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CRISPR Knockout of HRE Sequences in the C9orf72 Gene to Treat ALS, FTD, and HDL2

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ABSTRACT

Amyotrophic lateral sclerosis (ALS), familial frontotemporal dementia, and phenotypic Huntington syndrome-like disease (HDL2) have in common multiple hexanucleotide repeat expansions (HREs), in the C9orf72 gene. The C9orf72 protein aids in sending and receiving signals in multiple neurons. The C9orf72 protein is found primarily expressed in the brain and specific neurons, especially in the cerebral cortex. In ALS, FTD, and HDL2, the gene has a repeat sequence GGGGCC that is found in many patients with familial neurodegenerative conditions, resulting in the loss of function of many patients' motor neurons. The repeat sequence causes reduced production of the C9orf72 protein, called haploinsufficiency, and simultaneously a gain-of-function mutation by producing toxic dipeptide proteins, and excess RNA foci (RNA toxicity) in the brain. Different gene editing techniques in vitro and in vivo have shown the successful knockout of genes in the brain as well as on the C9orf72 gene and the connection to ALS, FTD, and Huntington-like syndrome in mice. CRISPR is a gene-editing tool used to target HREs. This review will focus on knocking down/out the HREs of the C9orf72 gene using various delivery strategies. Targeting the C9orf72 gene HREs, it would be expected that a decrease in protein aggregations that affect motor neurons, be able to prevent protein haploinsufficiency and prevent RNA toxicity, which are the main factors of these three neurodegenerative diseases. Using gene editing, treatment may be possible for patients with familial ALS, FTD, and HDL2 with the HRE sequences in the C9orf72 gene.

Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder that targets the function of motor neurons throughout the body, both upper (originating in the cerebral cortex, down the spinal cord) and lower (including the spinal cord and various parts of the body) neurons and leads to the loss of motor control (Grad et al., 2017; Hardiman et al., 2017). Most ALS cases occur sporadically (sALS), arising without a clear family history. Familial ALS (fALS) is genetically inherited and is caused by specific mutations in genes such as C9orf72, SOD1, TARDBP, and FUS (Grad et al., 2017; Hardiman et al., 2017). These four genes have been linked to 70% of cases of fALS, and the mutations of the C9orf72 gene have been linked to approximately 39.3% of cases of fALS (Hardiman et al., 2017; Kirby et al., 2016). The Mendelian pattern of inheritance follows autosomal dominant (AD) inheritance; where one copy of the mutated gene, inherited from either parent, is sufficient to cause fALS (Kirby et al., 2016). Autosomal recessive (AR) inheritance is a less common way of inheriting fALS and involves both copies of a specific gene to be affected (Kirby et al., 2016). In some cases, fALS can also follow an X-linked inheritance pattern, with the mutated gene located on the X chromosome (Kirby et al., 2016). Environmental and lifestyle factors have been inconclusively linked to ALS but have no substantial evidence to back up these claims (Hardiman et al., 2017). ALS is seen in individuals with upper motor neuron at disease onset, where lower motor neuron degeneration becomes more apparent in the later stages of the disease (Hardiman et al., 2017). Patients can present with symptoms of muscle weakness and muscle spasticity (Hardiman et al., 2017). Patients who present with lower motor neuron dysfunction have symptoms of cramps, muscle

wasting, and fasciculations, or twitches (Hardiman et al., 2017). Additional symptoms of ALS include progressive dysarthria, asymmetrical presentation of muscles, unexpected weight loss, and apathy among many more symptoms that are specific to each patient (Hardiman et al., 2017). Diagnosing ALS requires the identification of progressive weakness in motor neurons throughout the body; however, this disease can present differently in every patient (Hardiman et al., 2017). This review will focus on the genetically inherited types of ALS, fALS containing mutations linked to the C9orf72 gene.

Frontotemporal Dementia (FTD)

Frontotemporal Dementia (FTD) is categorized as a group of disorders that exhibit progressive deficits in executive function, behavior, and language (Bang et al., 2015). FTD is organized into several main variants: behavioral-variant frontotemporal dementia (bvFTD), non-fluent variant primary progressive aphasia (PPA), and sematic-variant primary progressive aphasia (svPPA) (Bang et al., 2015; Olney et al., 2017). Frontotemporal Lobar Dementia (FTLD) is another subtype of FTD which includes the neurodegenerative process of selective neuron loss and gliosis (Olney et al., 2017). Gliosis is an excess of glial cells that can cause scars in the frontal and temporal lobes, contributing to FTLD (Olney et al., 2017). Some types of FTD can be inherited, familial FTD (fFTD), and are described to result from mutations in MAPT, GRN, and like fALS, the C9orf72 gene (Olney et al., 2017). Around 40% of FTD cases have a family history of neurodegenerative diseases (Olney et al., 2017). FTD mainly follows an AD pattern of inheritance, though AR inheritance is less common (Olney et al., 2017). Like ALS, the majority of cases of FTD are sporadic, meaning caused by unclear family history (Olney et al., 2017). Symptoms of fFTD range from cognitive, behavioral, and language degeneration (Olney et al., 2017). The three types of fFTD are various markers and may present variably. Behavioral FTD (bvFTD) can present as changes in behavior, such as impulsivity, lack of empathy, social disinhibition, repetitive behaviors, reduced hygiene, and poor judgment (Olney et al., 2017). PPA has symptoms categorized under the language impairment of patients, including difficulty with word comprehension or loss of knowledge of words as well as difficulty with grammar and sentence construction (Bang et al., 2015; Olney et al., 2017). FTLD presents with symptoms that include a combination of cognitive, behavioral, and language deficiencies (Olney et al., 2017). Like ALS, this review will focus on familial FTD and the variants falling under this umbrella linked to the mutation in the C9orf72 gene.

Huntington's Disease Like 2 (HDL2)

Huntington disease-like 2 (HDL2) is diagnosed in a patient when Huntington disease (HD) is suspected, but the patient lacks the CAG repeat expansion mutation (Moss et al., 2014). Individuals who are suspected of having HD and show the symptoms, but lack the CAG expansion, are said to have HD-like disorders or HD phenocopy syndromes, referred to as HDL2 (Moss et al., 2014). To date, a small cohort of individuals have been diagnosed with HDL2 (Anderson et al., 2004). HDL2 cannot be phenotypically distinguished from HD, however, genotypically these diseases are different (Anderson et al., 2004). HD is inherited in an autosomal dominant manner, with a 50% chance of passing the mutated gene from an affected parent to their children (Moss et al., 2014). HDL2 is also inherited in an autosomal dominant way, although the mutations are on different chromosomes (Moss et al., 2014) To be diagnosed with HDL2, a positive family history of the disease should be identified, and the phenotypic symptoms and a repeat expansion of CTG trinucleotide repeats in the JPH3 gene, as well as HRES in the C9orf72 gene (Anderson et al., 2004). Both HD and HDL2 include symptoms that affect movement, cognition, and psychiatric well-being (Anderson et al., 2004). One of the distinctive motor symptoms is chorea, marked by involuntary and jerky movements that can affect various parts of the body (Anderson et al., 2004). Individuals with HD or HDL2 may also experience difficulties in sustaining voluntary movements and keeping

specific postures, a condition known as motor persistence (Moss et al., 2014). Cognitive decline is a common feature, encompassing challenges with memory, concentration, and executive functions (Moss et al., 2014). Psychiatric symptoms include mood disturbances such as mood swings, depression, anxiety, and impulsivity (Anderson et al., 2004; Moss et al., 2014). Personality changes, social withdrawal, and psychosis may also manifest (Anderson et al., 2004). As HDL2 progresses, individuals may experience chorea, hypokinesia, dysarthria, and hyperreflexia in the later stages of the disease (Anderson et al., 2004). 1.95% of patients who had the HRE in the C9orf72 gene exhibited the symptoms of HDL2 (Moss et al., 2014). Since the expansion in the C9orf72 gene has been identified as the likely cause of HDL2, treating the HRE will treat this disease as well as be a preventative measure. This review will touch on HDL2 caused by the expansion of the C9orf72 gene.

Gene of Interest C9orf72

The exact function of the C9orf72 protein is not fully understood, but research suggests that it may play a role in various cellular processes, including autophagy and vesicle trafficking (Smeyers et al., 2021). Autophagy is a cellular process responsible for the removal and recycling of damaged or unnecessary cellular components (Gendron & Petrucelli, 2018). Vesicle trafficking involves the transport of vesicles, which are small membrane-bound sacs within cells, crucial for various cellular functions (Gendron & Petrucelli, 2018). The C9orf72 gene is mainly expressed in the central nervous system, particularly in the brain and spinal cord (Smeyers et al., 2021). Within the brain, high levels of C9orf72 expression are seen in various regions, including the frontal cortex, hippocampus, and cerebellum (Smeyers et al., 2021). The gene is notably expressed in neurons (Smeyers et al., 2021). Hexanucleotide repeat expansions (HRE) are found in the noncoding region of the C9orf72 gene, where 400-2,000 repeats of GGGGCC lead to RNA toxicity and C9orf72 protein toxicity as well as C9orf72 protein haploinsufficiency (Kirby et al., 2016). All three of these mechanisms directly contribute to neurodegeneration in patients (Hardiman et al., 2017; Kirby et al., 2016). The HRE's commonly have two gain-of-function outcomes: RNA foci and protein toxicity (Leko et al., 2019). Firstly, the production of abnormal amounts of RNA molecules referred to as RNA foci, accumulate of which is believed to interfere with RNA-binding proteins and cause protein accumulations (Hardiman et al., 2017). These foci can sequester RNA-binding proteins, normal functions and accumulating in the brain (Hardiman et al., 2017). This interference may disrupt the regulation of RNA processing, including splicing and transport, leading to incorrect RNA profiles and protein production (Hardiman et al., 2017). Secondly, dipeptide repeat proteins generated from the HRE trigger neurotoxicity and interfere with nucleocytoplasmic transport (Hardiman et al., 2017). The dipeptide repeats proteins tend to aggregate and form inclusion bodies within cells (Hardiman et al., 2017). These inclusion bodies are dense, insoluble structures that can disrupt normal cellular processes and contribute to dysfunction (Kirby et al., 2016). Despite being in a non-coding part of the gene, the expanded hexanucleotide repeats are transcribed in both directions, forming repetitive RNAs through a nontraditional translation process known as repeat associated non-ATG (RAN) (McEachin et al., 2020). This process generates five distinct arginine-rich dipeptide repeat (DPR) proteins, which have been found to impact the transport of molecules within cells, affecting neuronal survival (McEachin et al., 2020) The HREs in the non-coding region of the gene also leads to a loss of function of C9orf72, resulting in haploinsufficiency (Smeyers et al., 2021). C9orf72 haploinsufficiency refers to a condition where there is a deficiency or reduced expression of the C9orf72 gene, resulting in lower-than-normal levels of the corresponding C9orf72 protein (Shi et al., 2018). This deficiency appears to affect different cellular processes, such as synaptic vesicle recycling, lysosomal accumulation, and autophagy-mediated RNA balance, potentially contributing to neuroinflammation (Kirby et al., 2016; Kortazar-Zubizarreta et al., 2023). Studies suggest that C9orf72 may play a role in synaptic function, and deficiency might impair processes related to synaptic vesicle recycling (Leko et al., 2019). This can impact communication between nerve cells in the brain (Leko et al., 2019). The C9orf72 protein also involves regulating autophagy, the dysfunction of which can contribute to the accumulation of damaged cellular components and protein aggregates, which are all common features in

neurodegenerative diseases (Shi et al., 2018). C9orf72 haploinsufficiency has also been associated with lysosomal dysfunction (Shi et al., 2018). Lysosomes are cellular organelles responsible for breaking down and recycling cellular waste (Shi et al., 2018). Dysfunction in this process can lead to the accumulation of material within cells (Shi et al., 2018). All three neurodegenerative diseases, fALS, fFTD, and HDL2 have been confirmed to be caused by the HRE in the C9orf72 gene (Shi et al., 2018).. CRISPR can be utilized to selectively target and cut off the hexanucleotide repeat expansion in the C9orf72 gene, associated with neurodegenerative diseases, through the precise editing of its DNA sequence using the Cas9 enzyme and guide RNA.

Gene Editing

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a groundbreaking genetic technology that allows the precise editing of DNA in living organisms (Lino et al., 2018; Paul & Montoya, 2020). CRISPR is derived from the adaptive immune system of bacteria and archaea, both use as defense mechanisms against viruses (Lino et al., 2018; Paul & Montoya, 2020). CRISPR works with engineered nucleases, which act as molecular scissors to cut DNA sequences into double-strand breaks (DSBs) at a specific locus in the genome (Lino et al., 2018; Paul & Montoya, 2020). CRISPR-associated protein (Cas) is a nuclease which are in bacteria and archaea and play a crucial role in the prokaryotic adaptive immune system (Bondy-Denomy, 2018). These proteins recognize and cleave the DNA or RNA of invading viruses, and in the context of gene editing, Cas proteins like Cas9 can be engineered to precisely modify specific DNA sequences, enabling targeted genetic alterations the genetic code (Bondy-Denomy, 2018). Cas9 is an RNA-guided endonuclease enzyme that plays a central role in the CRISPR-Cas9 gene-editing system (Bondy-Denomy, 2018). Scientists can design a synthetic guide RNA (gRNA) that directs the Cas9 nuclease to a specific DNA sequence, enabling the cleaving of DSBs (Lino et al., 2018; Paul & Montoya, 2020). Natural repair mechanisms then come into play, allowing researchers to introduce and seal desired changes to the DNA (Lino et al., 2018; Ma et al., 2014; Paul & Montoya, 2020). Briefly, the CRISPR-Cas9 gene-editing process involves several steps; first, researchers identify the target DNA sequence and design a synthetic guide RNA (sgRNA) that matches this sequence (Lino et al., 2018; Ma et al., 2014). The Cas9 protein is synthesized to bind to the sgRNA to form the sgRNA-Cas9 complex that will enter the cell as the endonuclease (Lino et al., 2018). The sgRNA guides Cas9 to the specific location on the DNA at the Protospacer Adjacent Motif (PAM) sequence, where Cas9 induces a double-strand break (Lino et al., 2018; Ma et al., 2014; Paul & Montoya, 2020). The PAM sequence is a short DNA sequence adjacent to the target sequence, which the Cas9 protein recognizes and binds to initiate the process of target DNA recognition and cleavage (Lino et al., 2018; Ma et al., 2014). The PAM sequence also signals where to unwind the DNA sequence 10-12 nucleotides after in a region called the seed sequence (Lino et al., 2018; Ma et al., 2014). The Cas9 protein cleaves the DNA to form a DSB, a type of DNA damage where both strands of the DNA double helix are broken at the same location (Lino et al., 2018; Paul & Montoya, 2020). There are two primary pathways through which cells repair DSBs: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR) (Lino et al., 2018). NHEJ is a rapid and error-prone process that occurs primarily during the G1 phase of the cell cycle (Lino et al., 2018). When a DSB happens, NHEJ components will recognize the broken ends of the DNA, remove any damaged DNA, and later ligate the ends together (Lino et al., 2018). HDR is the second pathway a cell can use to repair the DSB (Lino et al., 2018). Unlike NHEJ, HDR relies on a homologous DNA template such as an exogenous piece of DNA to precisely repair the broken DNA strands (Lino et al., 2018). This homologous template is provided by the scientist externally rather than relying on the sister chromatid for strands (Lino et al., 2018). The process begins with the resection of the broken DNA ends to generate single-stranded DNA overhangs (Lino et al., 2018). Next, homologous pairing with the complementary sequences in the provided template (Lino et al., 2018). DNA synthesis, using the template as a guide, fills in the gap, and the repaired DNA is ligated to complete the process (Lino et al., 2018; Paul & Montoya, 2020). HDR is highly accurate and enables the introduction of specific nucleotide changes, the addition or removal of genetic elements, or the insertion of

entirely new sequences at the targeted genomic locus (Lino et al., 2018; Paul & Montoya, 2020). The choice between NHEJ and HDR, and the success of the repair process, are influenced by various factors, including the cell type, the specific characteristics of the target locus, and the experimental design (Paul & Montoya, 2020). The importance of CRISPR lies in the revolutionary ability to edit genetic material precisely, enabling breakthroughs in genetic research and human disease (Lino et al., 2018; Paul & Montoya, 2020; Ran et al., 2013). CRISPR technology holds the potential to address the Hexanucleotide Repeat Expansions (HREs) associated with the C9orf72 gene, a key genetic factor in fALS, fFTD, and HDL2. Researchers can design guide RNA (gRNA) sequences specific to the expanded G4C2 repeat in the C9orf72 gene and with the CAS9 enzyme, deliver it using viral vectors like adeno-associated viruses (AAVs) or lentiviral vectors (LVs). Once inside the cells, the gRNA guides Cas9 to the target site, inducing double-strand breaks (DSBs). The natural repair mechanisms, particularly homology-directed repair (HDR) in the presence of a repair template, can then replace the expanded repeat with a normal genetic sequence. Through this process, CRISPR can facilitate the excision of HREs in the C9orf72 gene, potentially stopping the formation of toxic RNA foci and polypeptides associated with fALS and fFTD or returning proper protein production and restoring the haploinsufficiency. Although promising, the translation of CRISPR-based therapies for gene editing into clinical applications requires thorough testing.

Another way to target HRE in the C9orf72 gene is CRISPR interference. CRISPR interference, often abbreviated as CRISPRi, is a technique that utilizes the CRISPR-Cas9 system to interfere with the expression of specific genes without making permanent changes to the underlying DNA sequence (Larson et al., 2013; Pickles et al., 2023). In CRISPRi, the Cas9 protein is modified to be enzymatically inactive (known as deadCas9 or dCas9), meaning it cannot cut the DNA strands like the active Cas9 used in traditional CRISPR gene editing (Larson et al., 2013). The CRISPRi system works by guiding the dCas9 protein to a targeted gene using a specially designed guide RNA (gRNA). Instead of introducing double-strand breaks and triggering the cellular DNA repair machinery, as in traditional CRISPR gene editing, CRISPRi interferes with gene expression at the transcriptional level by obstructing the normal transcription process, leading to the suppression of gene expression (Larson et al., 2013; Pickles et al., 2023). CRISPR interference provides a reversible and tunable method to regulate gene activity. It is particularly useful for studying gene function, as researchers can selectively silence genes of interest like the C9orf72 gene (Larson et al., 2013; Pickles et al., 2023). Although promising, the translation of CRISPR-based therapies for gene editing into clinical applications requires thorough testing. CRISPR and CRISPRi are both gene editing tools that could be administered to excise the C9orf72 HREs successfully.

In Vitro Research Conducted

In the realm of in vitro research, investigations were undertaken to explore and understand the dynamics of gene editing through CRISPR technology, in the context of the HREs associated with the C9orf72 gene. The use of induced pluripotent stem cells (iPSCs) is valuable to in vitro research as cells can be derived from patients (Meijboom et al., 2022). In one study performed at the University of Massachusetts Medical School, iPSCs were differentiated into motor neurons and removed the C9orf72 HRE successfully (Meijboom et al., 2022). Both human-derived and murine-derived iPSCs were used to determine whether the removal of the HREs resulted in a dramatic reduction of the RNA foci and dipeptide proteins causing toxicity in the neurons (Meijboom et al., 2022). First, the scientists designed gRNAs flanking the outsides of the repeat HRE (Meijboom et al., 2022). The HRE is in an intronic region of the DNA, which was challenging to design as the exon was only 35 bp from the intronic region (Meijboom et al., 2022). Alternative splicing is used to produce three different mRNA versions of the C9orf72 gene (Meijboom et al., 2022). In HEK293T cells given AAVs, Sanger sequencing observed that gRNA combinations of gRNA 2,3 as well as gRNA 2,4 successfully had over 80% excision efficiency (Meijboom et al., 2022). With a small percentage of nucleotide insertions and deletions, the overall

excision of the HRE in the HEK293T cells indicated successful editing (Meijboom et al., 2022). These small insertions that occurred within the intron did not alter the amino acid composition of the C9orfF72 protein and were described to be unlikely to impact transcription levels (Meijboom et al., 2022). They then identified off-target sequences with less than three new mutations to different sequences. For coding sequences, 2 mismatch targets for gRNA 2, 3 were found, and three mismatch targets for gRNA 2, 4 (Meijboom et al., 2022). A small number of mismatch targets indicated the overall specificity of the genome editing in HEK293T cells, leading to researchers moving onto mouse primary cortical neurons (Meijboom et al., 2022).

The next step taken was using the same process containing the Cas9 plasmid and gRNA combinations of 2,3 and 2,4 and transducing them using AAV's into mouse primary cortical neurons in vitro. These murine neurons carry about 600 repeats (Meijboom et al., 2022). To validate results, the humanized mouse model of C9-500 was used (Meijboom et al., 2022). The C9-500 mouse does not express CAS-9 endogenously and contains one human copy of the C9orf72 gene with 500 repeats (Meijboom et al., 2022). Primary cortical neurons were harvested at embryonic day 15 and transduced with AAV9 expressing gRNA 2, 3 or gRNA 2, 4, at day 4 (Meijboom et al., 2022). In these experiments, a gRNA targeting the Rosa locus was encapsulated in AAV9 and employed as a control (Meijboom et al., 2022). Following PCR, there was no observed DNA amplification in untreated primary neurons or those treated with AAV9-Rosa (Meijboom et al., 2022). Conversely, template DNA isolated from neurons transduced with AAV9-gRNA 2,3 and AAV9-gRNA 2,4 yielded a band at approximately 320 bps, indicating effective excision of the HRE mutation in neurons (Meijboom et al., 2022). PCR results showed success (Meijboom et al., 2022). Again, to evaluate editing efficiency in primary neurons in a similar mouse model, AAV vectors were employed to introduce Cas9 and gRNA expression in primary neurons obtained from heterozygous BAC111 embryos with HRE's although these embryos endogenously contained Cas9 (Meijboom et al., 2022). The BAC111 mouse neuron contains 6–8 copies of exon 1–6 of the human C9ORF72 gene, including 600 HREs, and mirrors the molecular pathology of C9-ALS/FTD by developing RNA foci and toxic dipeptides (Meijboom et al., 2022). However, it differs from C9-ALS/FTD patients, who possess only one copy of the C9orf72 gene with a pathological expansion (Meijboom et al., 2022). In contrast, neurons treated with buffer or AAV9-Rosa did not exhibit such efficiency (Meijboom et al., 2022). Though the endogenous expression of Cas9 is not seen in humans, the success of the experiment in BAC111 embryos show the possibility of future treatment (Meijboom et al., 2022). In these two experiments, it is seen how the successful excision of the C9orf72 HRE is achieved, a step towards for human treatment in the future.

In Vivo Research Conducted

Successful attempts at in vitro research on mouse neurons and HEK293T cells led researchers to progress to live mice for in vivo work. Research conducted in vivo has been significant in showing that gene editing can be done in mouse brains. The mouse models previously discussed, including BAC111, have drawbacks to conducting research regarding ALS and FTD due to endogenously expressing Cas9 (Meijboom et al., 2022). However, two other mouse models, C9-500 and BAC112, were also used in this ALS/FTD study because they express multiple copies of the expanded C9orf72 gene without Cas9 expression (Meijboom et al., 2022). These BAC112 mice were used to show the effectiveness of gene editing through a dual vector system and had around 550 HREs in their brain (Meijboom et al., 2022). Although this mouse model does not exhibit the progressive neurodegeneration characteristic of ALS and FTD patients, it shows gain-of-function pathological features, including RNA foci and RAN translation leading to the presence of toxic polypeptides (Meijboom et al., 2022). In experiments involving young adult BAC112 mice aged 2–3 months, microinjections of AAV9-gRNA2,3 or AAV9-gRNA2,4 were delivered into the brains of the mouse models (Meijboom et al., 2022). BAC112, compared to the C9-500 mouse model used in the study additionally received AAV9-Cas9 (Meijboom et al., 2022). After eight weeks, the mice were sacrificed, and striatal tissues from both brain hemispheres were collected to assess gene-editing events (Meijboom et al., 2022). Results analyzed those primary neurons that had the presence of CRISPR-edited

DNA, excluding templates with the long GC-rich HRE sequences still intact (Meijboom et al., 2022). A significant decrease in the toxic dipeptide proteins was discovered, a 60% reduction in the C9-500 mouse (Meijboom et al., 2022). These findings confirm the successful in vivo excision of HRE in the BAC112 mouse model of C9orf72 ALS and FTD (Meijboom et al., 2022). After confirming the excision of the HRE, researchers investigated whether this process mitigated the toxic effects associated with gain-of-function pathologies in vivo (Meijboom et al., 2022). Poly-GR is a commonly used biomarker to see overall poly-dipeptide production (Meijboom et al., 2022). The results revealed a significant and substantial reduction in poly-GR levels following treatment with AAV9-gRNA2,3 and gRNA2,4 in the striatum of C9-500 mouse model by about 50% (Meijboom et al., 2022). Subsequently, researchers assessed the proportion of nuclei exhibiting HRE RNA foci, which causes RNA toxicity in neurons (Meijboom et al., 2022). Reductions were observed in BAC111/Cas9 mice, where the number of striatal nuclei with foci decreased from 44% in controls to 17–18% in treated animals (Meijboom et al., 2022). The successful excision of the HRE and knockdown of RNA foci in an in vivo experiment shows the success rate that CRISPR has removing the HRE of the C9orf72 gene (Meijboom et al., 2022). Since this experiment has been conducted successfully in mice, it is a viable idea to be conducted in different mammals such as the monkey.

CRISPRi Research Conducted

CRISPRi is another delivery method of CRISPR machinery. It can be used as a tool to target the HREs in C9orf72 gene. systeCRISPRi is a completely different strategy of how to edit genes, one that is viable and has been shown to be successful. A study by Pickles et al utilized a novel CRISPRi knockin mouse line, to investigate gene function in ALS and FTD (Pickles et al., 2023a). This group targeted Stmn2, which is found to be decreased in FTS/ALS patients (Pickles et al., 2023b). Stmn2, a microtubule-binding protein that is highly expressed in neurons, plays a major role in axonal growth and maintenance (Pickles et al., 2023b). The splicing of Stmn2 is under the regulation of TDP-43 (Pickles et al., 2023b). Thus, when there is a TDP-43 deficiency, an unusual exon is included in the RNA (Pickles et al., 2023b). This leads to the generation of a non-functional truncated Stmn2 variant and the loss of full-length Stmn2 in ALS and FTD cases (Pickles et al., 2023b). The CRISPRi machinery was incorporated into the Rosa26 locus, expressing dCas9 fused with the KRAB transcriptional repressor domain (Pickles et al., 2023a). AAV was utilized to deliver sgRNA, directing the CRISPRi machinery to the transcription start site of Stmn2 (Pickles et al., 2023a). Four sgRNAs targeting Stmn2 from a CRISPRi library of the entire mouse genome were tested (Pickles et al., 2023a). Compared to CRISPRi mice injected with enhanced Green Fluorescent Protein or non-transgenic mice (NT, lacking the CRISPRi machinery), there was a significant knockdown of the Stmn2 protein (Pickles et al., 2023a). Stmn2 protein knockdown ranged from 40% to 75%, showing successful results (Pickles et al., 2023a). A successful protein knockdown has been achieved by the CRISPRi complex by targeting the Stmn2 protein, therefore in theory, this approach could be used to target the excess protein production caused by HREs of the C9orf72 gene as a treatment or preventative measure against FTD and ALS.

Delivery Mechanisms and the Blood Brain Barrier

To deliver the elements of CRISPR to target HREs, delivery mechanisms need to be identified, like the dual gRNA, lentiviral vectors, adeno-associated viruses, microinjections, and lipid nanoparticles. An approach to successfully knocking out the repeat sequences in DNA is a dual gRNA approach. Two guide RNAs will bind and cleave complementary to opposite DNA strands of the target site (Piao et al., 2022). This method enhances the accuracy and effectiveness of CRISPR-mediated gene editing, enabling modifications of multiple genomic locations in a single experiment called double nicking (Piao et al., 2022). The double-nicking approach provides

several notable benefits because it only introduces a single-strand break into the section of DNA, not a DSB (Piao et al., 2022). Firstly, it enhances the specificity of the gene-editing process, reducing the likelihood of off-target effects compared to single guide RNA approaches (Piao et al., 2022). Additionally, the strategy promotes the use of the HDR pathway, allowing for more precise modifications with heightened efficiency (Piao et al., 2022). In fact, Ran et. all used double nicking to successfully promote HDR in cells (Ran et al., 2013). By inducing nicks instead of double-strand breaks, the approach minimizes reliance on the error-prone NHEJ repair pathway (Piao et al., 2022). Inserting a double guide RNA into the brain to knockdown the HRE in the C9of72 gene is a possible delivery mechanism of CRISPR.

Lentiviral vectors (LVs) are a subgroup of retroviruses known for their ability to infect both dividing and non-dividing cells (Dong & Kantor, 2021). Lentiviral vectors are employed for RNA interference (RNAi) against mRNA or gene knockout by incorporating short hairpin RNA (shRNA) or dual guide RNA sequences designed to target specific genes or mRNAs of interest (Dong & Kantor, 2021). These sequences with other necessary elements are integrated into the genetic material of a lentiviral vector (Dong & Kantor, 2021). After packaging the vector into lentiviral particles, these are used to infect target cells, facilitating the delivery and integration of the dual gRNA or shRNA into the host cell genome (Dong & Kantor, 2021; Perry & Rayat, 2021). Once integrated, the lentiviral vector promotes the expression of shRNA or gRNA for RNAi or CRISPR-Cas9 gene editing (Dong & Kantor, 2021; Moore et al., 2010). shRNA is an artificial RNA molecule that is structured as a stem-loop, with the stem usually being 19 to 22 base pairs in length (Dong & Kantor, 2021; Perry & Rayat, 2021). It mimics the structure of natural precursor microRNA (pre-miRNA) or small interfering RNA (siRNA) (Dong & Kantor, 2021). shRNA can be inserted into a lentiviral vector under the control of a promoter, which is then transcribed and processed within cells to form a short double-stranded RNA (Dong & Kantor, 2021). The guide strand of the processed shRNA, loaded onto the RNA-induced silencing complex (RISC), binds to the target gene mRNA, leading to degradation or inhibiting translation (Dong & Kantor, 2021; Perry & Rayat, 2021). shRNA is a valuable tool to use in RNA interference thus to study biology and human disease. CRISPR can be integrated into a lentiviral vector to be delivered to the brain to target the C9orf72 HRE.

Adeno-associated viruses (AAVs) are small, non-enveloped viruses that have become pivotal tools in genetic engineering and gene therapy (Naso et al., 2017). AAVs are known to have a nonpathogenic nature and depend on helper viruses for replication (Naso et al., 2017). The AAV genome consists of a single-stranded DNA molecule flanked by inverted terminal repeats (ITRs) (Moore et al., 2010). To package AAVs with dual gRNAs or shRNAs, a series of steps are followed (Dong & Kantor, 2021; Moore et al., 2010). Initially, specific, and effective gRNA or shRNA sequences targeting the gene or genes of interest are designed, and these sequences are cloned into an AAV vector containing expression elements such as promoters and regulatory elements (Naso et al., 2017). The AAV vector, along with helper plasmids expressing Rep and Cap genes necessary for replication and capsid formation, is transfected into packaging cells, through transient transfection or stable packaging cell lines (Naso et al., 2017). Then, cells produce AAV particles that contain the dual gRNA or shRNA constructs (Naso et al., 2017). Finally, the produced AAV particles are harvested, purified, and gene therapy or research purposes, allowing clinicians or researchers respectively to take advantage of AAV's to efficiently deliver genetic material to target cells (Naso et al., 2017). There is a possibility that AAVs can be used to deliver CRISPR to the brain and target the HRE in the C9orf72 gene. However, overcoming the blood brain barrier remains an obstacle to consider.

The blood-brain barrier (BBB) is a critical physiological barrier that separates the circulating blood from the central nervous system (CNS), consisting of the brain and spinal cord (Alahmari, 2021). The primary function of the BBB is to tightly regulate the passage of substances between the blood and the brain's extracellular fluid, maintaining a stable internal environment needed for proper neural function (Alahmari, 2021). The BBB is primarily composed of endothelial cells lining the cerebral blood vessels. These endothelial cells are interconnected by tight junctions, creating a physical barrier that restricts the free diffusion of molecules between cells (Alahmari, 2021). The tight junctions seal the intercellular clefts, restricting diffusion and rendering

the BBB impermeable to large or hydrophilic substances (Daneman & Prat, 2015). The membrane surrounding the endothelial cells provides structural support, and additional cells such as pericytes and astrocytes contribute to the goal to preserve the chemical components of the brain to keep it functioning normally (Alahmari, 2021). The semi-permeability of the BBB is crucial for regulating the entry of substances into the brain (Alahmari, 2021). It allows the passage of essential nutrients, oxygen, and small lipophilic molecules, while restricting the entry of larger molecules, pathogens, and most therapeutic agents (Alahmari, 2021; Daneman & Prat, 2015). By limiting the entry of pathogens, toxins, and large molecules, the BBB acts as a protective shield for the delicate neural environment (Alahmari, 2021; Daneman & Prat, 2015). This defense mechanism prevents infections and shields neurons from potential damage caused by circulating toxins (Alahmari, 2021; Daneman & Prat, 2015). Specialized transport mechanisms facilitate the regulated entry of essential substances (Alahmari, 2021; Daneman & Prat, 2015). These include carrier-mediated transport, receptor-mediated transcytosis, and active transport systems that ensure the delivery of nutrients and signaling molecules to the brain (Alahmari, 2021; Daneman & Prat, 2015). While the BBB is crucial for maintaining neural health, it poses significant challenges in delivering therapeutic agents, particularly those aimed at treating neurological disorders (Daneman & Prat, 2015). Large and complex therapeutic agents, such as proteins and gene-editing tools like CRISPR, face difficulties in crossing the BBB due to size limitations and selective permeability (Alahmari, 2021). Additionally, utilizing viral vectors or nanoparticles as delivery vehicles for therapeutics introduces challenges related to stability during transit through the BBB (Daneman & Prat, 2015; Zou et al., 2022). Maintaining the structure of the delivery vehicle is crucial for successful therapeutic delivery (Alahmari, 2021). The introduction of foreign substances into the CNS also raises concerns about potential immunogenicity and safety. Immune responses triggered by therapeutic agents may lead to inflammation and unintended side effects by the delivery of a foreign vector through the BBB (Alahmari, 2021; Daneman & Prat, 2015). The BBB poses a potentially difficult obstacle in sending different CRISPR-associated therapies into the brain. To overcome the challenge of delivering CRISPR through the BBB, the use of nanoparticles and microinjections has been proven to be efficient and trustworthy (Gonzalez-Perez et al., 2010; Scioli Montoto et al., 2020).

To deliver CRISPR based technologies like LVs, AAVs, and dual guide RNA, lipid nanoparticles are successful (Scioli Montoto et al., 2020). Solid lipid nanoparticles (SLN) are minute, spherical particles composed of solid lipids at room temperature, resembling perfect crystal lipid matrices capable of accommodating drugs or other molecules between fatty acid chains (Scioli Montoto et al., 2020). However, findings indicate that this description is not always applicable, as disc-like shapes or flat geometries of the SLNs have also been observed (Scioli Montoto et al., 2020). The structure allows loaded drugs to be predominantly attached to the carrier matrix surface rather than embedded into the solid core (Scioli Montoto et al., 2020). The second generation of lipid nanoparticles, known as nanostructured lipid carriers (NLC), is more effective at carrying drugs (Scioli Montoto et al., 2020). Being an advanced version of SLN, NLC incorporates liquid lipids (oils) at room temperature, causing structural rearrangements in the matrix (Scioli Montoto et al., 2020). This innovation allowed the release of the incorporated drug over time, dispensing it into the environment of choice (Scioli Montoto et al., 2020). The addition of oils in NLC reduces the crystalline degree of the lipid core in SLN, preventing drug expulsion, enhancing drug loading capacity, as well as enhancing physical and chemical long-term stability (Scioli Montoto et al., 2020). Both SLNs and NLCs in aqueous environments are hydrophobic, resulting in low or no hydration (Scioli Montoto et al., 2020). They cannot spontaneously dissolve or disperse in water, requiring energy input for dispersion preparation (Scioli Montoto et al., 2020). Various synthesis methods involve energy-providing steps such as ultrasonic waves, high pressures, high-speed homogenization, or microwaves to release the drug of choice (Scioli Montoto et al., 2020). One feature of the BBB is the efflux transporters from the ATP-binding cassette (ABC) superfamily (Scioli Montoto et al., 2020). These transmembrane proteins, located at the luminal membrane of endothelial cells that line the BBB, are responsible for pumping toxic substrates out of the intracellular space (Scioli Montoto et al., 2020). One of the notable consequences of these transporters is the occurrence of multi-drug resistance (Scioli Montoto et al., 2020). Encapsulating drugs

into SLNs and NLCs have been proposed as a strategy to avoid these transporters to allow for delivery of drugs (Scioli Montoto et al., 2020). The transport of substances across the BBB can occur through four main mechanisms: paracellular diffusion, which is reserved for small water-soluble substances; transcellular diffusion, more relevant for lipophilic molecules; carrier-facilitated diffusion and active transport, responsible for the passage of specific molecules; and endocytosis, reported for the passage of peptides and proteins like insulin and insulin-like growth factors (Scioli Montoto et al., 2020). Once inside endothelial cells, nanoparticles (NP) can undergo exocytosis to the other side (transcytosis) or be released into the intracellular space, facilitating access to the CNS (Scioli Montoto et al., 2020). Given that endocytosis of NPs by BBB endothelial cells is predominantly mediated by receptors and has been shown to be highly efficacious, efforts have been made to enhance drug accessibility through surface functionalization of SLNs (Scioli Montoto et al., 2020). Delivering CRISPR through these SLNs and NLCs is a promising approach to getting through the BBB and targeting the HREs in the C9orf72.

Another way to deliver CRISPR through the BBB is through microinjections directly into the brain (Gonzalez-Perez et al., 2010). Administering microinjections is a critical procedure for introducing drugs, viral vectors, or cell transplants into the brain (Gonzalez-Perez et al., 2010). However, a potential concern is potential brain lesions caused by the needle impact and insertion, which was found to occur in mouse brains (Gonzalez-Perez et al., 2010). This issue is heightened due to the small size of the mouse brain and the occasional need for multiple injections. In this study a method for using glass capillary needles with a 50-µm lumen is introduced, reducing brain damage and enabling precise targeting within the rodent brain (Gonzalez-Perez et al., 2010). This allows for the delivery of small volumes (ranging from 20 to 100 nl), decreases the risk of bleeding, and minimizes the passive diffusion of drugs into the brain parenchyma (Gonzalez-Perez et al., 2010). The successful experiment allows there to be a possibility of delivering CRISPR through these microinjections that can be used to target the HRE in the C9orf72 gene. Microinjections using a glass capillary tube have significantly improved injection techniques, enabling deep brain targeting with minimal collateral damage in small rodents. In the future there may be the possibility of delivering CRISPR directly into the human brain, specifically targeting the C9orf72 gene HRE.

Discussion

ALS, FTD, and HDL2 are neurodegenerative disorders characterized by progressive symptoms that affect a significant portion of the aging population, despite much ongoing research on these puzzling diseases. This review focused on three different treatments and the possible delivery mechanism of CRISPR into the brain passing through the BBB. In summation, the knockdown or knockout of HREs contained in the intronic sequence of the C9orf72 gene in the brain can be targeted to treat or prevent fALS, fFTD, and HDL2. Lentiviral vectors and adeno-associated vectors both can be used to target the HRE and knock down expression. Both LVs and AAVs offer valuable tools for investigating gene function and potential therapeutic applications by modulating gene expression levels. Both LVs and AAVs have shown promise in the knockdown of the C9orf72 gene in vivo and in vitro (Meijboom et al., 2022). In another approach, dual gRNA strategy enhances the efficiency of gene disruption, resulting in a more robust impact on gene expression. These approaches will eventually be able to be delivered through lipid nanoparticles to avoid the technicalities of the BBB, a selectively permeable barrier that guards the brain. Targeting the C9orf72 gene HRE can treat ALS, FTD, and HDL2 as the removal of it would treat these three diseases.

Limitations

CRISPR-based gene editing holds immense potential for advancing our understanding of neurobiology and developing innovative therapies for various neurological disorders. However, its application in the brain is accompanied by significant challenges and limitations that necessitate careful consideration and ongoing research. One of the primary challenges is the delivery of CRISPR components to the brain, given the existence of the blood-brain barrier (BBB). This protective barrier limits the passage of foreign substances, including gene-editing tools, from the bloodstream into the brain (Alahmari, 2021; Daneman & Prat, 2015; Zou et al., 2022). Furthermore, the issue of off-target effects remains a significant concern. CRISPR systems, while highly precise, may occasionally induce unintended genetic modifications at locations other than the target (Guo et al., 2023). The C9orf72 gene is expressed throughout the body and is present in many tissues, reflecting its broad expression profile throughout the body (Guo et al., 2023). In the intricate environment of the brain, where precise neural circuits govern complex functions, off-target effects could have unpredictable and potentially harmful consequences. Achieving a higher degree of specificity and minimizing off-target effects is a crucial area of ongoing research to enhance the safety and accuracy of CRISPR applications in the brain. Ensuring the precision of CRISPR editing in the brain is particularly challenging due to the complexity of neural networks and the multitude of cell types. The consequences of unintended genetic modifications in neurons or glial cells could disrupt normal brain functions and contribute to unforeseen side effects. Addressing this challenge requires advancements in CRISPR technology, such as the development of more specific Cas proteins or improved guide RNA design, to enhance the precision of gene editing within the intricate neural landscape. Additionally, the potential immune response triggered by the introduction of CRISPR components into the brain is another critical consideration. Immune reactions can lead to inflammation and tissue damage, posing risks to the delicate neural environment (Guo et al., 2023). Regarding delivering the drug successfully, long-term effects represent another aspect of the challenge. The durability and stability of CRISPR-mediated genetic modifications in the brain over an extended period are not fully understood. Comprehensive monitoring and assessment of the long-term consequences are essential to evaluate the safety and efficacy of CRISPR-based interventions in the dynamic and evolving neural landscape.

Future Directions

The future directions of delivering CRISPR into the brain hold promising avenues for the knockdown or knockout of the HRE in the C9orf72 gene and expanding to include other brain or motor neuron diseases. Researchers are actively exploring innovative delivery systems, such as enhanced viral vectors, to overcome the challenges posed by the blood-brain barrier (BBB) across diverse mammalian species (Guo et al., 2023). Refinements in viral vector engineering, including the use of adeno-associated viruses (AAVs) with improved tropism for neural tissues, are anticipated to enhance the precision and efficiency of CRISPR delivery. Additionally, the exploration of non-viral delivery methods, such as advanced nanoparticle formulations and focused ultrasound techniques, holds potential for achieving non-invasive and targeted delivery across various mammalian brains. Tailoring delivery systems to accommodate species-specific neuroanatomy and physiological differences will be crucial for translating CRISPR-based interventions into effective and safe therapeutic approaches for ALD, FTD, and HDL2 across diverse mammalian models. The evolving CRISPR delivery research will shape the future of precision medicine treatments of these 3 neurological conditions and allow us to study the effects through the minds of smaller mammals.

Germline and somatic CRISPR editing represent two different strategies of genetic modification. Germline editing targets reproductive cells or embryos, introducing heritable changes with the potential to eradicate genetic diseases across generations (Ormond et al., 2017). However, this approach has many complexities, including concerns about unforeseen consequences. Somatic editing focuses on non-reproductive cells, aiming to treat or cure individuals by correcting specific genetic abnormalities without impacting future generations (Ormond et al., 2017). Somatic editing holds significant therapeutic promise in the immediate future; and studies

have already been published that successfully knockout and knockdown the HRE in the C9orf72 gene (Meijboom et al., 2022). In the future, it is a possibility that HREs can be targeted in utero, as it has been done successfully in a mouse model (Shinmyo et al., 2016). As new research emerges, such technology could potentially be used to prevent disease in humans.

CRISPR-based gene knockdown in the brain offers a promising avenue for the treatment of various neurological conditions. By precisely reducing the expression of specific genes associated with additional neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington like diseases, CRISPR technology holds the potential to slow, halt, or even reverse disease progression. In genetic cases, where specific mutations contribute to abnormal neuronal activity, CRISPR knockdown can be applied to modulate the expression of implicated genes. The ability to achieve targeted and localized gene knockdown in the brain through CRISPR technologies represents a groundbreaking approach for developing precise therapeutic strategies, paving the way for innovative treatments to the molecular intricacies of neurological diseases. As research continues in the field of CRISPR, many different possibilities and treatments can be uncovered.

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