf, N. I., Forget, D., Guerrero, K., Tran, L. T., Choquet, K., et al. (2015). Recessive mutations in POLR1C cause a leukodystrophy by impairing biogenesis of RNA polymerase III. *Nat. Commun.* 6:7623. doi: 10.1038/ncomms8623

Tornøe, J., Kusk, P., Johansen, T. E., and Jensen, P. R. (2002). Generation of a synthetic mammalian promoter library by modification of sequences spacing transcription factor binding sites. *Gene* 297, 21–32. doi: 10.1016/s0378-1119(02)00878-8

Traka, M., Wollmann, R. L., Cerda, S. R., Dugas, J., Barres, B. A., and Popko, B. (2008). Nur7 is a nonsense mutation in the mouse aspartoacylase gene that causes spongy degeneration of the CNS. *J. Neurosci.* 28, 11537–11549. doi: 10.1523/jneurosci.1490-08.2008

Tress, O., Maglione, M., May, D., Pivneva, T., Richter, N., Seyfarth, J., et al. (2012). Panglial gap junctional communication is essential for maintenance of myelin in the CNS. *J. Neurosci.* 32, 7499–7518. doi: 10.1523/jneurosci.0392-12.2012

Tseng, Y. S., and Agbandje-McKenna, M. (2014). Mapping the AAV capsid host antibody response toward the development of second generation gene delivery vectors. *Front. Immunol.* 5:9. doi: 10.3389/fimmu.2014.00009

Tucci, F., Scaramuzza, S., Aiuti, A., and Mortellaro, A. (2020). Update on clinical *ex vivo* hematopoietic stem cell gene therapy for inherited monogenic diseases. *Mol. Ther.* 29, 489–504. doi: 10.1016/j.ymthe.2020.11.020

Ungari, S., Montepeloso, A., Morena, F., Cocchiarella, F., Recchia, A., Martino, S., et al. (2015). Design of a regulated lentiviral vector for hematopoietic stem cell gene therapy of globoid cell leukodystrophy. *Mol. Ther. Methods Clin. Dev.* 2:15038. doi: 10.1038/mtm.2015.38

Vagner, T., Mouravlev, A., and Young, D. (2015). A novel bicistronic sensor vector for detecting caspase-3 activation. *J. Pharmacol. Toxicol. Methods* 72, 11–18. doi: 10.1016/j.vascn.2014.11.006

van der Knaap, M. S., Barth, P. G., Stroink, H., van Nieuwenhuizen, O., Arts, W. F., Hoogenraad, F., et al. (1995). Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. *Ann. Neurol.* 37, 324–334. doi: 10.1002/ana.410370308

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

van der Knaap, M. S., Schiffmann, R., Mochel, F., and Wolf, N. I. (2019). Diagnosis, prognosis, and treatment of leukodystrophies. *Lancet Neurol.* 18, 962–972. doi: 10.1016/s1474-4422(19)30143-7

Vanderver, A., Bernard, G., Helman, G., Sherbini, O., Boeck, R., Cohn, J., et al. (2020). Randomized clinical trial of first-line genome sequencing in pediatric white matter disorders. *Ann. Neurol.* 88, 264–273. doi: 10.1002/ana.25757

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, 219–223. doi: 10.1016/j.spen.2012.10.001

Vanderver, A., Prust, M., Tonduti, D., Mochel, F., Hussey, H. M., Helman, G., et al. (2015). Case definition and classification of leukodystrophies and leukoencephalopathies. *Mol. Genet. Metab.* 114, 494–500. doi: 10.1016/j.ymgme.2015.01.006

Vanderver, A., Simons, C., Helman, G., Crawford, J., Wolf, N. I., Bernard, G., et al. (2016). Whole exome sequencing in patients with white matter abnormalities. *Ann. Neurol.* 79, 1031–1037. doi: 10.1002/ana.24650

von Jonquieres, G., Fröhlich, D., Klugmann, C. B., Wen, X., Harasta, A. E., Ramkumar, R., et al. (2016). Recombinant human myelin-associated glycoprotein promoter drives selective AAV-mediated transgene expression in oligodendrocytes. *Front. Mol. Neurosci.* 9:13. doi: 10.3389/fnmol.2016.00013

von Jonquieres, G., Froud, K. E., Klugmann, C. B., Wong, A. C., Housley, G. D., and Klugmann, M. (2014). Loss of central auditory processing in a mouse model of Canavan disease. *PLoS One* 9:e97374. doi: 10.1371/journal.pone.0097374

von Jonquieres, G., Mersmann, N., Klugmann, C. B., Harasta, A. E., Lutz, B., Teahan, O., et al. (2013). Glial promoter selectivity following AAV-delivery to the immature brain. *PLoS One* 8:e65646. doi: 10.1371/journal.pone.0065646

von Jonquieres, G., Spencer, Z. H. T., Rowlands, B. D., Klugmann, C. B., Bongers, A., Harasta, A. E., et al. (2018). Uncoupling N-acetylaspartate from brain pathology: implications for Canavan disease gene therapy. *Acta Neuropathol.* 135, 95–113. doi: 10.1007/s00401-017-1784-9

Vranckx, L. S., Demeulemeester, J., Debyser, Z., and Gijsbers, R. (2016). Towards a safer, more randomized lentiviral vector integration profile exploring artificial LEDGF chimeras. *PLoS One* 11:e0164167. doi: 10.1371/journal.pone.0164167

Wagenblast, E., Azkanaz, M., Smith, S. A., Shakib, L., McLeod, J. L., Krivdova, G., et al. (2019). Functional profiling of single CRISPR/Cas9-edited human long-term hematopoietic stem cells. *Nat. Commun.* 10:4730. doi: 10.1038/s41467-019-12726-0

Wake, H., Moorhouse, A. J., Jinno, S., Kohsaka, S., and Nabekura, J. (2009). Resting microglia directly monitor the functional state of synapses *in vivo* and determine the fate of ischemic terminals. *J. Neurosci.* 29, 3974–3980. doi: 10.1523/jneurosci.4363-08.2009

Waldman, A. T. (2018). Leukodystrophies. *Continuum (Minneap Minn)* 24, 130–149. doi: 10.1212/con.0000000000000560

Wang, D., Tai, P. W. L., and Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* 18, 358–378. doi: 10.1038/s41573-019-0012-9

Wang, D., Zhang, F., and Gao, G. (2020). CRISPR-based therapeutic genome editing: strategies and *in vivo* delivery by AAV Vectors. *Cell* 181, 136–150. doi: 10.1016/j.cell.2020.03.023

Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., et al. (2013). Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell* 12, 252–264. doi: 10.1016/j.stem.2012.12.002

Wang, Z., Ma, H. I., Li, J., Sun, L., Zhang, J., and Xiao, X. (2003). Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Ther.* 10, 2105–2111. doi: 10.1038/sj.gt.3302133

Watakabe, A., Ohtsuka, M., Kinoshita, M., Takaji, M., Isa, K., Mizukami, H., et al. (2015). Comparative analyses of adeno-associated viral vector serotypes 1, 2, 5, 8 and 9 in marmoset, mouse and macaque cerebral cortex. *Neurosci. Res.* 93, 144–157. doi: 10.1016/j.neures.2014.09.002

Weinstock, N. I., Kreher, C., Favret, J., Nguyen, D., Bongarzone, E. R., Wrabetz, L., et al. (2020). Brainstem development requires galactosylceramidase and is critical for pathogenesis in a model of Krabbe disease. *Nat. Commun.* 11:5356. doi: 10.1038/s41467-020-19179-w

Weismann, C. M., Ferreira, J., Keeler, A. M., Su, Q., Qui, L., Shaffer, S. A., et al. (2015). Systemic AAV9 gene transfer in adult GM1 gangliosidosis mice reduces lysosomal storage in CNS and extends lifespan. *Hum. Mol. Genet.* 24, 4353–4364. doi: 10.1093/hmg/ddv168

Weleber, R. G., Pennesi, M. E., Wilson, D. J., Kaushal, S., Erker, L. R., Jensen, L., et al. (2016). Results at 2 years after gene therapy for RPE65-Deficient leber congenital amaurosis and severe early-childhood-onset retinal dystrophy. *Ophthalmology* 123, 1606–1620. doi: 10.1016/j.ophtha.2016.03.003

Westhaus, A., Cabanes-Creus, M., Rybicki, A., Baltazar, G., Navarro, R. G., Zhu, E., et al. (2020). High-throughput *In Vivo*, *Ex Vivo*, and *In Vivo* screen of adeno-associated virus vectors based on physical and functional transduction. *Hum. Gene Ther.* 31, 575–589. doi: 10.1089/hum.2019.264

Wilmott, P., Lisowski, L., Alexander, I. E., and Logan, G. J. (2019). A User's guide to the inverted terminal repeats of adeno-associated virus. *Hum. Gene Ther. Methods* 30, 206–213. doi: 10.1089/hgtb.2019.276

Wilson, J. M., and Flotte, T. R. (2020). Moving forward after two deaths in a gene therapy trial of myotubular myopathy. *Hum. Gene Ther.* 31, 695–696. doi: 10.1089/hum.2020.182

Windrem, M. S., Schanz, S. J., Morrow, C., Munir, J., Chandler-Militello, D., Wang, S., et al. (2014). A competitive advantage by neonatally engrafted human glial progenitors yields mice whose brains are chimeric for human glia. *J. Neurosci.* 34, 16153–16161. doi: 10.1523/jneurosci.1510-14.2014

Wolf, N. I., Vanderver, A., van Spaendonk, R. M., Schiffmann, R., Brais, B., Bugiani, M., et al. (2014). Clinical spectrum of 4H leukodystrophy caused by POLR3A and POLR3B mutations. *Neurology* 83, 1898–1905. doi: 10.1212/wnl.0000000000001002

Wu, Y., Dissing-Olesen, L., MacVicar, B. A., and Stevens, B. (2015). Microglia: dynamic mediators of synapse development and plasticity. *Trends Immunol.* 36, 605–613. doi: 10.1016/j.it.2015.08.008

Xiao, Y., Muhuri, M., Li, S., Qin, W., Xu, G., Luo, L., et al. (2019). Circumventing cellular immunity by miR142-mediated regulation sufficiently supports rAAV-delivered OVA expression without activating humoral immunity. *JCI Insight* 5:e99052. doi: 10.1172/jci.insight.99052

Xie, Q., Bu, W., Bhatia, S., Hare, J., Somasundaram, T., Azzi, A., et al. (2002). The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10405–10410. doi: 10.1073/pnas.162250899

Xu, Z., Rao, Y., Huang, Y., Zhou, T., Feng, R., Xiong, S., et al. (2020). Efficient strategies for microglia replacement in the central nervous system. *Cell Rep.* 33:108443. doi: 10.1016/j.celrep.2020.108443

Yang, B., Li, S., Wang, H., Guo, Y., Gessler, D. J., Cao, C., et al. (2014). Global CNS transduction of adult mice by intravenously delivered rAAVrh.8 and rAAVrh.10 and nonhuman primates by rAAVrh.10. *Mol. Ther.* 22, 1299–1309. doi: 10.1038/mt.2014.68

Yoon, S. Y., Hunter, J. E., Chawla, S., Clarke, D. L., Molony, C., O'Donnell, P. A., et al. (2020). Global CNS correction in a large brain model of human alpha-mannosidosis by intravascular gene therapy. *Brain* 143, 2058–2072. doi: 10.1093/brain/awaa161

Yu, H., Koilkonda, R. D., Chou, T. H., Porciatti, V., Ozdemir, S. S., Chiodo, V., et al. (2012). Gene delivery to mitochondria by targeting modified adenoassociated virus suppresses Leber's hereditary optic neuropathy in a mouse model. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1238–E1247. doi: 10.1073/pnas.1119577109

Zhang, H., Yang, B., Mu, X., Ahmed, S. S., Su, Q., He, R., et al. (2011). Several rAAV vectors efficiently cross the blood-brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. *Mol. Ther.* 19, 1440–1448. doi: 10.1038/mt.2011.98

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., et al. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J. Neurosci.* 34, 11929–11947. doi: 10.1523/jneurosci.1860-14.2014

Zhang, Y., Sloan, S. A., Clarke, L. E., Caneda, C., Plaza, C. A., Blumenthal, P. D., et al. (2016). Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron* 89, 37–53. doi: 10.1016/j.neuron.2015.11.013

Zhong, G., Wang, H., He, W., Li, Y., Mou, H., Tickner, Z. J., et al. (2020). A reversible RNA on-switch that controls gene expression of AAV-delivered therapeutics *in vivo*. *Nat. Biotechnol.* 38, 169–175. doi: 10.1038/s41587-019-0357-y

Zhong, L., Li, B., Mah, C. S., Govindasamy, L., Agbandje-McKenna, M., Cooper, M., et al. (2008). Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7827–7832. doi: 10.1073/pnas.0802866105

Zinn, E., Pacouret, S., Khaychuk, V., Turunen, H. T., Carvalho, L. S., Andres-Mateos, E., et al. (2015). In Silico reconstruction of the viral evolutionary lineage

yields a potent gene therapy vector. *Cell Rep.* 12, 1056–1068. doi: 10.1016/j.celrep.2015.07.019

Zinn, E., and Vandenberghe, L. H. (2014). Adeno-associated virus: fit to serve. *Curr. Opin. Virol.* 8, 90–97. doi: 10.1016/j.coviro.2014.07.008

Zotova, E., Bharambe, V., Cheaveau, M., Morgan, W., Holmes, C., Harris, S., et al. (2013). Inflammatory components in human Alzheimer's disease and after active amyloid-β42 immunization. *Brain* 136(Pt 9), 2677–2696. doi: 10.1093/brain/awt210

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., et al. (1998). Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J. Virol.* 72, 9873–9880. doi: 10.1128/jvi.72.12.9873-9880.1998

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

REVIEW
published: 12 August 2021
doi: 10.3389/fncel.2021.682106

White Matter Pathology as a Barrier to Gangliosidosis Gene Therapy

Anne S. Maguire[1,2] *and Douglas R. Martin*[1,2]*

[1] Scott-Ritchey Research Center, Auburn University College of Veterinary Medicine, Auburn, AL, United States, [2] Department of Anatomy, Physiology, and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, AL, United States

The gangliosidoses are a family of neurodegenerative lysosomal storage diseases that have recently seen promising advances in gene therapy. White matter deficits are well established components of gangliosidosis pathology that are now receiving more attention because they are partially refractory to correction by gene therapy. After a brief synopsis of normal myelinogenesis, this review outlines current viewpoints on the origin of white matter deficits in the gangliosidoses and potential obstacles to treating them effectively by gene therapy. Dysmyelinogenesis (failure of myelin sheaths to form properly) is proposed as the predominant contributor to white matter pathology, but precise mechanistic details are not well understood. The involvement of neuronal storage deficits may extend beyond secondary demyelination (destruction of myelin due to axonal loss) and contribute to dysmyelinogenesis. Preclinical studies in animal models of the gangliosidoses have substantially improved lifespan and quality of life, leading to the initiation of several clinical trials. However, improvement of white matter pathology has lagged behind other metrics and few evidence-based explanations have been proposed to date. Research groups in the field are encouraged to include myelin-specific investigations in future gene therapy work to address this gap in knowledge.

Keywords: GM1 gangliosidosis, GM2 gangliosidosis, Tay-Sachs disease, Sandhoff disease, AAV gene therapy, white matter, myelin, oligodendrocytes

OPEN ACCESS

Edited by:
Dominic J. Gessler, University of Minnesota, United States

Reviewed by:
Yoshio Bando, Akita University, Japan
Dan Wang, University of Massachusetts Medical School, United States
David Pleasure, University of California, Davis, United States

***Correspondence:**
Douglas R. Martin
martidr@auburn.edu

Specialty section:
This article was submitted to Cellular Neuropathology, a section of the journal Frontiers in Cellular Neuroscience

Received: *17 March 2021*
Accepted: *23 July 2021*
Published: *12 August 2021*

Citation:
Maguire AS and Martin DR (2021) White Matter Pathology as a Barrier to Gangliosidosis Gene Therapy. Front. Cell. Neurosci. 15:682106. doi: 10.3389/fncel.2021.682106

INTRODUCTION

GM1 and GM2 gangliosidosis (GM1 and GM2) are devastating lysosomal storage diseases that result in the neurodegenerative decline and death of children before 5 years of age in most cases. This family of autosomal recessive diseases causes the dysfunction of the lysosomal enzymes β-galactosidase (βgal) or β-*N*-acetylhexosaminidase (Hex) resulting in the buildup of GM1 or GM2 ganglioside, respectively, in neuron cell bodies. GM1 results from mutation of the *GLB1* gene and affects one in 100,000–200,000 live births (Brunetti-Pierri and Scaglia, 2008). Three clinically similar subtypes of GM2 exist: Tay-Sachs disease (TSD), Sandhoff disease (SD), and GM2 activator deficiency, which result from mutation of *HEXA*, *HEXB*, and *GM2A* genes, respectively. While TSD and SD affect one in 200,000–400,000 live births (Meikle et al., 1999), GM2 activator deficiency occurrence has only been recorded in a handful of cases across the world. Regardless of genetic origin, dysfunction of the βgal or Hex enzymes causes a cascade of central nervous system (CNS) symptoms in affected patients such as delay or loss of developmental milestones, difficulty swallowing, seizures, and ultimately death. Only palliative treatment is available currently, but clinical trials with gene therapy began in 2019. The animal studies that preceded clinical trials have shown dramatic improvement in clinical metrics, but complete normalization has yet to be

achieved reproducibly. To maximize patient benefit, and because gene therapy manufacturing is expensive and labor-intensive, it is crucial to make sure that each dose is as effective as possible.

While gene therapy usually results in enzyme activity at or above effective levels (10–20% of normal) and substantially reduced neuronal storage, lifespans and other measures of success are still not at normal levels. This leads researchers to suspect that other deficits persist despite gene therapy, such as inflammation and myelin pathology. Indeed, white matter deficits are increasingly considered therapeutic targets in neurodegenerative diseases such as Alzheimer's disease (Nasrabady et al., 2018), Parkinson's disease (Luo et al., 2017), and other closely related lysosomal storage diseases (Takikita et al., 2004; Buccinnà et al., 2009; Provenzale et al., 2015). Similarly, white matter pathology in the gangliosidoses has been characterized since the 1970s and is now receiving attention as a phenomenon potentially separate from gray matter deficits. While early gene therapy efforts focused on enzyme activity restoration and storage reduction, recent studies have implied that myelin pathology is at least partially refractory to gene therapy. Therefore, to improve the efficacy of treatment, the field would benefit from future gene therapy studies incorporating white matter metrics. After a brief synopsis of normal myelinogenesis, this review encompasses three overlapping areas of study in the gangliosidoses (**Figure 1**): white matter pathology, the current state of gene therapy success, and the effect of gene therapy on white matter pathology.

Normal Myelinogenesis

Normal myelinogenesis involves three major processes: oligodendrocyte development, myelin sheath assembly, and axonal communication. Oligodendrocytes, the glial cells responsible for myelinating axons within the CNS, have four developmental stages: oligodendrocyte precursor cell (OPC), preoligodendrocyte, immature oligodendrocyte, and mature oligodendrocyte (Barateiro and Fernandes, 2014). Common protein markers used for identification of these stages include NG2 (neural/glial antigen 2) and PDGF-Rα (platelet-derived growth factor receptor α) for OPCs and preoligodendrocytes, with the additional expression of O4 and GRP17 marking a transition between the two cell types. O4 expression is retained by immature oligodendrocytes, which also produce GalC (galactocerebroside) and CNPase (2′,3′-cyclic nucleotide 3′-phosphodiesterase). MBP (myelin basic protein), PLP (proteo-lipid protein), and MAG (myelin-associated glycoprotein) are markers of mature oligodendrocytes (Barateiro and Fernandes, 2014). In humans, this process is initiated at 10 weeks post-conception when OPCs first appear, and it reaches completion perinatally with the appearance of substantial numbers of MBP-positive mature oligodendrocytes (Barateiro and Fernandes, 2014).

Myelin sheath components include specific families of lipids in addition to the proteins present on the mature oligodendrocyte cell membrane (such as MBP and PLP). In humans, the appearance of these substances follows a prescribed sequence consisting of major lipids and proteins that can be detected biochemically. Cholesterol synthesized by oligodendrocytes is a major component and critical precursor of myelin sheaths, as well as a rate-limiting factor in their formation (Saher et al., 2005). Formation of phospholipids precedes the emergence of sphingomyelin, which is followed by the near-simultaneous appearance of cerebrosides, sulfatides, MBP and PLP. The latter four substances are the primary components of adult myelin (Kinney et al., 1994). The onset of this sequence varies substantially between anatomical regions, but ranges from mid-gestation (for the tracts that are the earliest to myelinate) to early infancy (for the later-developing tracts) (Kinney et al., 1994).

Recent evidence has revealed that neuroaxonal communication with oligodendrocytes and myelin sheaths is critical to the development of mature myelin and the maintenance of plastic neuronal circuitry (Almeida and Lyons, 2017; Chorghay et al., 2018; Turner, 2019). Indeed, emerging concepts such as activity-dependent myelination and the existence of an axomyelinic synapse (as reviewed in Chorghay et al., 2018) imply that axonal interactions must be considered when investigating oligodendrocyte and myelin development. For example, glutamate released from axons after action potentials likely binds to receptors on both OPCs and oligodendrocytes, with the former hastening OPC maturation and the latter facilitating myelin sheath plasticity and maintaining CNS circuitry (Chorghay et al., 2018). Still undergoing active research is the timing of activity-dependent myelination mechanisms, which appear to be highly variable throughout brain regions and developmental experiences. A detailed understanding of this concept would provide invaluable insight into the pathology of diseases, such as the gangliosidoses, with concurrent developmental and degenerative components.

WHITE MATTER PATHOLOGY IN THE GANGLIOSIDOSES

White matter deficits have long been reported in the gangliosidoses, with occasional speculation as to their origin. There are three proposed mechanisms for how myelin deficits occur as part of any CNS pathology: dysmyelinogenesis (failure to form properly), primary demyelination (destruction after proper formation), or secondary demyelination (loss after axonal degeneration) (**Figure 2**). An emerging theme in the gangliosidosis literature suggests that dysmyelinogenesis is the main contributor to white matter pathology, with primary and secondary demyelination playing less important roles. Last reviewed by Folkerth (1999), this hypothesis is supported by early deficits in white matter tract development and abnormal myelin sheath structure. A detailed understanding of the mechanism for myelin pathology remains elusive in the current literature, with evidence available to support interference with all major components of myelinogenesis: oligodendrocytes, myelin sheaths, and neuroaxonal complexes. The use of inducible mouse models of gangliosidosis, which permit shutdown of Hex expression at different ages, may provide insight into myelin pathogenesis at critical developmental stages (Sargeant et al., 2012).

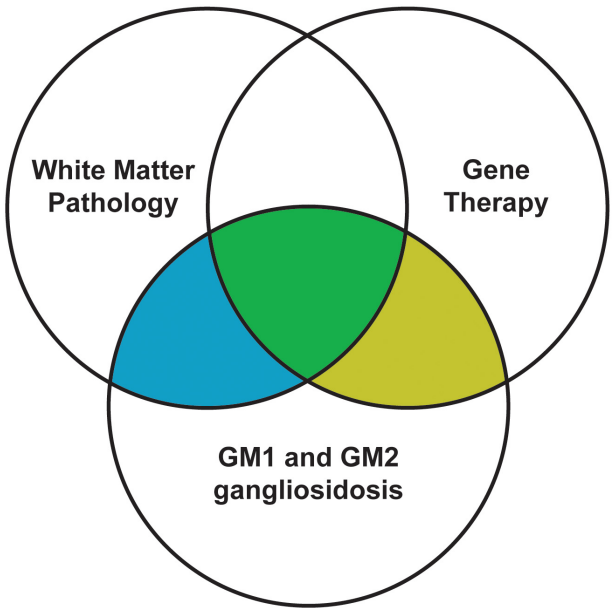


FIGURE 1 | Scope of this review. Filled areas are included in this review, while unfilled areas are covered elsewhere.

Early Deficits in White Matter Tract Development

Though a generalized "loss of white matter" is routinely reported as part of the diagnostic process in case studies of the gangliosidoses, several publications examine these deficits in more detail to elucidate the timing of the onset of dysmyelinogenesis. The most common methods include magnetic resonance imaging (MRI) and histochemical staining (Weil or Luxol Fast Blue). In cat, dog, and sheep models of both GM1 and GM2, cerebellar and cerebral cortical white matter is universally considered decreased compared to normal counterparts (Kaye et al., 1992; Kroll et al., 1995; Porter et al., 2011; Gray-Edwards et al., 2020), while results for non-cortical white matter are mixed. In GM1 (Folkerth et al., 2000) and GM2 (Haberland et al., 1973) patients, tracts that would normally develop prenatally are less affected than tracts that develop postnatally, so a perinatal onset of dysmyelinogenesis is hypothesized.

Abnormal Myelin Sheath Architecture

The myelin sheath in GM1 and GM2 has been critically evaluated from two perspectives: quantification of its major components and evidence of its breakdown (**Table 1**). The most thoroughly investigated myelin sheath components are cerebrosides and sulfatides, which have been shown *via* high performance thin layer chromatography (HPTLC) to be decreased in CNS tissue across mice, dogs, cats, and human patients with both GM1 and GM2 (Haberland et al., 1973; Kaye et al., 1992; Chakrabarti et al., 1996; Broekman et al., 2007; Baek et al., 2009, 2010; Heinecke et al., 2015; Rockwell et al., 2015; **Table 1**). Collectively, these studies implicate that such deficits occur in most major regions of the CNS. A notable exception was observed in Gray-Edwards et al. (2017a), who found an increase in cerebrosides and sulfatides in cerebrospinal fluid (CSF) of cats with GM1, potentially indicating leakage due to myelin breakdown in tissue.

The consensus on cerebroside and sulfatide deficiencies in GM1 and GM2 leads to the question of whether there are similar changes in the major myelin proteins that emerge at the same time in normal myelinogenesis. Unfortunately, only two studies have investigated protein and mRNA levels for MBP, PLP, MAG, and CGT (UDP-galactose:ceramide galactosyltransferase), and more region-specific work is needed to draw concrete conclusions about the onset of white matter pathogenesis. For example, protein levels of MBP and PLP are decreased both in the frontal lobe of humans with GM1 (van der Voorn et al., 2004) and the cerebrum of mice with GM2 (Cachón-González et al., 2014), but no other regions have undergone quantitative analysis. The mRNA levels of MBP, MAG, and CGT in the olfactory bulb, cerebrum, cerebellum, brainstem, and spinal cord of GM2 mice were either at or below normal levels, with the only consistencies across all three markers occurring in the cerebrum (normal levels) or brainstem (below normal levels) (Cachón-González et al., 2014). Future studies should examine whether a correlation exists between protein/mRNA levels and specific white matter tract deficiencies that have previously been established (Folkerth, 1999).

Histological and ultrastructural assessments of myelin sheath integrity inconsistently note pathological changes (**Table 1**), but animal model studies only examine one or two CNS regions at this level (Cummings et al., 1985; Kroll et al., 1995; Heinecke et al., 2015; Gray-Edwards et al., 2020). More informative are the comprehensive evaluations of autopsy cases of GM1 (Folkerth et al., 2000) and GM2 (Haberland et al., 1973) patients that compared major CNS regions and white matter tracts. Both studies noted abnormally thin sheaths that were otherwise structurally intact. In GM2 patients, sudanophilic breakdown products were found throughout the CNS in a pattern that roughly corresponded with neuronal loss (Haberland et al., 1973). These breakdown products were presumed to be degenerating myelin and axons, prompting the proposed mechanism that secondary demyelination occurs after neuronal loss caused by storage or metabolic insults (Haberland et al., 1973).

In conclusion, the evidence for early deficiencies in the major components of myelin with few indications of their destruction supports the hypothesis that dysmyelinogenesis is the predominant mechanism of white matter pathology, with minor contributions from primary and/or secondary demyelination. This conclusion could be modified as more investigations are conducted into myelin proteins and sheath degeneration across CNS regions in animal models.

Oligodendrocyte Pathology

Pathological changes to oligodendrocytes have been found across species (**Table 1**), and CNS regions. Oligodendrocyte number is qualitatively decreased upon evaluation of limited regions of GM1 human tissue (Folkerth et al., 2000; van der Voorn et al., 2004), but unchanged from normal levels when immunohistochemistry (IHC) staining is quantified throughout

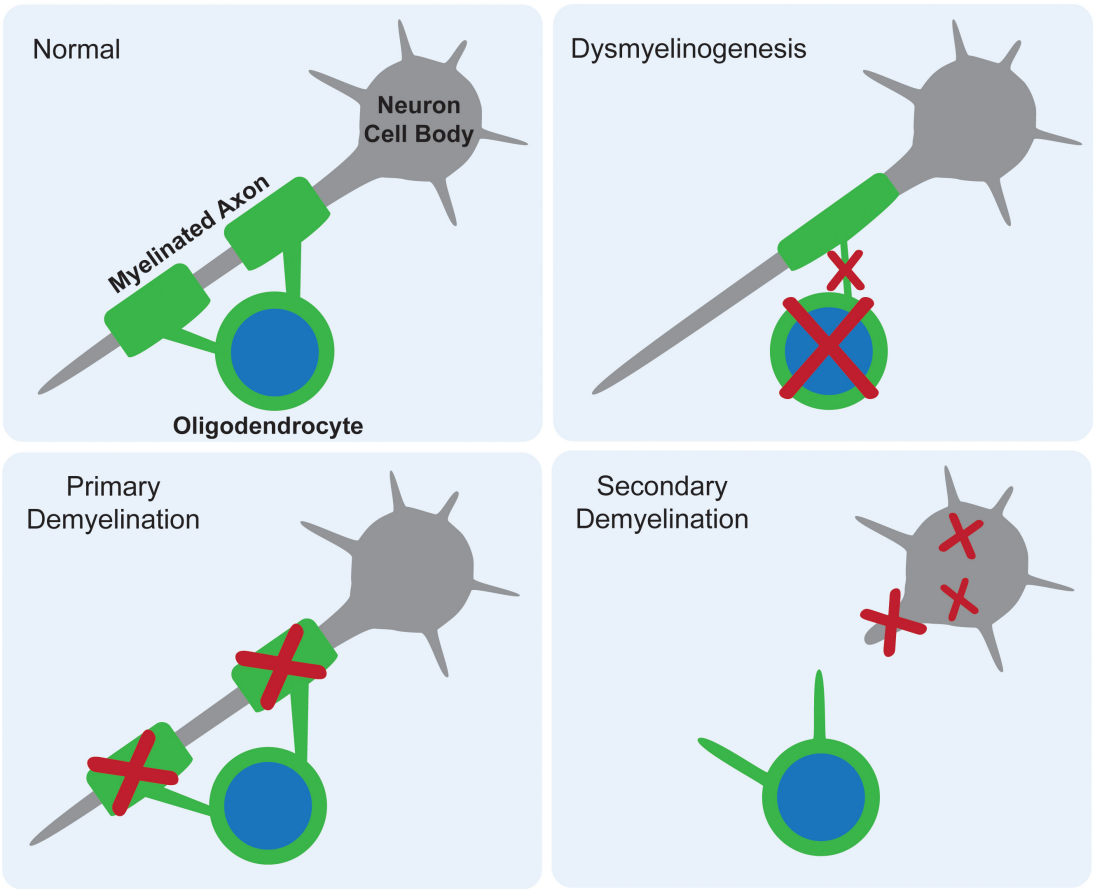


FIGURE 2 | Proposed mechanisms for myelin deficits in the central nervous system. In normal tissue, each oligodendrocyte modifies its own cell membrane to myelinate axons. If dysmyelinogenesis occurs, pathology within the oligodendrocyte, early myelin structures, or neuroaxonal communication leads to an abnormally myelinated axon. In primary demyelination, the oligodendrocyte is functional and myelin is formed properly, but then degraded. In secondary demyelination, neuroaxonal dysfunction leads to axonal loss, resulting in the overall loss of myelinated axons.

TABLE 1 | Trends in pathological changes to oligodendrocytes, axons, and myelin in GM1 and GM2 gangliosidosis.

	Myelin			Oligodendrocytes			Axons			
	Cerebrosides/ sulfatides	**Sheath integrity**	**Protein/mRNA markers**	**Storage**	**Number**	**Cell injury**	**Degenerative changes**	**Axon number**	**Cell body number**	**Number myelinated**
GM1										
Human		NC[1] ↓[1]	↓[2]	NC[1–3]	↓[1,2]	↑[2]	NC[1,2]	↓[2]		↓[1,2]
Mouse	↓[4–6]	↓[4]								
Cat	↑[7]*	↓[8]		NC[8]	NC[8]	↑[8]				
Dog	↓[9]						↑[9]			↓[9]
GM2										
Human	↓[3,10,11]	↓[10]			NC[10]		↑[10]			↓[10]
Mouse	↓[3]		↓[12]				NC[13] ↑[12]		↓[12,14]	
Cat	↓[3,15]	NC[16]					↑[16]	NC[16]		NC[16]
Sheep							↑[17]			
Dog		NC[18]		↑[18]			↑[18,19]		NC[18]	

**Measured cerebrosides/sulfatides in CSF rather than CNS parenchyma.*
↑, increased from normal; ↓, decreased from normal; NC, no change from normal.
References: [1]Folkerth et al., 2000, [2]van der Voorn et al., 2004, [3]Baek et al., 2009, [4]Heinecke et al., 2015, [5]Broekman et al., 2007, [6]Baek et al., 2010, [7]Gray-Edwards et al., 2017a, [8]Gray-Edwards et al., 2020, [9]Kaye et al., 1992, [10]Haberland et al., 1973, [11]Sandhoff et al., 1971, [12]Cachón-González et al., 2014, [13]Walkley, 2000, [14]Sargeant et al., 2011, [15]Rockwell et al., 2015, [16]Kroll et al., 1995, [17]Porter et al., 2011, [18]Cummings et al., 1985, and [19]Sanders et al., 2013.

the brain of GM1 cats (Gray-Edwards et al., 2020). The only study to investigate oligodendrocytes in GM2 found a similar number in affected human patients compared to normal (Haberland et al., 1973). Evidence of oligodendrocyte cell injury is apparent in GM1 through TUNEL staining (van der Voorn et al., 2004) or ultrastructural examination (Gray-Edwards et al., 2020). Despite these abnormalities in number and pathological changes, the consensus appears to be that ganglioside storage does not occur in oligodendrocytes (Folkerth et al., 2000; van der Voorn et al., 2004; Baek et al., 2009; Gray-Edwards et al., 2020), with the only exception reported in one Japanese spaniel with GM2 (Cummings et al., 1985). Authors that observe oligodendrocyte pathology consider it to play a substantial role in the development of white matter deficits, but specific mechanisms of action and the extent of concurrent dysfunctional neuroaxonal communication have yet to be delineated.

Neuroaxonal Dystrophy

Neuronal pathology incited by ganglioside storage likely contributes to myelin pathology through deficiencies in axonal-oligodendrocyte communication, secondary demyelination, or both. While the separate concepts of neuroaxonal dystrophy in GM1/GM2 (**Table 1**) and axon-led activity-dependent myelination (Chorghay et al., 2018) are well-established in the literature, they have not been thoroughly investigated together as a potential mechanism for white matter deficits in the gangliosidoses. Common methods for investigating neuroaxonal pathology include quantitative IHC (NeuN stain), and qualitative evaluation of hematoxylin and eosin (H&E) staining, silver staining, and ultrastructure. In most species with gangliosidosis, myelinated and total numbers of axons are decreased (Haberland et al., 1973; Kaye et al., 1992; Folkerth et al., 2000; van der Voorn et al., 2004). This could be due to degenerative changes within axons, which are inconsistently found throughout the CNS (Haberland et al., 1973; Cummings et al., 1985; Kaye et al., 1992; Kroll et al., 1995; Folkerth et al., 2000; Walkley, 2000; van der Voorn et al., 2004; Porter et al., 2011; Sanders et al., 2013; Cachón-González et al., 2014), or secondary to the decrease in cell body number noted in mice with GM2 (Sargeant et al., 2011; Cachón-González et al., 2014). Regardless of cause, the contribution of axonal loss to secondary demyelination and/or dysfunctional activity-dependent myelination represents an intriguing area of future study.

GENE THERAPY IN THE GANGLIOSIDOSES

Gene therapy for GM1 and GM2 has achieved outstanding preclinical results using several vector backbones, capsids, delivery routes, and animal models (**Table 2**). Full reviews of the status of gene therapy in the gangliosidoses have recently been published elsewhere (Bradbury et al., 2015b; Kelly et al., 2017; Rha et al., 2021). Initial studies in the gangliosidoses involved lentivirus and adenovirus (Guidotti et al., 1999; Bourgoin et al., 2003; Takaura et al., 2003; Kyrkanides et al., 2005, 2007), but many recent studies focus on adeno-associated virus (AAV). Intracranial administration sites included the striatum or thalamus with or without the cerebellum (Cachon-Gonzalez et al., 2006; Baek et al., 2010; Sargeant et al., 2011; Cachón-González et al., 2012, 2014; Bradbury et al., 2013; McCurdy et al., 2014, 2015), structures with important functional roles and generally well connected with other regions of the CNS. Concerns about the risk of cerebellar surgery in affected children led to studies that substituted the lateral ventricle [intracerebroventricular (ICV)] for cerebellum injection sites (Rockwell et al., 2015; Gray-Edwards et al., 2018, 2020). Recently, injections of the cisterna magna and/or intravenous routes have shown promising results and led to clinical trials in humans. Taghian et al. (2020) describe a novel technique for administering vector intrathecally in two TSD patients as part of an expanded access trial. Also, a phase I/II clinical trial for GM2 gangliosidosis (ClinicalTrials.gov Identifier NCT04669535) and three trials for GM1 children are underway or almost so (NCT03952637; NCT04713475; and NCT04273269).

The degree of lifespan extension in AAV-treated animals often correlates with other metrics used to evaluate gene therapy success. Common avenues of investigation include enzyme activity, ganglioside storage, lipid content, inflammatory response, and other histopathological changes. These assessments are also often used to investigate potential mechanisms of gangliosidosis pathogenesis. Recently, biomarkers such as neurological status, MRI, MRS, CSF, and blood chemistry markers have emerged as valuable tools for assessing treatment effectiveness in-life (Bradbury et al., 2015a; Regier et al., 2016; Gray-Edwards et al., 2017b). Some of these biomarkers correlate well between human patients and animal models, and are therefore expected to play important roles in clinical trials (Regier et al., 2016).

GENE THERAPY AND MYELIN IN THE GANGLIOSIDOSES

Though supporting evidence is needed, investigations into white matter deficits within gene-therapy treated animal models of the gangliosidoses imply that myelin deficits are refractory to gene therapy treatment. Cachón-González et al. (2014) found that myelin protein content in treated mice remained abnormally low regardless of age at treatment and petitioned for further investigation with a larger cohort of animals. However, early treatment seemed to provide the best opportunity to preserve myelin. SD mice treated at 12 weeks old consistently demonstrated MBP and PLP at levels 40% of normal, while those treated at 4 and 8 weeks old showed higher levels, albeit with high variability between animals (Cachón-González et al., 2014). Similarly, treatment age of SD cats was inversely correlated with survival, with those treated early in the post-symptomatic period faring better than those treated late (lifespan 3.5 and 1.5 times that of untreated SD cats, respectively) (McCurdy et al., 2021). Gray-Edwards et al. (2020) found that AAV treatment only partially corrected pale LFB staining and myelin integrity loss evident on TEM. A surprising result was that

TABLE 2 | Studies involving animal models treated with gene therapy.

First Author	Year	Disease	Species	Route	Vector
Guidotti[1]	1999	GM2 (TSD)	Mouse	Intravenous, intramuscular	Adenovirus
Takaura[2]	2003	GM1	Mouse	Intravenous	Adenovirus
Bourgoin[3]	2003	GM2 (SD)	Mouse	Intracranial (intracerebral)	Adenovirus
Kyrkanides[4]	2005	GM2 (SD)	Mouse	Intraperitoneal	Lentivirus
Cachon-Gonzalez[5]	2006	GM2 (SD)	Mouse	Intracranial (striatum)	AAV1
Kyrkanides[6]	2007	GM2 (SD)	Mouse	Intraperitoneal	Lentivirus
Broekman[7]	2007	GM1	Mouse	Intracranial (ICV)	AAV1
Broekman[8]	2009	GM1	Mouse	Intracranial (hippocampus)	AAV1
Baek[9]	2010	GM1	Mouse	Intracranial (thalamus and DCN)	AAV1
Sargeant[10]	2011	GM2 (SD)	Mouse	Intracranial (striatum)	AAV1
Cachon-Gonzalez[11]	2012	GM2 (SD)	Mouse	Intracranial (striatum, hippocampus, and cerebellum)	AAV1
Bradbury[12]	2013	GM2 (SD)	Cat	Intracranial (thalamus)	AAV1, rh8
Cachon-Gonzalez[13]	2014	GM2 (SD)	Mouse	Intracranial (striatum and cerebellum)	AAV1
McCurdy[14]	2014	GM1	Cat	Intracranial (thalamus and DCN)	AAV1, rh8
McCurdy[15]	2015	GM2 (SD)	Cat	Intracranial (thalamus and DCN)	AAVrh8
Rockwell[16]	2015	GM2 (SD)	Cat	Intracranial (thalamus and ICV)	AAVrh8
Weismann[17]	2015	GM1	Mouse	Intravenous	AAV9
Walia[18]	2015	GM2 (SD)	Mouse	Intravenous	AAV9
Gray-Edwards[19]	2015	GM2 (SD)	Cat	Intracranial (thalamus, DCN, and ICV)	AAV1, rh8
Bradbury[20]	2015	GM2 (SD)	Cat	Intracranial (thalamus)	AAVrh8
Tropak[21]	2016	GM2 (SD)	Mouse	Intravenous	AAV9
Bradbury[22]	2017	GM2 (SD)	Cat	Intracranial (thalamus, DCN, and ICV)	AAV1, rh8
Gray-Edwards[23]	2017	GM1	Cat	Intracranial (thalamus and DCN)	AAV1, rh8
Gray-Edwards[24]	2017	GM1	Cat	Intracranial (thalamus and DCN)	AAVrh8
Gray-Edwards[25]	2018	GM2 (TSD)	Sheep	Intracranial (thalamus and ICV)	AAVrh8
Gray-Edwards[26]	2020	GM1	Cat	Intracranial (thalamus and ICV)	AAVrh8
Taghian[27]	2020	GM2 (TSD)	Sheep/Human	Intrathecal (CM)	AAV9
Lahey[28]	2020	GM2 (SD)	Mouse	Intravenous	AAV9, PHP.B
McCurdy[29]	2021	GM2 (SD)	Cat	Intracranial (thalamus and DCN)	AAVrh8

SD, Sandhoff disease; TSD, Tay-Sachs disease; ICV, intracerebroventricular; DCN, deep cerebellar nuclei; CM, cisterna magna; AAV, adeno-associated virus.
References: [1]Guidotti et al., 1999, [2]Takaura et al., 2003, [3]Bourgoin et al., 2003, [4]Kyrkanides et al., 2005, [5]Cachon-Gonzalez et al., 2006, [6]Kyrkanides et al., 2007, [7]Broekman et al., 2007, [8]Broekman et al., 2009, [9]Baek et al., 2010, [10]Sargeant et al., 2011, [11]Cachón-González et al., 2012, [12]Bradbury et al., 2013, [13]Cachón-González et al., 2014, [14]McCurdy et al., 2014, [15]McCurdy et al., 2015, [16]Rockwell et al., 2015, [17]Weismann et al., 2015, [18]Walia et al., 2015, [19]Gray-Edwards et al., 2015, [20]Bradbury et al., 2015a, [21]Tropak et al., 2016, [22]Bradbury et al., 2017, [23]Gray-Edwards et al., 2017b, [24]Gray-Edwards et al., 2017a, [25]Gray-Edwards et al., 2018, [26]Gray-Edwards et al., 2020, [27]Taghian et al., 2020, [28]Lahey et al., 2020, and [29]McCurdy et al., 2021.

oligodendrocyte number was not abnormally low in affected untreated animals, but increased in cats treated with AAV, while ultrastructural oligodendrocyte pathology was partially corrected. This leads to an intriguing hypothesis that treated animals produce more oligodendrocytes to compensate for early pathology, which could be further explored through cell death assays specific to oligodendrocytes. Though more myelin-specific work is needed in future gene therapy studies, current evidence indicates that myelin deficits occur before the typical age of AAV treatment or that AAV is ineffective at treating the underlying cause of myelin deficits. One explanation may be that oligodendrocytes are poorly treated by most AAV serotypes (Powell et al., 2016).

It is interesting to consider whether certain routes of AAV delivery are better able to treat myelin pathology than others. While no controlled experiments of myelin preservation have been performed to compare delivery routes, results from feline studies show that myelin integrity generally reflects the overall efficacy of the treatment. For example, to date SD cats have been treated most effectively by injection of the thalamus and deep cerebellar nuclei (DCN) or thalamus and lateral ventricle, with both routes achieving similar increases in survival (∼4.5 times greater than untreated). Animals treated by these routes also had similar preservation of white matter on anatomical MRI, and cerebroside levels in various regions of the brain were similar (McCurdy et al., 2015; Rockwell et al., 2015; unpublished data). Studies are underway in both the SD and GM1 cat models to directly compare intravenous versus CSF-based delivery methods.

While white matter-specific experiments are more informative, insight into how gene therapy affects myelin pathology can be obtained through indirect methods. Baek et al. (2010) found that cerebroside and sulfatide levels in AAV-treated mice were not fully normalized, which implicates early involvement of myelin structural components. *In vivo* MRS measurements of GPC + PCh, an indicator of membrane

turnover, were almost completely normalized in AAV-treated cats, which could indicate that refractory pathology does not involve the increased turnover of myelin sheaths (Gray-Edwards et al., 2017b, 2020). Finally, the contribution of secondary demyelination can be indirectly measured through neuron density, which appears to be well-corrected by AAV treatment of SD mice (Sargeant et al., 2011; Cachón-González et al., 2014). These findings implicate an early myelin-specific pathological mechanism that is relatively independent of the loss of neurons and axons.

DISCUSSION

In conclusion, a few investigations have implied that white matter pathology in the gangliosidoses is refractory to gene therapy. Promising avenues of investigation include oligodendrocyte cell death assays and protein, mRNA, and IHC quantification. Separation of white and gray matter to increase sensitivity to myelin-specific changes is recommended. Though gene therapy has achieved substantial success in improving lifespan, enzyme activity, and other metrics of success, current evidence implies that persistent white matter pathology begins prior to the age of typical gene therapy treatment. This is supported by evidence that the principal mechanism of myelin pathology in the gangliosidoses is dysmyelinogenesis, that likely occurs perinatally. Performing more myelin-centric assays will allow future gene therapy strategies to be refined to correct early-onset refractory white matter deficits. Should it be confirmed that early white matter defects hinder therapy of the gangliosidoses, this will further support the consensus across most lysosomal diseases that early treatment is crucial for long-term benefit. Early diagnosis through programs such as newborn screening will be a critical component of an optimal treatment strategy.

AUTHOR CONTRIBUTIONS

AM and DM contributed to the conception of the review. AM wrote the first draft of the manuscript and created the tables and figures. Both authors revised the manuscript and approved the submitted version.

FUNDING

NIH R01 NS093941 supported the research and position of AM during the course of this study. Support also was provided by a Graduate Research Fellowship from the Auburn University Center for Neuroscience Initiative to AM.

REFERENCES

Almeida, R. G., and Lyons, D. A. (2017). On myelinated axon plasticity and neuronal circuit formation and function. *J. Neurosci.* 37:10023. doi: 10.1523/JNEUROSCI.3185-16.2017

Baek, R. C., Broekman, M. L. D., Leroy, S. G., Tierney, L. A., Sandberg, M. A., D'Azzo, A., et al. (2010). AAV-mediated gene delivery in adult GM1-gangliosidosis mice corrects lysosomal storage in CNS and improves survival. *PLoS One* 5:e13468. doi: 10.1371/journal.pone.0013468

Baek, R. C., Martin, D. R., Cox, N. R., and Seyfried, T. N. (2009). Comparative analysis of brain lipids in mice, cats, and humans with Sandhoff disease. *Lipids* 44, 197–205. doi: 10.1007/s11745-008-3268-0

Barateiro, A., and Fernandes, A. (2014). Temporal oligodendrocyte lineage progression: In vitro models of proliferation, differentiation and myelination. *Biochim. Biophys. Acta* 1843, 1917–1929. doi: 10.1016/j.bbamcr.2014.04.018

Bourgoin, C., Emiliani, C., Kremer, E. J., Gelot, A., Tancini, B., Gravel, R. A., et al. (2003). Widespread distribution of β-hexosaminidase activity in the brain of a Sandhoff mouse model after coinjection of adenoviral vector and mannitol. *Gene Ther.* 10, 1841–1849. doi: 10.1038/sj.gt.3302081

Bradbury, A. M., Cochran, J. N., McCurdy, V. J., Johnson, A. K., Brunson, B. L., Gray-Edwards, H., et al. (2013). Therapeutic response in feline Sandhoff disease despite immunity to intracranial gene therapy. *Mol. Ther.* 21, 1306–1315. doi: 10.1038/mt.2013.86

Bradbury, A. M., Gray-Edwards, H. L., Shirley, J. L., McCurdy, V. J., Colaco, A. N., Randle, A. N., et al. (2015a). Biomarkers for disease progression and AAV therapeutic efficacy in feline Sandhoff disease. *Exp. Neurol.* 263, 102–112. doi: 10.1016/j.expneurol.2014.09.020

Bradbury, A. M., Gurda, B. L., Casal, M. L., Ponder, K. P., Vite, C. H., and Haskins, M. E. (2015b). A review of gene therapy in canine and feline models of lysosomal storage disorders. *Hum. Gene Ther. Clin. Dev.* 26, 27–37. doi: 10.1089/humc.2015.002

Bradbury, A. M., Peterson, T. A., Gross, A. L., Wells, S. Z., McCurdy, V. J., Wolfe, K. G., et al. (2017). AAV-mediated gene delivery attenuates neuroinflammation in feline Sandhoff disease. *Neuroscience* 340, 117–125. doi: 10.1016/j.neuroscience.2016.10.047

Broekman, M. L. D., Baek, R. C., Comer, L. A., Fernandez, J. L., Seyfried, T. N., and Sena-Esteves, M. (2007). Complete correction of enzymatic deficiency and neurochemistry in the GM1-gangliosidosis mouse brain by neonatal adeno-associated virus–mediated gene delivery. *Mol. Ther.* 15, 30–37. doi: 10.1038/sj.mt.6300004

Broekman, M. L. D., Tierney, L. A., Benn, C., Chawla, P., Cha, J. H., and Sena-Esteves, M. (2009). Mechanisms of distribution of mouse β-galactosidase in the adult GM1-gangliosidosis brain. *Gene Ther.* 16, 303–308. doi: 10.1038/gt.2008.149

Brunetti-Pierri, N., and Scaglia, F. (2008). GM1 gangliosidosis: Review of clinical, molecular, and therapeutic aspects. *Mol. Genet. Metab.* 94, 391–396. doi: 10.1016/j.ymgme.2008.04.012

Buccinnà, B., Piccinini, M., Prinetti, A., Scandroglio, F., Prioni, S., Valsecchi, M., et al. (2009). Alterations of myelin-specific proteins and sphingolipids characterize the brains of acid sphingomyelinase-deficient mice, an animal model of Niemann-Pick disease type A. *J Neurochem.* 109, 105–115. doi: 10.1111/j.1471-4159.2009.05947.x

Cachon-Gonzalez, M. B., Wang, S. Z., Lynch, A., Ziegler, R., Cheng, S. H., and Cox, T. M. (2006). Effective gene therapy in an authentic model of Tay-Sachs-related diseases. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10373–10378. doi: 10.1073/pnas.0603765103

Cachón-González, M. B., Wang, S. Z., McNair, R., Bradley, J., Lunn, D., Ziegler, R., et al. (2012). Gene transfer corrects Acute GM2 gangliosidosis—potential therapeutic contribution of perivascular enzyme flow. *Mol. Ther.* 20, 1489–1500. doi: 10.1038/mt.2012.44

Cachón-González, M. B., Wang, S. Z., Ziegler, R., Cheng, S. H., and Cox, T. M. (2014). Reversibility of neuropathology in Tay-Sachs-related diseases. *Hum. Mol. Genet.* 23, 730–748. doi: 10.1093/hmg/ddt459

Chakrabarti, A. K., Dasgupta, S., Gadsden, R. H., Hogan, E. L., and Banik, N. L. (1996). Regulation of brain m calpain Ca2+ sensitivity by mixtures of membrane lipids: activation at intracellular Ca2+ level. *J. Neurosci Res.* 44, 374–380.

Chorghay, Z., Káradóttir, R. T., and Ruthazer, E. S. (2018). White matter plasticity keeps the brain in tune: axons conduct while glia wrap. *Front. Cell. Neurosci.* 12:428. doi: 10.3389/fncel.2018.00428

Cummings, J., Wood, P., Walkley, S. U., De Lahunta, A., and DeForest, M. (1985). GM 2 gangliosidosis in a Japanese spaniel. *Acta Neuropathol.* 67, 247–253.

Folkerth, R. D. (1999). Abnormalities of developing white matter in lysosomal storage diseases. *J. Neuropathol. Exp. Neurol.* 58, 887–902. doi: 10.1097/00005072-199909000-00001

Folkerth, R. D., Alroy, J., Bhan, I., and Kaye, E. M. (2000). Infantile G(M1) gangliosidosis: complete morphology and histochemistry of two autopsy cases, with particular reference to delayed central nervous system myelination. *Pediatr. Dev. Pathol.* 3, 73–86. doi: 10.1007/s100240050010

Gray-Edwards, H. L., Brunson, B. L., Holland, M., Hespel, A.-M., Bradbury, A. M., McCurdy, V. J., et al. (2015). Mucopolysaccharidosis-like phenotype in feline Sandhoff disease and partial correction after AAV gene therapy. *Mol. Genet. Metab.* 116, 80–87. doi: 10.1016/j.ymgme.2015.05.003

Gray-Edwards, H. L., Jiang, X., Randle, A. N., Taylor, A. R., Voss, T. L., Johnson, A. K., et al. (2017a). Lipidomic evaluation of feline neurologic disease after AAV gene therapy. *Mol. Ther.* 6, 135–142. doi: 10.1016/j.omtm.2017.07.005

Gray-Edwards, H. L., Maguire, A. S., Salibi, N., Ellis, L. E., Voss, T. L., Diffie, E. B., et al. (2020). 7T MRI predicts amelioration of neurodegeneration in the brain after AAV gene therapy. *Mol. Ther. Methods Clin. Dev.* 17, 258–270. doi: 10.1016/j.omtm.2019.11.023

Gray-Edwards, H. L., Randle, A. N., Maitland, S. A., Benatti, H. R., Hubbard, S. M., Canning, P. F., et al. (2018). Adeno-associated virus gene therapy in a sheep model of Tay–Sachs disease. *Hum. Gene Ther.* 29, 312–326. doi: 10.1089/hum.2017.163

Gray-Edwards, H. L., Regier, D. S., Shirley, J. L., Randle, A. N., Salibi, N., Thomas, S. E., et al. (2017b). Novel biomarkers of human GM1 gangliosidosis reflect the clinical efficacy of gene therapy in a feline model. *Mol Ther.* 25, 892–903. doi: 10.1016/j.ymthe.2017.01.009

Guidotti, J. E., Mignon, A., Haase, G., Caillaud, C., McDonell, N., Kahn, A., et al. (1999). Adenoviral gene therapy of the tay-sachs disease in hexosaminidase a-deficient knock-out mice. *Hum. Mol. Genet.* 8, 831–838. doi: 10.1093/hmg/8.5.831

Haberland, C., Brunngraber, E., Witting, L., and Brown, B. (1973). The white matter in GM2 gangliosidosis. *Acta Neuropathol.* 24, 43–55. doi: 10.1007/BF00691417

Heinecke, K. A., Luoma, A., d'Azzo, A., Kirschner, D. A., and Seyfried, T. N. (2015). Myelin abnormalities in the optic and sciatic nerves in mice with GM1-Gangliosidosis. *ASN Neuro* 7:1759091415568913. doi: 10.1177/1759091415568913

Kaye, E. M., Alroy, J., Raghavan, S. S., Schwarting, G. A., Adelman, L. S., Runge, V., et al. (1992). Dysmyelinogenesis in animal model of GM1 gangliosidosis. *Pediatr. Neurol.* 8, 255–261. doi: 10.1016/0887-8994(92)90361-2

Kelly, J. M., Bradbury, A., Martin, D. R., and Byrne, M. E. (2017). Emerging therapies for neuropathic lysosomal storage disorders. *Prog. Neurobiol.* 152, 166–180. doi: 10.1016/j.pneurobio.2016.10.002

Kinney, H. C., Karthigasan, J., Borenshteyn, N. I., Flax, J. D., and Kirschner, D. A. (1994). Myelination in the developing human brain: Biochemical correlates. *Neurochem. Res.* 19, 983–996. doi: 10.1007/BF00968708

Kroll, R. A., Pagel, M. A., Roman-Goldstein, S., Barkovich, A. J., D'Agostino, A. N., and Neuwelt, E. A. (1995). White matter changes associated with feline GM2 gangliosidosis (Sandhoff disease): correlation of MR findings with pathologic and ultrastructural abnormalities. *AJNR Am. J. Neuroradiol.* 16, 1219–1226.

Kyrkanides, S., Miller, J. H., Brouxhon, S. M., Olschowka, J. A., and Federoff, H. J. (2005). β-hexosaminidase lentiviral vectors: transfer into the CNS via systemic administration. *Mol. Brain Res.* 133, 286–298. doi: 10.1016/j.molbrainres.2004.10.026

Kyrkanides, S., Miller, J.-N. H., Tallents, R. H., Brouxhon, S. M., Centola, G. M., and Olschowka, J. A. (2007). Intraperitoneal inoculation of Sandhoff mouse neonates with an HIV-1 based lentiviral vector exacerbates the attendant neuroinflammation and disease phenotype. *J. Neuroimmunol.* 188, 39–47. doi: 10.1016/j.jneuroim.2007.05.010

Lahey, H. G., Webber, C. J., Golebiowski, D., Izzo, C. M., Horn, E., Taghian, T., et al. (2020). Pronounced therapeutic benefit of a single bidirectional AAV vector administered systemically in Sandhoff mice. *Mol Ther.* 28, 2150–2160.

Luo, C., Song, W., Chen, Q., Yang, J., Gong, Q., and Shang, H.-F. (2017). White matter microstructure damage in tremor-dominant Parkinson's disease patients. *Neuroradiology* 59, 691–698. doi: 10.1007/s00234-017-1846-7

McCurdy, V. J., Johnson, A. K., Gray-Edwards, H. L., Randle, A. N., Bradbury, A. M., Morrison, N. E., et al. (2021). Therapeutic benefit after intracranial gene therapy delivered during the symptomatic stage in a feline model of Sandhoff disease. *Gene Ther.* 28, 142–154. doi: 10.1038/s41434-020-00190-1

McCurdy, V. J., Johnson, A. K., Gray-Edwards, H. L., Randle, A. N., Brunson, B. L., Morrison, N. E., et al. (2014). Sustained normalization of neurological disease after intracranial gene therapy in a feline model. *Sci. Transl. Med.* 6:231ra248. doi: 10.1126/scitranslmed.3007733

McCurdy, V. J., Rockwell, H. E., Arthur, J. R., Bradbury, A. M., Johnson, A. K., Randle, A. N., et al. (2015). Widespread correction of central nervous system disease after intracranial gene therapy in a feline model of Sandhoff disease. *Gene Ther.* 22, 181–189. doi: 10.1038/gt.2014.108

Meikle, P. J., Hopwood, J. J., Clague, A. E., and Carey, W. F. (1999). Prevalence of lysosomal storage disorders. *JAMA* 281, 249–254. doi: 10.1001/jama.281.3.249

Nasrabady, S. E., Rizvi, B., Goldman, J. E., and Brickman, A. M. (2018). White matter changes in Alzheimer's disease: a focus on myelin and oligodendrocytes. *Acta Neuropathol. Commun.* 6:22. doi: 10.1186/s40478-018-0515-3

Porter, B. F., Lewis, B. C., Edwards, J. F., Alroy, J., Zeng, B. J., Torres, P. A., et al. (2011). Pathology of GM2 gangliosidosis in jacob sheep. *Vet. Pathol.* 48, 807–813. doi: 10.1177/0300985810388522

Powell, S. K., Khan, N., Parker, C. L., Samulski, R. J., Matsushima, G., Gray, S. J., et al. (2016). Characterization of a novel adeno-associated viral vector with preferential oligodendrocyte tropism. *Gene Ther.* 23, 807–814. doi: 10.1038/gt.2016.62

Provenzale, J. M., Nestrasil, I., Chen, S., Kan, S.-H., Le, S. Q., Jens, J. K., et al. (2015). Diffusion tensor imaging and myelin composition analysis reveal abnormal myelination in corpus callosum of canine mucopolysaccharidosis I. *Exp. Neurol.* 273, 1–10. doi: 10.1016/j.expneurol.2015.07.021

Regier, D. S., Kwon, H. J., Johnston, J., Golas, G., Yang, S., Wiggs, E., et al. (2016). MRI/MRS as a surrogate marker for clinical progression in GM1 gangliosidosis. *Am. J. Med. Genet. A.* 170, 634–644. doi: 10.1002/ajmg.a.37468

Rha, A. K., Maguire, A. S., and Martin, D. R. (2021). GM1 gangliosidosis: mechanisms and management. *Appl. Clin. Genet.* 14, 209–233.

Rockwell, H. E., McCurdy, V. J., Eaton, S. C., Wilson, D. U., Johnson, A. K., Randle, A. N., et al. (2015). AAV-mediated gene delivery in a feline model of Sandhoff disease corrects lysosomal storage in the central nervous system. *ASN Neuro* 7:1759091415569908. doi: 10.1177/1759091415569908

Saher, G., Brügger, B., Lappe-Siefke, C., Möbius, W., Tozawa, R.-I., Wehr, M. C., et al. (2005). High cholesterol level is essential for myelin membrane growth. *Nat. Neurosci.* 8, 468–475. doi: 10.1038/nn1426

Sanders, D. N., Zeng, R., Wenger, D. A., Johnson, G. S., Johnson, G. C., Decker, J. E., et al. (2013). GM2 gangliosidosis associated with a HEXA missense mutation in Japanese Chin dogs: A potential model for Tay Sachs disease. *Mol. Genet. Metab.* 108, 70–75. doi: 10.1016/j.ymgme.2012.11.008

Sandhoff, K., Harzer, K., Wässle, W., and Jatzkewitz, H. (1971). Enzyme alterations and lipid storage in three variants of tay-sachs disease. *J. Neurochem.* 18, 2469–2489. doi: 10.1111/j.1471-4159.1971.tb00204.x

Sargeant, T. J., Drage, D. J., Wang, S., Apostolakis, A. A., Cox, T. M., and Cachón-González, M. B. (2012). Characterization of inducible models of Tay-Sachs and related disease. *PLoS Genet.* 8:e1002943. doi: 10.1371/journal.pgen.1002943

Sargeant, T. J., Wang, S., Bradley, J., Smith, N. J. C., Raha, A. A., McNair, R., et al. (2011). Adeno-associated virus-mediated expression of -hexosaminidase prevents neuronal loss in the Sandhoff mouse brain. *Hum. Mol. Genet.* 20, 4371–4380. doi: 10.1093/hmg/ddr364

Taghian, T., Marosfoi, M. G., Puri, A. S., Cataltepe, O. I., King, R. M., Diffie, E. B., et al. (2020). A safe and reliable technique for CNS delivery of AAV vectors in the cisterna magna. *Mol. Ther.* 28, 411–421. doi: 10.1016/j.ymthe.2019.11.012

Takaura, N., Yagi, T., Maeda, M., Nanba, E., Oshima, A., Suzuki, Y., et al. (2003). Attenuation of ganglioside GM1 accumulation in the brain of GM1

gangliosidosis mice by neonatal intravenous gene transfer. *Gene Ther.* 10, 1487–1493. doi: 10.1038/sj.gt.3302033

Takikita, S., Fukuda, T., Mohri, I., Yagi, T., and Suzuki, K. (2004). Perturbed myelination process of premyelinating oligodendrocyte in niemann-picktype C mouse. *J. Neuropathol. Exp. Neurol.* 63, 660–673. doi: 10.1093/jnen/63.6.660

Tropak, M. B., Yonekawa, S., Karumuthil-Melethil, S., Thompson, P., Wakarchuk, W., Gray, S. J., et al. (2016). Construction of a hybrid β-hexosaminidase subunit capable of forming stable homodimers that hydrolyze GM2 ganglioside in vivo. *Mol. Ther.* 3:15057. doi: 10.1038/mtm.2015.57

Turner, R. (2019). Myelin and modeling: bootstrapping cortical microcircuits. *Front. Neural Circuits* 13:34. doi: 10.3389/fncir.2019.00034

van der Voorn, J. P., Kamphorst, W., van der Knaap, M. S., and Powers, J. M. (2004). The leukoencephalopathy of infantile GM1 gangliosidosis: oligodendrocytic loss and axonal dysfunction. *Acta Neuropathol.* 107, 539–545. doi: 10.1007/s00401-004-0848-9

Walia, J. S., Altaleb, N., Bello, A., Kruck, C., Lafave, M. C., Varshney, G. K., et al. (2015). Long-term correction of sandhoff disease following intravenous delivery of rAAV9 to mouse neonates. *Mol. Ther.* 23, 414–422. doi: 10.1038/mt.2014.240

Walkley, S. U. (2000). Gangliosides as modulators of dendritogenesis in normal and storage disease-affected pyramidal neurons. *Cereb Cortex* 10, 1028–1037. doi: 10.1093/cercor/10.10.1028

Weismann, C. M., Ferreira, J., Keeler, A. M., Su, Q., Qui, L., Shaffer, S. A., et al. (2015). Systemic AAV9 gene transfer in adult GM1 gangliosidosis mice reduces lysosomal storage in CNS and extends lifespan. *Hum. Mol. Genet.* 24, 4353–4364. doi: 10.1093/hmg/ddv168

Conflict of Interest: DM reports stock options from Lysogene and personal fees from Axovant outside the submitted work.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership