How can gene therapy be used to treat PLA2G6 Associated Neurodegeneration?

Aadya Jain[1], Jacob Odell[2]

[1]Dulles High School, TX 77478,
[2]Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca NY 14853
Corresponding author: Aadya Jain [aadya.jain@gmail.com]

Abstract:

Neurodegenerative diseases rank among the most prevalent and challenging health burdens globally, characterized by progressive neuronal degeneration. Mutations in the *PLA2G6* gene, encoding the iPLA2β enzyme critical for cellular phospholipid metabolism underlie severe neurodegenerative syndromes such as infantile neuroaxonal dystrophy (INAD) and dystonia-parkinsonism. Advances in gene therapy, notably CRISPR-Cas9 technology, offer a promising approach to target and correct underlying genetic mutations. CRISPR-Cas9 enables precise modification of the mutated PLA2G6 gene, potentially restoring normal enzyme function and halting disease progression. However, effective delivery of CRISPR components to the central nervous system remains a significant challenge, necessitating innovative strategies such as intracranial viral vector delivery for targeted gene editing. This paper explores the molecular mechanisms of PLA2G6-related neurodegenerative diseases, discusses the application of CRISPR-Cas9 for genetic correction, and evaluates intracranial viral vector delivery as a feasible delivery method. By exploring these approaches, this research aims to contribute to the development of novel therapies aimed at addressing the genetic causes of neurodegenerative diseases associated with *PLA2G6* mutations, potentially offering new hope for patients affected by these devastating conditions.

Keywords: PLA2G6, Parksinson's, CRISPR-Cas9, neurodegeneration, gene therapy

Introduction:

Third to cancer and heart disease, neurodegenerative diseases take over the lives of about 50 million individuals around the world every year as of 2023 (Dimmer 2024). These diseases are a group of incurable conditions where the nerve cells or glia or both within the nervous system begin to deteriorate until they lead to progressive dysfunction. While the causes range from inherited or De novo gene variants, or age, neurodegenerative diseases prove detrimental to the cognitive or behavioral functions of the human body (Davenport et al., 2023). However, neurodegenerative is just an overarching topic filled with diverse disorders affecting different ages, genders, races, and environments. Older populations more commonly suffer from Alzheimer's, Huntingtons', and Parkinson's diseases, where either build-up of different proteins, progressive nerve loss, or loss in a specific type of neuron eventually lead to the person's demise (Davenport et al. 2023). Unfortunately, these types of diseases have even more severe effects on toddlers and children. An infant begins to create connections through their senses and makes many neural pathways that help develop into language, communication, and memories. Their frontal lobes begin to mature as the toddlers figure out how to walk, use a spoon, and write their names or ride a bike. Entering early elementary school is when development switches to be more social and the kids learn how to interact with each other and acquire a more logical way of thinking (Hodel 2018). These early developmental stages are

crucial for a person to fulfill a 'normal lifestyle' as they mature into adults and build stronger relationships, start businesses, and have more responsibility over what they say and do. Therefore, the effect of neurodegenerative disease on these young, prosperous minds is far more severe as it interferes with brain development. The irreversible damage caused inhibits normal developmental processes thus limiting the already compromised life of the children. Most neurodegenerative disorders have a sudden onset, and even if symptoms were to be recognized earlier, there is no way to stop the disease from occurring (Davenport et al., 2023). The outcomes for these young patients may already be determined, which raises the question: how can a disease that is genetically coded and irreversible even be controlled? Each disease has its path and origin, and starting at the root of the problem can help in possibly devising better preventive measures and treatments.

What is the PLA2G6 gene?

Each neurodegenerative disease can be traced to its prospective genes. Alzheimer's is associated with mutations in the *APP, APOE, PSEN1*, and *PSEN2* genes and many others, Huntington's begins with a mutation on the *HTT* gene, and Frontotemporal Dementia results from the mutation of gene *C9orf72 causes mostly ALS.* Some forms of Parkinson's disease begin on the *PLA2G6* gene. The *PLA2G6* gene is located on 22q13.11 and is in charge of coding a protein of the A2 phospholipase family (Guo et al., 2018). The calcium-dependent or independent enzymes secreted are important with immune responses, cell proliferation, and recreating membranes of phospholipids. The protein encoded by this gene is a lipase which also breaks down fat and is distributed towards different organs. (Guo et al., 2018).

The majority of human genes are composed of a series of exons and introns. An exon is a section of DNA within a gene that contains the genetic code used to create protein-encoding parts of the messenger RNA (mRNA) molecule. These exons are retained after non-coding regions called introns are removed through RNA splicing (Aspden et al., 2023). Alterations in the DNA sequence within an exon can result in abnormalities in the protein sequence. These changes may contribute to various effects, potentially including the onset or progression of diseases, consequently promoting more cognitive and mobility-depriving diseases like Dystonia or Parkinsons, classified as PLAN (PLA2G6- Associated- Neurodegeneration). The 4 main types of PLAN are Infantile Neuroaxonal Dystrophy (INAD), Atypical Neuroaxonal dystrophy (ANAD), Adult-onset Dystonia-Parkinsonism (DP), and Autosomal Recessive Early-Onset Parkinsonism (AREP) (Guo et al., 2018). While INAD, which affects infants younger than 2, and ANAD are more youth-focused, DP and AREP affect people until around the age of 40. INAD patients show psychomotor decline and impaired functions such as hearing and swallowing. Those diagnosed with ANAD suffer from seizures, and psychomotor regression and often have autism. Both DP and AREP are triggered by Parkinson's which encourages a further decline (Guo et al., 2018). Traditional medication and methods prove ineffective towards these symptoms, as the disease is ultimately terminal. With advancing technology, more effective and promising techniques are coming into play when trying to target mutations and hopeful treatments and preventative measures. Given that PLAN is not a complete mystery, where both the underlying issues and origin are apparent, solutions like gene therapy emerge as a possible, effective cure for these diseases.

	INAD	ANAD	DP	AREP

Age of onset	6 months-3 years	Early childhood - 18 years	20-40 years	under 40 years
Initial Symptoms	Psychomotor deterioration	Cerebellar ataxia	Parkinsonism	Parkinsonism
Progression	Rapid	Slower than INAD	Slow	Slow
Important Signs	Cerebellar ataxia, hypotonia, hyporeflexia, nystagmus, strabismus	Cerebellar ataxia, hypermyotonia	Hypermyotonia, extrapyramidal signs	Hypermyotonia in lower limbs
Brain MRI Signs	Brain iron accumulation in globus pallidus, substance Niagara, cerebellar atrophy	Brain iron accumulation in two sides of basal ganglia, cerebellar atrophy	A substantial reduction in data	A substantial reduction in data
Patient Lifetime	Short, before age 10	Slightly longer than INAD	Long	Long
Treatment	Symptomatic Treatment	Symptomatic Treatment	Dopaminergic Agents	Dopaminergic Agents

An Intro to CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are used to edit the genomes of organisms by targeting their DNA sequence. CRISPR/Cas9 (CRISPR-associated protein 9**)** originated from a naturally occurring system found in certain bacteria, which functions as part of their immune system. CRISPR, facilitated by the CAS proteins, is a part of the bacterial immune system, which uses fragments of the virus's DNA and keeps a "genetic memory" of past infections. When a bacteriophage infects the cell, it inserts its genetic material which gets replicated by the host cells' machinery (Lander 2016). Through the lytic cycle, once enough phages are accumulated in the cell, the cell eventually 'pops' and releases all the phages into the environment, which leads to the destruction of the cell. To combat this fate, many bacteria have developed their own CRISPR system to build immunity. The CRISPR structure is composed of spacers and repeats. The repeats are short, identical sequences of clustered base arrangements and play a role in the adaptation phase of the CRISPR response. Other Cas proteins recognize these repeats and insert spacers between them, but they do not contribute to guiding the Cas9 protein to target and cut the DNA (Lander 2016). The spacers are transcribed into CRISPR RNA (crRNA), a fundamental component of

the CRISPR-Cas system that originates from the CRISPR array in bacterial DNA. The crRNA is the RNA sequence complementary to the target DNA site and has a complementary base pair towards the target DNA sequence. The tracrRNA interacts with the Cas9 protein and stabilizes its binding to the sgRNA, helping to stabilize and orientate it within the complex. This interaction is important to ensure the proper assembly of the Cas9-sgRNA ribonucleoprotein (RNP) complex, which is necessary for precise DNA targeting and cleavage (Liao & Beisel, 2023). Together with tracrRNA, they guide the Cas9 enzyme to modify specific DNA sequences, allowing precise genome editing in scientific and medical applications across different organisms. Spacers are complementary to the specific segments of phage DNA (Liao & Beisel, 2023). This structure stores the phage DNA to build up a defense for when they try to attack again and is passed down to the following generations. When the bacterium encounters a viral or plasmid threat, the crRNA guides the Cas proteins to the corresponding sequences in the foreign DNA (Lander 2016). The Cas proteins, particularly Cas9 in the case of the popular CRISPR-Cas9 system, act as molecular scissors, cutting the invading DNA at precise locations dictated by the crRNA sequence. This targeted DNA cleavage disables the virus or plasmid, rendering it unable to replicate and infect the bacteria further.

The Cas9 protein is one of the most important components of this process. When Cas9 is paired with crRNA and tracrRNA, it is responsible for recognizing and attacking foreign genetic material (Lander 2016). This protein acts almost like scissors and will now use the crRNA to scan for any sequences that are complementary to crRNA, or in other words, match previously encountered phages or viruses. Once detected, Cas9 binds to the DNA and starts scanning it using the crRNA. When it finds a match, it positions its nuclease domains to make a double-strand break in the target DNA, initiating the destruction of the infected code. This process helps the bacteria acquire immunity against these diseases and incorporates this immunity into the bacteria's original genome. Think of Cas9 as a zipper that progressively unwinds the DNA while searching for a match, rather than simply binding to the target DNA directly (Liao & Beisel, 2023). Cas9 is now being implemented in today's medical world and used to treat mutated DNA which is responsible for neurodegenerative diseases.

As a gene editing tool, the CRISPR system is a two-part system that utilizes the Cas9 protein and the guide RNA (sgRNA) (Liao & Beisel, 2023). Like the crRNA, the sgRNA's role is to help guide the Cas9 to the correct target DNA sequence. The sgRNA combines two different RNA functions, CRISPR RNA (crRNA) and trans-activating RNA (tracrRNA), into a chimeric RNA complex (Liao & Beisel, 2023). This chimeric RNA is formed synthetically in the lab by combining the essential parts of the crRNA and tracrRNA into a single hairpin-shaped RNA molecule. This simplifies the delivery and use of the CRISPR system. Once the Cas9 protein recognizes a region of DNA complementary to the gRNA, it creates a double-stranded break (DSB) at the targeted site (Cui et al.,2018). At the DSB, the gene will be edited or mutated, depending on the outcome of the repair through either non-homologous end joining (NHEJ) or homology-directed repair (HDR)(Xue & Greene, 2021). This targeted alteration can lead to specific changes in gene function, such as disabling a gene to study its role in cellular processes or disease, correcting mutations responsible for genetic disorders, or inserting new genetic material to introduce desired traits. The proceeding DSB repair process, whether through error-prone non-homologous end joining (NHEJ) or precise homology-directed repair (HDR), then determines the success and specificity of the genetic modification intended by the researcher(Xue & Greene, 2021). Both NHEJ and HDR are possible within a population of cells intended for editing. Researchers can influence the preference for one repair pathway over the

other using various techniques, but these methods are not 100% effective. This variability represents a significant limitation, so it is important to note that the repair process may not always yield the desired outcome (Xue & Greene, 2021). The NHEJ process is first initiated when a DSB is detected in the DNA. NHEJ then fixes the DSB and rejoins the broken ends of the DNA, however, is limited in precision when trying to ligate broken DNA ends. NHEJ does not have any specific template to follow, and instead uses the proteins, Ku70, Ku80, and DNA-PKcs which bind to the ends and help align the DNA (Lieber 2010). This could result in random insertions or deletions (indels) of base pairs. The indels can further alter the sequence of the protein encoded by this gene, and in many cases lead to a loss of function that ablates the function of the protein (Lieber 2010). This underscores the importance of developing and utilizing genome editing techniques that prioritize accuracy and minimize off-target effects, such as optimizing CRISPR-Cas9 systems for improved specificity and reliability. Unlike NHEJ, which is error-prone and can lead to random indels at the repair site, HDR offers a more precise method of genome editing. HDR utilizes a homologous template DNA strand to guide the repair of double-stranded breaks (DSBs)(Xue & Greene, 2021). This process occurs when the cell is in the S or G2 phase of the cell cycle. HDR is thus more accurate, as it uses the template to precisely replace the damaged DNA sequence. This allows for the insertion of specific sequences, correction of mutations, or more targeted genetic modifications at the targeted genomic locus(Xue & Greene, 2021). The success and specificity of HDR-mediated genetic modifications depend on the availability and quality of the homologous template DNA, which can be modified and perfected in the lab as well as the efficiency of the HDR machinery within the cell To achieve a precise gene edit, researchers can use a custom-designed template DNA, engineered by researchers to carry specific genetic sequences needed for precise genome editing(Xue & Greene, 2021). This template is used during cell repair processes to insert new sequences, correct mutations, or make targeted genetic modifications at desired genomic locations. This approach allows for controlled and accurate genetic alterations, essential for both research and therapeutic applications.

Researchers raised the question if these methods could be applied to other diseases and gene-related issues, which began the long journey of gene editing and revolutionized the fields of genetics and biomedicine. This targeted method could not only correct the mutations and “edit the gene” but perhaps even eliminate the disease at its root. The paper will begin to identify both the positive and adverse potential of gene editing for the PLA2G6 gene and different strategies to further its research.

Body:

CRISPR for PLA2G6

CRISPR-Cas9 technology offers a promising therapeutic strategy for genetic disorders by precisely targeting and correcting mutations. By utilizing specially designed guide RNAs and the cell’s endogenous repair pathways, CRISPR can accurately address point mutations or replace defective segments within the *PLA2G6* gene. This gene-editing method has the potential to restore the normal function of the iPLA2β enzyme, thereby addressing the root cause of neurodegenerative diseases like INAD and dystonia-parkinsonism (Guo et al., 2018). To implement CRISPR in PLA2G6, it is important to understand the nature of the mutations and how this further affects the body's normal function. As mentioned before, the iPLA2β enzyme hydrolyzes the phospholipids that maintain the cell membrane’s structure and function. This protein contains many important sites which include eight N-terminal ankyrin repeats, a

caspase-3 cleavage site, an ATP-binding domain, a serine lipase consensus sequence (GXSXG), a bipartite nuclear localization sequence, and a C-terminal calmodulin-binding domain (Guo et al., 2018). These sites are responsible for providing the cell with energy, controlling apoptosis, breaking down phospholipids, and calcium regulation.

One of the common mutations that occurs within PLA2G6 is a missense mutation. Missense mutations, which result in single amino acid substitutions, can be targeted using CRISPR technology (Engel et al., 2010). By specifically addressing the mutated nucleotide sequence, CRISPR enables the introduction of a correct DNA template through HDR. This process replaces the errored sequence with the accurate one, thereby reinstating normal protein function. Frameshift mutations are mutations that introduce a premature stop codon, interfering with the normal stop codons and subsequently altering the reading sequence of amino acids (Engel et al., 2010). A faulty amino acid sequence results in a faulty or even dysfunctional protein. They can have detrimental effects and lead to genetic disorders, diseases, or loss of function of essential proteins depending on where and how they occur within the genome. For mutations causing frameshift that disrupt protein function, CRISPR offers the capability to either remove or correct the defective exonic regions (Engel et al., 2010). This correction ensures the production of a functional protein. However, the difference in the sites will make it hard to create one generalized therapy that targets a specific location. Additionally, it is important to consider that INAD and ANAD are observed at much younger ages, while DP and AREP typically affect adults, who may also face problems like Parkinson's (Guo et al., 2018). While a younger person will find it easier to adapt to the changes because their bodies are still growing, adults will be more of a challenge because they have a more developed and complex structure, and having something go wrong can make them lose the function they already have. The same could be said with infants and they are much more vulnerable to complications. Therapy is being given so early on.

Intracranial Viral Vector Delivery

Intracranial viral vector delivery is a sophisticated technique in neuroscience where viral vectors are employed to introduce genetic material into specific regions of the brain (Naso et al., 2017). This method typically involves selecting appropriate viral vectors like adeno-associated viruses (AAV) or lentiviruses, which are modified to carry desired DNA or RNA constructs. AAVs are small, non-enveloped viruses with a single-stranded DNA genome that can be engineered to carry therapeutic genes. These vectors are especially notable for their use in stable genetic manipulation (Naso et al., 2017). AAVs are non-pathogenic and do not replicate efficiently in human cells, making them safe for therapeutic use. Their ability to provide long-term gene expression in non-dividing cells makes them viable for treating genetic diseases, offering a promising avenue for precise and sustained genetic correction.

The typical AAV vector consists of a small viral genome packaged within a protein capsid derived from wild-type AAVs. The viral vector is then injected using microsyringes, ensuring accurate placement and controlled delivery volumes to minimize tissue damage (Naso et al., 2017). Once inside the cell, the AAV vector enters the nucleus, where its DNA cargo is released and can integrate into the host cell's genome or remain episomal (staying in the nucleus without integrating into the cell’s chromosomal DNA), depending on the AAV serotype and target cell type. AAV2, for example, is adept at targeting neurons, which is crucial for addressing neurological disorders, such as those associated with the PLA2G6 gene (Naso et al., 2017).

AAV2 has a natural affinity for neuronal cells which allows it to cross the blood-brain barrier and bind to the receptor in these cells (Shi et al., 2004). AAV2 directly delivers into the nucleus host cells and the DNA remains episomal, which allows it to facilitate long-term gene expression without integrating into the host's genome, further improving the safety of the AAV as there is no chance of random integration of the vector into the genome. For treating disorders related to the PLA2G6 gene, such as PLA2G6-associated neurodegeneration (PLAN), it is easy to mistakenly assume that AAV2s or lentiviruses could be engineered to carry corrected versions of the PLA2G6 gene and still be effective (Wang et al., 2019). Despite their potential, AAVs have limitations due to their restricted capacity for carrying genetic material, which is approximately 5,000 base pairs (Wang et al., 2019). This constraint poses challenges when working with larger and more complex genes or therapeutic constructs, such as those used in CRISPR-Cas9 systems. For instance, the Cas9 enzyme alone occupies 4,000 base pairs, and additional space is required to guide RNAs and promoter sequences before the therapeutic gene can even be delivered (Trapani, 2019). To overcome this limitation, a system involving the use of dual AAV vectors can be explored. This approach splits the therapeutic gene or components of CRISPR into two different AAV vectors which contain portions of the total genetic material. Once injected into the target cell, the two separate vectors combine to form a functional unit (Trapani 2019). With conditions like PLAN, a dual AAV vector proves to be an alternative where a single AAV vector is not feasible due to its size.

It is also important to consider that AAVs prefer transient expressions of CRISPR components to treat conditions including PLAN. Transient expression of CRISPR refers to the temporary introduction and activity of CRISPR components, such as the Cas9 enzyme and guide RNAs, within a cell (Wang et al.,2019). Unlike stable expression, where the CRISPR system is permanently integrated into the cell's genome, transient expression ensures that the CRISPR components are only present for a limited time. After the AAV delivers the CRISPR system and the genetic modification is completed, the CRISPR components are rapidly degraded or removed from the cell, leaving behind only the therapeutic benefits and reducing the potential for unintended off-target effects (Wang et al.,2019). This approach is used to achieve genetic modifications without the risk of long-term CRISPR activity, which can reduce the likelihood of off-target effects and potential disruptions to normal cellular functions.

Consequences of Gene Therapy/CRISPR in the Brain

CRISPR technology, while transformative for gene editing, faces challenges such as off-target effects, where the guide RNA may inadvertently recognize and bind to unintended DNA sequences, leading to unintended edits. The specificity of the guide RNA is critical, yet it is possible for it to cross-react with similar sequences elsewhere in the genome. To address these issues, molecular evolution has produced higher-fidelity versions of Cas9 that offer improved accuracy, reducing off-target activity. Additionally, advances in CRISPR systems include the use of Cas proteins like Cas11 and Cas13, which have distinct enzymatic properties compared to traditional Cas9, and various algorithms have been developed to screen for and minimize off-target effects. Researchers are exploring these novel Cas proteins and algorithms to refine the precision of gene editing. For applications in neurons, where HDR is less effective due to the cells' capacity for self-repair and limited HDR efficiency, base editors present a promising alternative. Base editors do not rely on Cas9's "scissors" but instead enable precise nucleotide changes without introducing double-strand breaks, offering a more reliable method for achieving targeted genetic modifications in neuronal cells.

The immunogenicity of the Cas9 protein poses a potential concern for CRISPR-based therapies targeting genes such as PLA2G6. Since it is derived from a bacteria, the Cas9 protein might trigger an immune response in the body, potentially leading to inflammation, swelling, and fluid buildup in the brain. This could undermine the effectiveness of the treatment by introducing unwanted side effects or complications. Consequently, while the immune response remains a critical consideration, advances in minimizing Cas9's immunogenicity through protein engineering or other strategies could enhance the safety and efficacy of CRISPR therapies for PLA2G6-related conditions. One approach is to explore Cas9 proteins derived from commensal bacteria, such as those naturally present in the gut. These proteins could be better tolerated by the immune system due to their familiar presence in the body, potentially reducing the risk of adverse immune reactions compared to entirely foreign Cas9 proteins.

The Blood-Brain Barrier (BBB) stands between the brain and the bloodstream, creating many challenges (Stanimirovic et al., 2018). The BBB is mainly composed of endothelial cells, which form the lining of blood vessels in the brain; Astrocyte End-Feet, star-shaped glial cells that provide support and maintain BBB integrity; Pericytes which regulate blood flow and permeability; and finally the basal membrane, a thin, extracellular matrix layer which separates the endothelial cells from the surrounding tissue. These cells protect the brain from toxins and pathogens while regulating nutrient and waste exchange. They furthermore restrict the movement of large hydrophilic therapeutics and contain polarized transporters that prevent unwanted lipophilic synthetic molecules from entering the brain(Stanimirovic et al., 2018). Biologics are therapeutic products derived from biological sources, including vaccines, recombinant proteins, gene therapy, and living cells. These large, complex molecules were once thought impractical for BBB penetration. Techniques like mannitol-induced osmotic BBB disruption and focused ultrasound have also shown limited clinical benefits. The BBB's restrictive nature, the passage of substances from the bloodstream into the brain is, thus complicating the delivery of CRISPR/Cas9 therapies intended for neurological disorders. Intravenously delivered CRISPR/Cas9 components can inadvertently end up in the liver, where they are taken up by hepatocytes instead of reaching their intended target in the brain. This misdelivery is a significant challenge in gene therapy, leaving researchers to look for technologies to overcome this (Teleanu et al., 2022).

One solution is through Lipid Nanoparticles (LNPs). Lipid nanoparticles are formed through the self-assembly of lipid components, including ionizable lipids, structural lipids, helper lipids, and cholesterol, into a spherical structure that encapsulates nucleic acids (Khare et al., 2023). Ionizable lipids help capture and release these nucleic acids in the cytoplasm. Structural lipids, such as phospholipids, form the lipid bilayer that maintains the nanoparticle's structural integrity, while helper lipids support the stability and release properties of the LNPs (Khare et al., 2023). Cholesterol is often included to enhance the fluidity and stability of the lipid bilayer. After formation, the LNPs are purified to remove unencapsulated materials and solvents, using methods like ultracentrifugation or filtration. These steps ensure that the LNPs are well-suited for delivering therapeutic agents effectively, minimizing unintended side effects, and improving targeting precision.

LNPs address the BBB challenge through several strategies. Firstly, they can be engineered with surface modifications that include specific ligands or antibodies, which enhance their ability to cross the BBB and target brain cells (Khare et al., 2023). These modifications help direct the nanoparticles away from the liver and toward their intended destinations. Additionally, adjusting the size and lipid composition of LNPs can influence their biodistribution, potentially

reducing liver accumulation and improving targeting efficiency. Controlled release mechanisms also play a critical role. LNPs can be designed to release their content, including the CRISPR/Cas9 components, in a controlled manner once they reach the target cells (Khare et al., 2023). This controlled release minimizes off-target effects and enhances therapeutic outcomes. Exploring alternative delivery methods, such as localized injection directly into brain tissues, also holds promise for bypassing systemic circulation and reducing liver uptake. This approach can be contextualized for targeting specific neuronal conditions linked to PLA2G6, allowing for more precise and effective gene editing. The goal here is to deliver a DNA cargo that encodes the Cas9 enzyme, gRNA, and repair template, ensuring targeted and efficient genetic modification within the affected brain regions.

Uterus Gene Therapy

Uterus gene therapy, when applied to treating conditions in developing fetuses, offers a promising approach to addressing genetic disorders before birth. This therapeutic strategy involves delivering therapeutic genes directly into the uterine environment, aiming to correct genetic mutations or modify gene expression in the fetus (Bose et al., 2021). For conditions that manifest early in development, such as certain neurodegenerative diseases, this approach has the potential to halt or prevent the progression of disease before symptoms even appear. In practice, uterus gene therapy for fetuses might involve several key methods. One approach is to use viral vectors or nanoparticles to deliver therapeutic genes to the fetal tissues through the amniotic fluid or directly into the uterine environment (Bose et al., 2021). By correcting genetic mutations or providing essential proteins, gene therapy could prevent the development of neurodegenerative diseases that would otherwise progress after birth. For neurodegenerative diseases like PLA2G6-related disorders, which are characterized by progressive damage to neurons, early intervention is crucial. However, the utero gene therapy, though tempting, cannot be implemented due to the size of the PLA2G6 gene.

Screening eggs, or preimplantation genetic testing (PGT), is a crucial technique used to identify genetic abnormalities in embryos before they are implanted during in vitro fertilization (IVF). This process begins with ovarian stimulation to produce multiple eggs, which are then retrieved and fertilized in the laboratory (Ryche & Berckmoes, 2020). The resulting embryos are cultured until they reach the blastocyst stage, at which point a few cells are biopsied for genetic testing. Advanced techniques such as next-generation sequencing or array comparative genomic hybridization are used to detect specific genetic mutations, including those associated with neurodegenerative diseases like PLA2G6-related disorders.

While egg screening and uterine gene therapy offer considerable advantages, such as preventing genetic disorders and supporting informed reproductive choices, they also come with challenges and ethical dilemmas (Ryche & Berckmoes, 2020). Despite the sophistication of modern genetic testing, there is still a risk of false positives or negatives that could impact critical decisions. Ethical issues include the potential for discarding embryos with genetic abnormalities and the broader implications of selecting embryos based on genetic traits, which raises questions about the limits of reproductive technology. Additionally, these advancements could exhibit socioeconomic disparities, as wealthier individuals may have greater access to and benefit from genetic modifications, potentially increasing inequality in reproductive health outcomes.

Brain Organoids For Testing

Brain organoids, which are three-dimensional, self-organizing clusters of brain-like cells derived from stem cells, offer a revolutionary approach to studying neurodegenerative diseases, including those caused by *PLA2G6* gene mutations (Wang et al.,2022). These organoids replicate many aspects of brain development and function, providing a more accurate model of human brain tissue than traditional two-dimensional cell cultures or animal models.

Brain organoids are created through a sophisticated process that starts with pluripotent stem cells, which can differentiate into any cell type in the body. These stem cells, either derived from embryonic sources or induced pluripotent stem cells (iPSCs) reprogrammed from adult cells, are cultured in a three-dimensional matrix that mimics the brain's extracellular environment. (Wang et al., 2022). This matrix supports the cells and encourages their self-organization into neural progenitor cells. These progenitors are then exposed to specific growth factors and signaling molecules that guide their differentiation into various brain cell types, including neurons, astrocytes, and oligodendrocytes. As the cells continue to grow and differentiate, they spontaneously form complex, multi-layered structures that resemble different regions of the brain. This process of self-organization results in brain organoids with features akin to those of the human brain (Wang et al.,2022). Over several weeks to months, these organoids mature, exhibiting characteristics such as electrical activity and neuronal connectivity. Researchers then characterize the organoids using techniques like microscopy, electrophysiology, and gene expression analysis to study their development and function. Through this intricate process, brain organoids provide a valuable model for investigating brain development, disease mechanisms, and potential therapeutic approaches. Once implanted, the brain organoid is observed for integration and functionality through techniques such as imaging and histological analysis, allowing researchers to assess how the organoid affects brain function and pathology (Wang et al.,2022). This approach enables detailed investigation into disease mechanisms and the testing of potential treatments in a system that closely mimics human brain tissue.

By incorporating PLA2G6 gene mutations into brain organoids, researchers can observe the direct effects of these mutations on neuronal development, functionality, and pathology in a controlled environment. Using brain organoids to study PLA2G6 mutations allows for a detailed investigation of disease mechanisms at a cellular level. For example, researchers can assess how these mutations impact neuronal differentiation, synaptic connectivity, and overall brain architecture. Moreover, brain organoids can be employed to test potential therapeutic strategies, including CRISPR-based gene editing, to correct PLA2G6 mutations and evaluate their effectiveness and safety. This application is particularly valuable because organoids offer a platform for high-throughput screening of drugs or gene therapies, providing insights into how such interventions might mitigate disease symptoms or modify disease progression.

Mouse Models

CRISPR-Cas9 technology can also be effectively utilized to create and manipulate mouse models. This method allows scientists to introduce specific mutations into the genetic material of mice, enabling the study of these mutations' effects and the exploration of potential treatments. For instance, in the case of the PLA2G6 gene, researchers can introduce a knock-in mutation by targeting exon 7, a protein-coding region of the gene that is often mutated in human

patients. The process begins with the creation of a knock-in target vector, which includes the DNA sequence to be inserted. Through a method known as homologous recombination, this vector aligned precisely with the corresponding sequence in the mouse genome, allowing for the accurate insertion of the mutation. This technique provides valuable insights into the role of the PLA2G6 gene and the potential therapeutic strategies for conditions associated with its mutations via study of the resulting mouse.

Scientists initiated a study where they knocked in mutated PLA2G6 genes into mice. While studying the effects of the mutation, scientists observed a reduction in dopamine neurons as the mice aged. Dopamine is crucial for various physiological functions, including prolactin production in the hypothalamus, which influences immune and metabolic functions. It also plays a vital role in cognition, attention, motor control, and overall mood, with its deficiency being linked to diseases such as Parkinson's. Additionally, mitochondria, the organelles responsible for providing cellular energy, storing calcium, and regulating apoptosis, were found to be impaired (Chiu 2019). Increased apoptosis levels in mitochondria were noted with the presence of more misfolded proteins. This was assessed using transmission electron microscopy, which employs light beams to create stains, revealing impaired mitochondrial activity and decreased ATP production associated with the mutated gene. Further analysis of the mice showed the activation of apoptosis pathways and downregulation in dopaminergic genes, resulting in the reduced creation of dopamine neurons. Researchers also examined altered mRNA levels of various proteins that either promote apoptosis or decrease the production of essential proteins for dopamine neuron survival, such as Mapk1 (Chiu 2019).

These mouse models were also used to simulate symptoms and explore potential treatment avenues for the future. For example, the researchers measured the motor abilities of the mice by timing their descent from a pole, observing spontaneous movements, and assessing their balance on a rotating apparatus (Chiu 2019). The main findings indicated that as the mice aged, there was a correlated decrease in velocity and distance, increased incoordination, and reduced activity. These symptoms align with those observed in diseases such as Parkinson's, which result from neurodegeneration due to mutations in the PLA2G6 gene. Additionally, the scientists tested various drugs to assess their potential therapeutic effects. One such drug was methyl L-DOPA, which was introduced to the mice to evaluate its impact. Compared to mice injected with saline, those treated with methyl L-DOPA showed significant improvements (Chiu 2019). This finding suggests that methyl L-DOPA could be a promising treatment option for mitigating symptoms associated with PLA2G6-related neurodegeneration.

More recently, advancements in gene therapy, particularly using AAV vectors, have shown significant promise for treating PLAN, with a strong emphasis on the use of mouse models. Researchers have developed an AAV-based gene therapy construct called AAV-EF1a-PLA2G6, which utilizes the elongation factor EF-1 alpha (EF1a) promoter to broadly express PLA2G6 across various cell types (Lin et al., 2023). When this construct was delivered to patient-derived neural progenitor cells (NPCs), it expressed PLA2G6 at low levels (10-20% of endogenous levels), which was sufficient to partially alleviate defects in Vps35 levels, lysosomal expansion, and mitochondrial abnormalities. Crucially, in Pla2g6 KO/G373R mice—a model for INAD—delivery of AAV-EF1a-PLA2G6 delayed the onset of motor defects, helped maintain

body weight, and prolonged lifespan (Lin et al., 2023). This approach underscores the importance of mouse models in evaluating the efficacy of AAV-mediated gene therapy for PLAN and suggests a promising avenue for further optimization and development of treatments for PLA2G6-associated neurodegeneration.

Discussion

PLAN currently lacks prescribed treatments or drugs, but exploring avenues in gene therapy may ultimately provide a solution. Advancements in CRISPR-Cas9 technology, intracranial viral vector delivery, and innovative approaches such as in-utero gene therapy and brain organoids present treatment to PLAN diseases. CRISPR-Cas9 holds the potential to precisely correct mutations in the PLA2G6 gene, potentially restoring normal iPLA2β enzyme function and addressing conditions like INAD and dystonia-parkinsonism. Advances in AAV vector techniques are enhancing targeted gene delivery to the brain, and overcoming the challenges of the blood-brain barrier. While challenges like the size constraints of AAV vectors, potential off-target effects, and the immunogenicity of CRISPR components need ongoing refinement, these implications could be resolved with continued research. Uterus gene therapy offers the possibility of preventing disease onset before birth, although it requires careful delivery and ethical consideration. Brain organoids provide valuable models for studying PLA2G6 mutations and testing new therapies, and CRISPR-enhanced mouse models are shedding light on disease progression and the efficacy of treatments like methyl L-DOPA.

Gene therapy using AAV9-PLA2G6 has shown promising results in a mouse model of PLAN, effectively preventing neurodegeneration, halting disease progression, and even reversing existing neurological damage. Treated mice exhibited significant improvements in motor function and lifespan, indicating the potential for this therapy to address both neurological and systemic aspects of the disease (Lin et al., 2023). While current treatments primarily focus on managing symptoms, this research opens up new possibilities for targeted therapies, including the development of gene therapy for human trials and cardiolipin-focused interventions. Future therapies could look to combine gene therapy with existing symptomatic treatments for a more comprehensive approach to patient care (Lin et al., 2023).

Researchers should also focus on developing advanced genetic counseling methods, including tests to evaluate the risk of PLA2G6-associated neurodegeneration in individuals and their offspring. Enhancing carrier testing, prenatal testing for at-risk pregnancies, and preimplantation genetic testing can be invaluable, particularly if pathogenic variants are known in the family. Early identification of genetic risk can profoundly influence family planning decisions, helping prospective parents gauge the likelihood of their child having the condition and make informed choices. This proactive approach can also equip families with the tools for early intervention and support, potentially alleviating the emotional and financial strain of managing a neurodegenerative disease with significant impacts.

In studying PLA2G6-associated neurodegeneration (PLAN), prioritizing the development of patient-derived brain organoids is crucial. These organoids should be analyzed for dopamine pathway changes to assess the impact of potential gene therapies. These organoids could consist of patient-derived cells and incorporating multiple cell types into these organoids allows for a comprehensive evaluation of potential therapies. Conducting preclinical trials with these

organoids allows researchers to assess the effectiveness of treatments before moving to animal models. Following successful validation in organoids, preclinical testing in mouse models is essential to confirm safety and efficacy. Additionally, genetic and molecular profiling, long-term efficacy studies, and comprehensive safety testing are necessary to ensure a thorough evaluation. These steps will advance effective treatments from experimental phases to clinical trials, addressing the needs of patients with advanced PLAN. Advancing treatments through these rigorous experimental phases will offer hope and improve the quality of life for those affected by these debilitating conditions.

References

1. Aspden, J. L., Wallace, E. W. J., & Whiffin, N. (2023, April 12). Not all exons are protein-coding: Addressing a common misconception. Cell genomics. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10112331/
2. Chiu, C.-C., Lu, C.-S., Weng, Y.-H., Chen, Y.-L., Huang, Y.-Z., Chen, R.-S., Cheng, Y.-C., Huang, Y.-C., Liu, Y.-C., Lai, S.-C., Lin, K.-J., Lin, Y.-W., Chen, Y.-J., Chen, C.-L., Yeh, T.-H., & Wang, H.-L. (2018, August 8). Park14 (D331Y) PLA2G6 causes early-onset degeneration of substantia nigra dopaminergic neurons by inducing mitochondrial dysfunction, ER stress, mitophagy impairment and transcriptional dysregulation in a knockin mouse model - molecular neurobiology. SpringerLink. https://link.springer.com/article/10.1007/s12035-018-1118-5
3. Cui, Y., Xu, J., Cheng, M., Liao, X., & Peng, S. (2018, April 11). Review of CRISPR/Cas9 sgrna design tools - interdisciplinary sciences: Computational Life Sciences. SpringerLink. https://link.springer.com/article/10.1007/s12539-018-0298-z
4. Davenport F;Gallacher J;Kourtzi Z;Koychev I;Matthews PM;Oxtoby NP;Parkes LM;Priesemann V;Rowe JB;Smye SW;Zetterberg H; (n.d.). Neurodegenerative Disease of the brain: A survey of interdisciplinary approaches. Journal of the Royal Society, Interface. https://pubmed.ncbi.nlm.nih.gov/36651180/
5. De Rycke, M., & Berckmoes, V. (2020, July 31). Preimplantation Genetic Testing for monogenic disorders. Genes. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7463885/
6. Engel, L. A., Jing, Z., O'Brien, D. E., Sun, M., & Kotzbauer, P. T. (2010, September 23). Catalytic function of PLA2G6 is impaired by mutations associated with infantile neuroaxonal dystrophy but not dystonia-parkinsonism. PloS one. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2944820/
7. Gregory, A. (2017, March 23). PLA2G6-associated neurodegeneration. GeneReviews® [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK1675/#:~:text=Routine%20pharmacologic%20treatment%20of%20spasticity,constipation%3B%20control%20of%20secretions%20with
8. Guo Y, Tang B and Guo J (2018) PLA2G6-Associated Neurodegeneration (PLAN): Review of Clinical Phenotypes and Genotypes. Front. Neurol. 9:1100. doi: 10.3389/fneur.2018.01100
9. hme0988. (2024, April 11). Uncovering the culprits of Neurodegenerative Disorders. News Center. https://news.feinberg.northwestern.edu/2024/04/11/uncovering-the-culprits-of-neurodegenerative-disorders/

10. Hodel, A. S. (2018, June). Rapid infant prefrontal cortex development and sensitivity to early environmental experience. Developmental review : DR. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6157748/
11. Lander, E. S., 1, 2, 3, & ago, T. years. (2016, January 14). *The heroes of CRISPR*. Cell. https://www.sciencedirect.com/science/article/pii/S0092867415017055
12. Liao, C., & Beisel, C. L. (2021, November 23). The tracrrna in CRISPR Biology and Technologies. Annual review of genetics. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7614092/
13. Lieber, M. R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annual review of biochemistry. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3079308/
14. Lin, G., Tepe, B., McGrane, G., Tipon, R. C., Croft, G., Panwala, L., Hope, A., Liang, A. J., Zuo, Z., Byeon, S. K., Wang, L., Pandey, A., & Bellen, H. J. (2023, January 16). Exploring therapeutic strategies for infantile neuronal axonal dystrophy (INAD/Park14). eLife. https://elifesciences.org/articles/82555
15. Nemudryi, A. A., Valetdinova, K. R., Medvedev, S. P., & Zakian, S. M. (2014, July). Talen and CRISPR/Cas genome editing systems: Tools of discovery. Acta naturae. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4207558/
16. Stanimirovic, D. B., Sandhu, J. K., & Costain, W. J. (2018a, October 10). Emerging technologies for delivery of Biotherapeutics and gene therapy across the blood–brain barrier - biodrugs. SpringerLink. https://link.springer.com/article/10.1007/s40259-018-0309-y
17. Teleanu, R. I., Preda, M. D., Niculescu, A.-G., Vladâcenco, O., Radu, C. I., Grumezescu, A. M., & Teleanu, D. M. (2022, May 4). Current strategies to enhance delivery of drugs across the blood–brain barrier. MDPI. https://www.mdpi.com/1999-4923/14/5/987
18. Trapani, I. (2019, April 9). Adeno-associated viral vectors as a tool for large gene delivery to the retina. Genes. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6523333/
19. U.S. National Library of Medicine. (n.d.). National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/
20. Wang, D., Tai, P. W. L., & Gao, G. (2019, May). Adeno-associated virus vector as a platform for gene therapy delivery. Nature reviews. Drug discovery. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6927556/
21. Wang, Y.-W., Hu, N., & Li, X.-H. (2022, July 1). Genetic and epigenetic regulation of brain organoids. Frontiers in cell and developmental biology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9283755/
22. WR;, N. M. B. W. (n.d.-a). Adeno-associated virus (AAV) as a vector for gene therapy. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy. https://pubmed.ncbi.nlm.nih.gov/28669112/
23. Xue, C., & Greene, E. C. (2021, July). DNA repair pathway choices in CRISPR-Cas9-mediated genome editing. Trends in genetics : TIG. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8187289/