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Special Edition: Highlights of 2023

Expression of Single mRNA Constructs Encoding Both CRISPR-CAS9 Protein and Guide RNAs for Future Gene Therapy Applications

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The Columbia Undergraduate Science Journal (CUSJ) was founded in 2006 by students who were passionate about showcasing undergraduate excellence in scientific research. Since then, CUSJ has remained Columbia's premier publication for original scientific research and scholarly reviews, and is managed by an editorial board of undergraduates with a vast scope of interests across all disciplines. The editorial board also manages the Columbia Junior Science Journal (CJSJ), a publication designed to introduce high school students to research and Columbia Scientist, a publication aimed at increasing scientific engagement and thought at all academic levels. In addition to our publications, the CUSJ team is dedicated to fostering the scientific community, both within Columbia and in the surrounding Morningside Heights and Harlem communities. To this end, the board frequently plans outreach and networking events relevant to young and early career scientists, including an annual Research Symposium poster session each spring.

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Letter from the Editor-in-Chief

Dear Readers

It is with great pleasure that the CUSJ team presents you with the 17th volume of the Columbia Under-graduate Science Journal. Since 2006, CUSJ has worked to elevate scientific journalism among under-graduates and provide a forum for showcasing their achievements.

To honor our journal's 18-year legacy, we took a step back to conduct a major refresh of editorial commit-tee members, policies, and practices. We pride ourselves on following a strict code of ethics, a rigorous peer review system, and an inclusive author support program. Maintaining these standards requires annual updates to our practices, allowing us to publish more works and provide our authors effective sup-port. We appreciate your patience with us this year, and we look forward to offering our dear readers and student researchers a better journal each year. It is imperative that we put 100% of our best efforts into editing and publishing exceptional work, despite the committee improvements we spent extra time work-ing on this year. Therefore, we thought it was best to publish a short special edition for the 2023 Columbia Undergraduate Science Journal, solely featuring the strongest submissions from the year.

On behalf of the entire editorial team, I'd like to thank every student who submitted their hard work. The Columbia Undergraduate Science Journal is a space to celebrate and promote budding scientists like all of you, and we're looking forward to reading more of your work in the future. I'd also like to give a special congratulations to our published authors. Your innovative ideas and thoughtful experiments are what keep our journal at the forefront of undergraduate scientific discourse, inspiring and informing both peers and professionals alike.

I want to give a special shout-out to the CUSJ editorial committee. I onboarded a lot of new members this year, and they did a phenomenal job editing their first journal. I'd also like to thank the club's other Editors-In-Chief on our e-board for supporting me as I trained new members and worked on revamping our editing and publication process for the new year. I couldn't have done it without your support. Finally, I extend my gratitude to our Faculty Advisory Board and the advisors at Columbia Libraries, whose assis-tance has been instrumental in enabling us to deliver research of the utmost quality.

It was an honor serving as the Editor-In-Chief for the 2022-2023 edition of CUSJ, and I am looking for-ward to coming back stronger with the 2023-2024 edition.

Thank you for reading CUSJ!
Sincerely,
Kynnedy Simone Smith
Editor-In-Chief, Columbia Undergraduate Science Journal

CUSJ
COLUMBIA UNDERGRADUATE SCIENCE JOURNAL

Table of Contents

Administrative

Selected Works

CUSJ
COLUMBIA UNDERGRADUATE SCIENCE JOURNAL

Masthead

Editors

Faculty Advisory Board

Expression of Single mRNA Constructs Encoding Both CRISPR-CAS9 Protein and Guide RNAs for Future Gene Therapy Applications

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KEYWORDS: CRISPR-Cas9, Gene Editing, mRNA

ABSTRACT: The basis of many life-threatening diseases is disruption in key genes. In many cases, repairing these disruptions can prevent or reverse disease. The development of CRISPR-Cas9 technology, which consists of Cas9 nuclease directed to specific genomic locations by guide RNA (gRNA), has significantly progressed in the past decade and has shown signs of promise for treating diseases such as Alzheimer's and cystic fibrosis. One integral issue of gene editing therapy is the method and effectiveness of delivery. Current approaches such as lentiviral and adeno-associated virus vectors suffer from either stable, constant expression of CRISPR components that causes unintended gene editing or an inability to efficiently carry large cargoes such as two independent genes: Cas9 and guide RNA. To begin to bypass these cargo limitations, we created a CRISPR-Cas9 mRNA structure that encompasses all of the necessary components for gene editing on a single RNA. These constructs consist of a promoter, followed by a Cas9 open reading frame, a triplex region from *MALAT1* that protects the Cas9 open reading frame, and then either 1, 2, or 4 gRNAs that target specific reporters, with each gRNA between two self-cleaving ribozyme sequences. These constructs successfully drove Cas9 editing of two distinct reporters in human cells and thus open the door for many more experiments such as incorporation into various delivery constructs to further develop this technology for gene editing therapy.

INTRODUCTION

CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9) gene editing technology has been shown to be a promising solution to genetic diseases. Cas9 proteins are naturally found in *Streptococcus thermophilus* as a defense system against invading viruses, but now scientists use this system to perform gene editing in many organisms.[1] In the CRISPR system, guide RNAs bind to a specific region of DNA as well as Cas9 nuclease, guiding Cas9 to the targeted DNA region to cause double-stranded breaks in an organism's genome. The cell's own non-homologous end joining DNA repair system is used to repair the break made by Cas9 which induces changes to targeted sequences in the genome (Figure 1).[2] This system has been extensively researched and expanded upon for the past decade, and more systems have been developed that use Cas9 as well such as base and prime editors. In these systems, various proteins have been fused to nuclease-deficient Cas9 to modify the genome in several different ways.

The advent of base editing technology grants the ability to perform specific types of point mutations to nucleotides. The first effective iteration of this technology, a cytosine base editor, arose in 2016 where Komor et al. was able to fuse rat derived deaminase protein to the amino terminal end of an inactive Cas9 protein.[3] Instead of causing double stranded breaks, this editor is able to deaminate cytosine to uracil. Since DNA replication machinery does not recognize uracil, the replication results in a C-G to T-A mutation.[4] Through further research, this editing system was able to become more specific and effective. An adenine base editor was also developed using a similar method to the cytosine base editor.[4] Like Cas9, cytosine and adenine base editors can cause targeted mutations, however these base editors are more specific due to the ability to specify the point mutation. In a gene therapy context, adenine base editors are being used to mutate premature stop codons, which are thought to cause faulty gene expression in up to a third of genetic diseases.[5]

Despite the incredible capabilities of base editors, they are unable to perform more than specific point mutations. However, another Cas9 related system, prime editors, can perform any type of mutations that can be encoded in a gRNA.[4] Simply speaking, these systems are created by fusing a reverse transcriptase to an inactive Cas9 protein. A prime editing guide RNA then directs targeted transition and transversion mutations.[4] Although there is great potential for this system, it still requires much more research to improve its efficacy and specificity. Other applications include gRNA targeted modulation of transcription or epigenetic marks by fusing transcriptional or epigenetic regulators to inactive Cas9.[6] These methods can be used to up or downregulate genes like those related to cancer, therefore showing a lot of promise for therapeutic uses.[4]

In several mouse models, researchers have been able to reduce the severity of

CRISPR-Cas9 Gene Editing
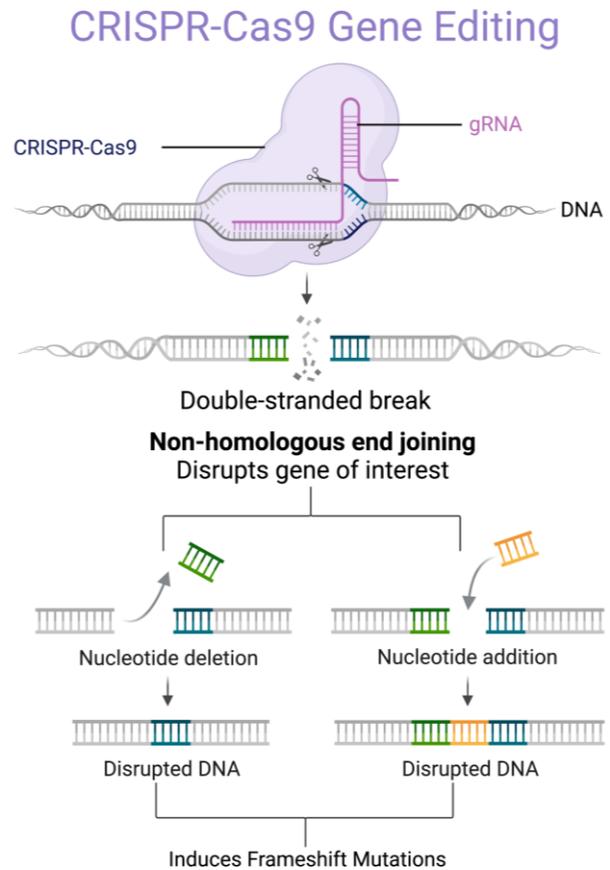
Figure 1: Illustration of how CRISPR-Cas9 performs gene editing.

neurodegenerative diseases by editing genes with CRISPR.[7] Recent studies have demonstrated Cas9 to be promising in decreasing the effects of Alzheimer's and Parkinson's disease through the mutation of the *APP* and *LRRK2* gene respectively.[7] A study from Yang et al. has shown that CRISPR technology can be used to ameliorate the symptoms of Huntington's disease in mouse models through mutations in the *HTT* gene.[8] In addition, clinical trials for CRISPR related therapies have already begun to treat cystic fibrosis by mutating the *CFTR* gene.[9] As a result, CRISPR, while still being heavily researched and developed, shows extremely promising capabilities for mitigating genetic diseases.

CRISPR-Cas9 Delivery
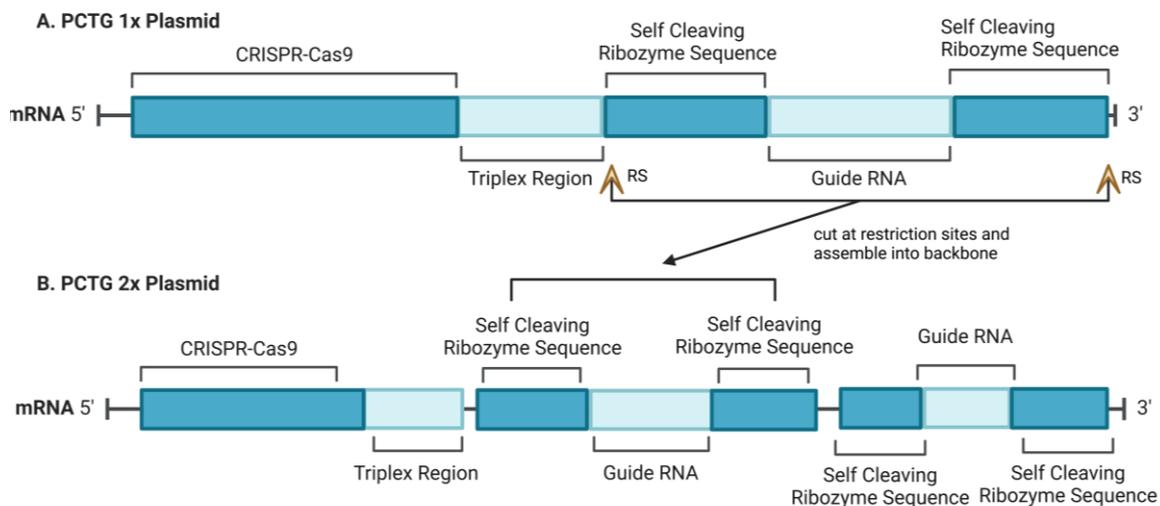
Figure 2: Sequence of PCTG plasmids involved with Cas9 gene editing. 1x refers to one copy of the gRNA between two self-cleaving ribozyme sequences. The PCTG 2x plasmids have two ribozyme + gRNA + ribozyme sequences subsequently and the PCTG 4x (not shown) plasmid has four.

One major hurdle to fully realizing the potential of Cas9-based approaches is delivery to tissues and cells. There are two primary ways currently used to deliver Cas9 and gRNA: viral and non-viral delivery. With viral delivery, there are methods that use adeno-associated viruses (AAVs) or lentiviral vectors to encapsulate both Cas9 and gRNA genetic information and deliver it to cells through a mechanism similar to viral infection. On the other hand, non-viral methods utilize other macromolecules like lipid nanoparticles (LNPs), protein polymers, and Au nanoparticles to deliver gRNA and Cas9 mRNA, protein, or DNA.[10] Delivering the protein form of CRISPR-Cas9 along with gRNA is efficient and doesn't result in much off-target editing, however it is very expensive and there is risk of endotoxin contamination.[10] Cas9 mRNA + gRNA delivery has similar
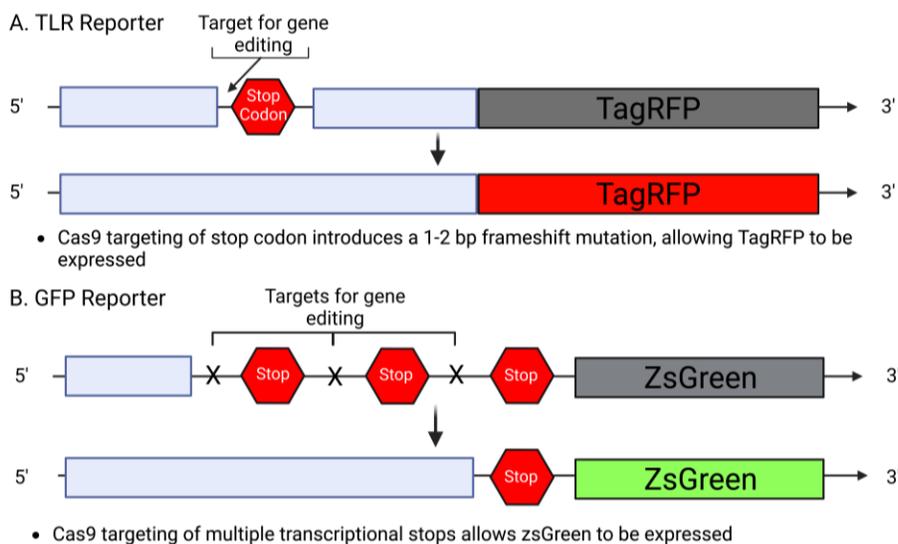
Figure 3: Schematic of RFP and GFP Reporters Used. Gene editing of TLR reporter causes a frameshift which makes RFP. GFP reporter is activated through editing multiple sites to delete transcriptional stops.

benefits to the protein form; however, it requires two components to get into particles and cells.[10]

Alternatively, Cas9 delivery can also be performed through complexing and conjugating with proteins. Ramakrishna et al. were able to design a cell-penetrating protein (CPP) that was conjugated to Cas9 and combined with a CPP complexed to a gRNA, resulting in efficient endogenous gene editing.[11] This technology has been proven effective in HEK293T cells, embryonic stem cells, embryonic carcinoma cells, and dermal fibroblasts.[11] Additionally, this system was able to produce less off-target editing events, however it is not perfect and it is difficult *in vivo* without a protective lipid nanoparticle.[11]

While there have been studies showing effective delivery of CRISPR-Cas9 into tissues using lentiviral and adeno-associated virus vectors, these methods of long-term expression can be problematic if used as a therapeutic[12,13]. For example, lentiviruses involve integration into the genome and potentially permanent, constant expression of Cas9 along with a gRNA[14]. Despite the specificity of Cas9, the technology is not guaranteed to always edit targeted gene sequences, and this can potentially lead to malignant or deleterious mutations.

In many of the previous methods, the size of cargo is also a significant barrier for the efficient delivery of gene editing technology. For instance, AAV packaging limits require separate viruses to be made for Cas9 and gRNA. Lentiviral packaging limitations lead to reduced efficacy when carrying large Cas9 and gRNA genes together. Several non-viral approaches depend on two different components (Cas9 and gRNA) to get into the same particles and cells. Therefore, to increase the efficiency of delivery, we engineered both the gRNA and Cas9 segments to be expressed from a single piece of RNA. With this construct design, cargo size would be significantly reduced and in the case of RNA delivery methods, two molecules reduced to one. This configuration has the potential to open the door for more efficient and a greater variety of delivery methods.

METHODS

Plasmids

pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454 ; http://n2t.net/addgene:8454 ; RRID:Addgene_8454).[15] lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961 ; http://n2t.net/addgene:52961 ; RRID:Addgene_52961).[16] pAAVS1-TLR targeting vector was a gift from Ralf Kuehn (Addgene plasmid # 64215 ; http://n2t.net/addgene:64215 ; RRID:Addgene_64215).[17] pCAG-loxP-STOPloxP-ZsGreen was a gift from Pawel Pelczar (Addgene plasmid # 51269 ; http://n2t.net/addgene:51269 ; RRID:Addgene_51269).[18]

Cloning of constructs encoding Cas9 and gRNA on a single RNA

A pCMV-Cas9 plasmid was cloned by replacing the VSV-G open reading frame in pCMV-VSV-G with Cas9 open reading frame from lentiCRISPR v2 using PCR followed by HiFi assembly (New England Biolabs). To clone in gRNAs, pCMV-Cas9 was linearized downstream of the Cas9 stop codon using StuI, then *MALAT1* Triplex (from Campa et al.)[19] + hammerhead (HH) ribozyme DNA and guide RNA sequence + hepatitis delta virus (HDV) ribozyme DNA were ordered from Integrated DNA Technologies as gBlocks with 20 bp overhangs between all three DNA fragments for HiFi assembly. XmaI, NheI, and HindIII sites as well as homologous regions for HiFi assembly were created in these DNAs in order to allow for expansion of the gRNA cassette according to the approach of Spakman et al. (Figure 2).[20]

The result of these steps was pCMV-Cas9-*MALAT1* Triplex-HH ribozyme-gRNA-HDV ribozyme, or PCTG-1x, where the 1x

denotes a single gRNA. In the text, gRNA targeting is added to the name: PCTG-TLR-1x encodes gRNA targeting traffic light reporter (GGTAGCGGGCGAAGCACTGC) and PCTG-GFP-1x encodes gRNA targeting GFP reporter (sgTOM from Wei et al.).[20,21]

     To create 2x and 4x gRNA constructs, we used the approach of Spakman et al. 2020 but with different restriction enzymes. Briefly, 1x constructs were linearized with NheI and separately the entire HH ribozyme-gRNA-HDV ribozyme region was cut out with XmaI and HindIII then gel purified. This creates overhangs that allow the entire HH ribozyme-gRNA-HDV ribozyme region to be cloned upstream of the other copy of the gRNA cassette with HiFi assembly to create 2x gRNA constructs. The same expansion was repeated starting with the 2x construct to get the 4X construct encoding 4 identical gRNAs. Plasmid sequences were confirmed by Sanger sequencing.

Transfection of PCTG-TLR and PCTG-GFP plasmids

     The day before transfection, HEK293T cells were seeded in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum at roughly 25% confluency in 6-well plates and incubated overnight at 37°C and 5% CO_2. The next day, PCTG-TLR 1x, 2x, 4x, or PCTG-GFP 1x and 2x were transfected into HEK293T cells with either pAAVS1-TLR or pCAG-loxP-STOPloxP-ZsGreen using JetOptimus transfection reagent. Plates were then left to incubate for 48 hours at 37°C and 5% CO_2.

Imaging of all constructed plasmids for gene editing activity

     All samples were imaged after 48 hours incubation using the Lionheart FX automated microscope with the 4X objective. The plates transfected with pAAVS1-TLR reporter were imaged for RFP and the plates that received pCAG-loxPSTOPloxP-ZsGreen reporter were imaged for GFP. Gen5
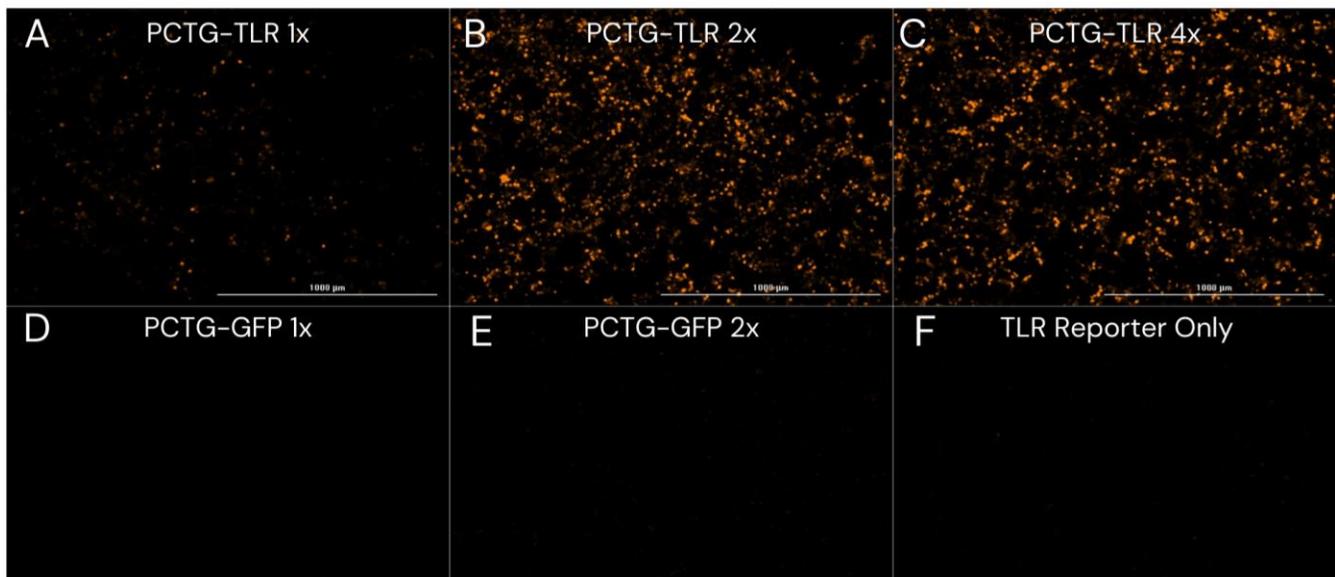
Figure 4: PCTG-TLR constructs efficiently edit TLR reporter. HEK293T cells were transfected with indicated Cas9-gRNA plasmid and traffic light reporter plasmid, then imaged for TagRFP after 48 hours. Fluorescence imaging was done with the Lionheart FX automated microscope using the 4X objective. Each red particle shown represents one cell expressing TLR. Each panel represents 1 of 16 fields imaged. Brightfield images were taken of all wells and showed similar cellular confluency (data not shown).
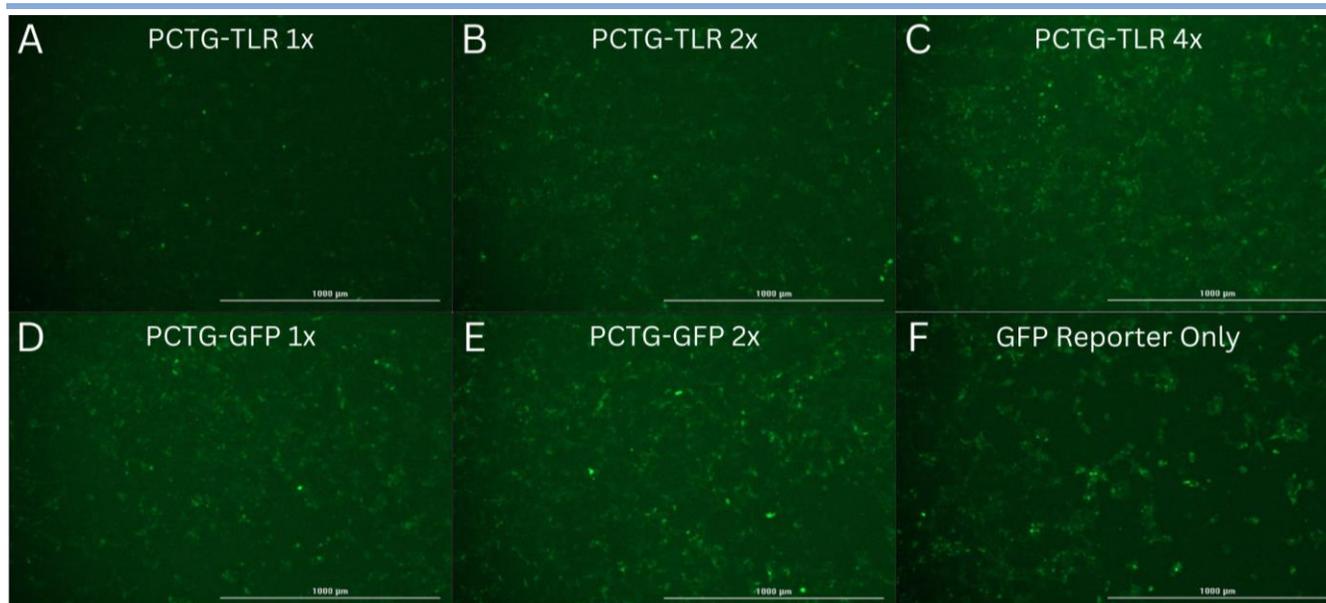
Figure 5: PCTG-GFP-2X construct edits GFP reporter. HEK293T cells were transfected with indicated Cas9-gRNA plasmid and green fluorescent protein reporter plasmid, then imaged for GFP after 48 hours. Fluorescence imaging was done with the Lionheart FX automated microscope using the 4X objective. Each bright green particle shown represents one cell expressing GFP. Each panel represents 1 of 16 fields imaged. Brightfield images were taken of all wells and showed similar cellular confluency (data not shown).

software was used to count RFP+ or GFP+ cells as well as calculate intensity of expression per cell. After imaging, a two-tailed, two sample equal variance, statistical t-test was performed to determine significance of RFP or GFP expression (n=2). Figures 1, 2, and 3 were made with BioRender software.

RESULTS

In order to test Cas9 and gRNA single RNA configurations, we created constructs that contain Cas9 open reading frame followed by an RNA region that forms a protective triplex.[22] This sequence is then followed by gRNA sequence sandwiched between self-cleaving ribozymes (Figure 2). In the cell, these self-cleaving ribozymes cleave out gRNA so that it can bind to Cas9 and the targeted DNA. The triplex is needed because these ribozyme cleavages would expose the 3' end of the Cas9 open reading frame to nucleases in the absence of the triplex.[22] We created versions expressing 1, 2, or 4 gRNAs that target traffic light reporter (TLR), or 1 or 2

gRNAs targeting the STOP region of pCAG-loxPSTOPloxP-ZsGreen (Figure 3). These reporters were targeted because both are well characterized and are also integrated into mouse lines, and thus are useful gRNAs for future *in vivo* experiments.[23]

To test targeted editing of TLR, we transfected each of these constructs into HEK293T cells along with traffic light reporter (TLR). Traffic light reporter encodes an upstream stop codon as well as an out-of-frame TagRFP. Upon Cas9 editing upstream of the early stop codon, a portion of editing events will cause the upstream stop codon to go out-of-frame and bring TagRFP in frame, resulting in RFP expression (Figure 3). Cells expressing TLR gRNAs exhibit RFP expression, which indicates successful gene editing by Cas9 that targeted the RFP reporter (Figures 4A-C). The quantification of RFP+ cells also demonstrate a significant number of cells underwent gene editing (Figure 6A). When comparing the difference between 1x, 2x, and 4x TLR gRNAs, there is a significant increase in
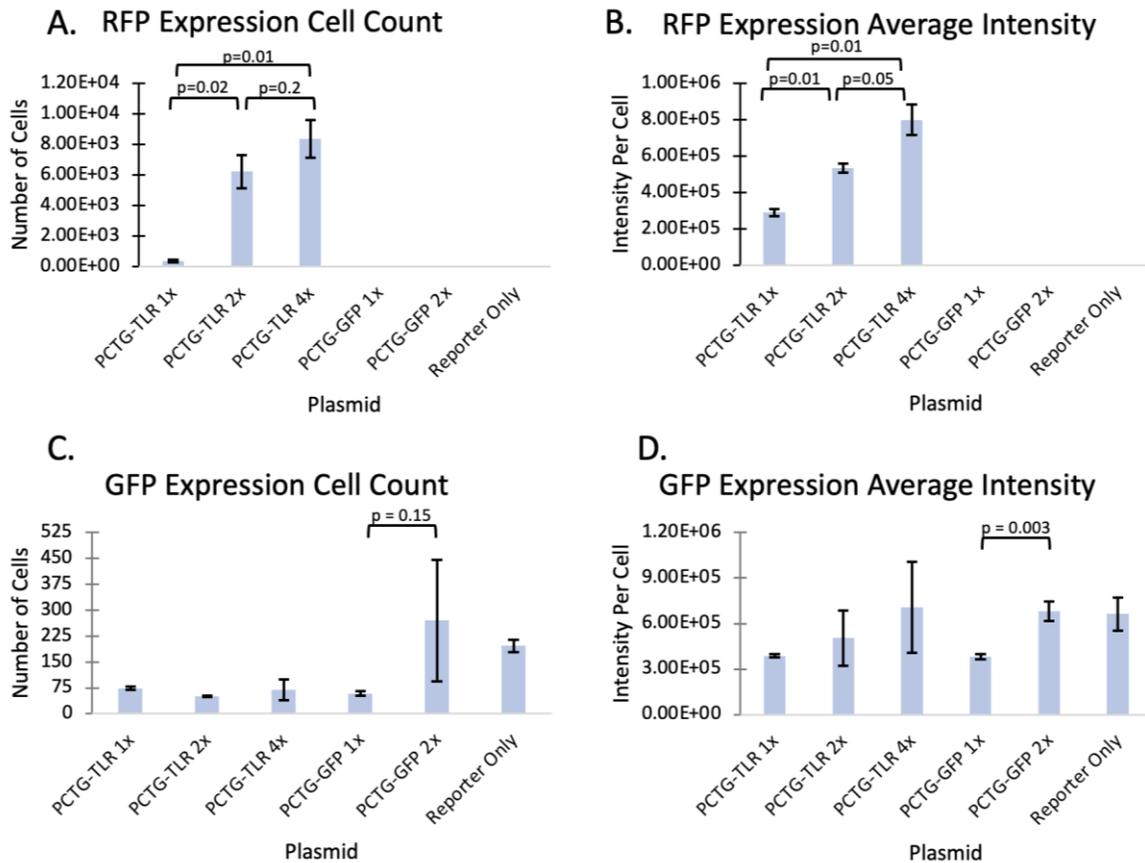
Figure 6: Quantitative analysis of imaging from figures 4 and 5. Corresponds with Figure 4: TagRFP+ cell counts (A) obtained over 16 images for each sample were obtained using Gen5 software. Average RFP intensity per cell (B) was also obtained using Gen5 software. Corresponds with Figure 5: GFP+ cell counts (C) obtained over 16 images for each sample were obtained using Gen5 software. Average GFP intensity per cell (D) was also obtained using Gen5 software. These data are the result of two independent experiments (n=2).

the number of cells that displayed gene editing (Figure 6A). Additionally, the magnitude of RFP expression per cell increased corresponding to the number of guide RNA sequences, meaning that 1x TLR gRNA showed the lowest magnitude, followed by 2x, and finally 4x demonstrated the largest magnitude of fluorescence (Figures 4A-C and 6B). Importantly, cells not expressing gRNA (Figure 4F) or expressing constructs encoding GFP targeting gRNA (Figure 4D-E) do not result in RFP expression, indicating that editing of TLR is selective.

Similarly, constructs expressing Cas9 and gRNAs targeted to the STOP region of pCAG-loxPSTOPloxP-ZsGreen were tested and demonstrated selective gene editing as well. pCAG-loxPSTOPloxP-ZsGreen encodes a ZsGreen reporter downstream of 3 successive transcription termination sites. Normally ZsGreen expression would be low but targeting Cas9 to regions between transcription termination sites with gRNA results in removal of termination sites and ZsGreen expression (Figure 3). Cells expressing Cas9 and TLR gRNAs along with reporter exhibited some GFP (Figures 5A–C), but co-expression of Cas9 and 2 GFP gRNAs resulted in increased GFP expression (Figures 5E and 6C-D).

When quantified, there was no statistical significance between the GFP reporter only negative control (Figure 5F) and the PCTG-GFP 2x version of the plasmid with

regards to both number of cells that express GFP and the magnitude of expression/gene editing (Figure 6C-D). This reporter has a higher background signal in the absence of editing as well as a higher threshold for signal (multiple Cas9 have to hit the same reporter DNA). In addition, the reporter only control is a transfection with only one plasmid and neither Cas9 nor gRNA, meaning that the reporter only control is not the best direct comparison. However, there was a significant increase in both number of GFP+ cells, as well as GFP intensity per cell when comparing PCTG-GFP 2x to PCTG-GFP 1x or any of the PCTG-TLR constructs (Figure 6C-D) indicating some level of selective gene editing. For future research, it will be valuable to create a PCTG-GFP 4x plasmid and assess whether or not it demonstrates an increase in gene editing activity similar to the PCTG-TLR plasmids.

DISCUSSION

The single RNA encoding Cas9 + guide RNA constructs were successful in performing gene editing on two different reporters. The plasmids containing TLR guide RNA were able to produce significant gene editing only on TLR and the duplication of guide RNA sequences was shown to improve not only the number of cells that had gene editing but also increased the magnitude of gene editing per cell as well.

With regards to the GFP guide RNA containing plasmid, there was a significant increase in the number of cells with gene editing between the 2x version of PCTG-GFP when compared to the 1x version or any of the PCTG-TLR versions, demonstrating the increase in number of gRNAs is also able to increase the level of selective gene editing in cells. While this result was clearly weaker in magnitude than the TLR system, there are some limitations to using this GFP reporter that likely account for this. First, there are nonzero amounts of background green fluorescence for negative controls (Figure 5).

This higher background is likely worse in the reporter only control in which only one plasmid is being transfected as opposed to all other samples which get two plasmids. In addition, the GFP system requires multiple editing events per cell to get GFP (Figure 3), making the signal weaker than the single editing event required for the TLR system.

An article written by McCarty et al. reviews construct designs somewhat similar to ours, including one encoding Cas12a.[19,24] We selected Cas9 for our configuration due to the wealth of knowledge about this system and Cas9's high activity relative to other Cas proteins. In addition, the selected self-cleaving ribozymes that process gRNAs are well-characterized and regulated cleavage can be engineered.[25,26] Since we found that this kind of construct design works, we can potentially also test other designs, such as one where different gRNA sequences are encoded on one RNA strand which would allow for simultaneous gene editing at multiple gene loci.[24] This kind of structure could be promising for gene therapy in the future and is worth considering.

CONCLUSION

These results are significant because after demonstrating that these Cas9/gRNA single RNA constructs function in HEK293T cells, future experiments can now be designed to test whether this configuration improves delivery of Cas9/gRNA using systems that have been shown to be limited by cargo size like AAVs, lentiviruses, and mRNA delivered by nano-lipid particles. AAVs are known to have limited cargo capacity despite being very effective for delivery[27]. Combining our configuration with smaller promoters, more compact Cas enzymes, and regulatable ribozymes may even allow a single AAV or more efficient lentiviruses to be produced.[28,29]

With regards to nonviral delivery, lipid nanoparticles are already known to produce efficient delivery of cargo, as well as target

specific cells. While nonviral delivery systems are generally able to hold larger cargos, it is still valuable to test this single RNA construct's effectiveness in lipid nanoparticles and whether it demonstrates improvement in gene editing frequency and magnitude since current methods often rely on getting two separate molecules, gRNA and Cas9, into particles.

In the future, these delivery approaches could be directly used in animal models too as the guide RNAs we have used in this study target commercially available TLR and Ai9 mice. Beyond this, testing for effective modification of disease associated genes using other gRNAs would provide valuable insight for the prospect of using our single RNA structure for gene therapy applications. In short, this technology has the potential to improve both existing and emerging Cas9/gRNA delivery methods.

AUTHOR INFORMATION

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Author Contributions

Elvis Lang conceived experiments, performed the cloning, and performed data analysis. He also wrote the manuscript and generated all the figures used. Dr. John Tilton provided direction and guidance during this project. Dr. Thomas Sweet also provided direction and including with the manuscript, conceiving experiments, and also did some of the plasmid design, cell culture work, and imaging for this project.

Funding Sources

ABBREVIATIONS

CRISPR: clustered regularly interspaced short palindromic repeats
Cas9: CRISPR associated protein 9
gRNA: guide RNA

AAV: adeno associated virus
RNP: ribonucleoprotein complex
LNP: lipid nanoparticles
CPP: cell penetrating protein
HEK293T: human embryonic kidney 293T
TLR: traffic light reporter
RFP: red fluoresce ent protein
GFP: green fluorescent protein
PCTG: pCMV-triplex-gRNA
CMV: cytomegalovirus

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Molecular Mechanisms and Clinical Features of Huntington Disease: A Fatal Neurodegenerative Disorder with Autosomal Dominant Inheritance

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ABSTRACT: Huntington disease (HD) is a fatal genetic disorder that affects the movement and cognition of affected individuals. It is inherited in an autosomal dominant manner, meaning that each child of a parent with HD has a 50% chance of inheriting the mutated gene. The mutation involves an expansion of a trinucleotide repeat (CAG) in the HD gene, which is located on the short arm of chromosome 4p16.3. The HD gene encodes a protein called huntingtin, which has an unknown function. The number of CAG repeats determines the severity and onset of the disease. Normal individuals have 26 or fewer repeats, while HD patients have 40 or more repeats. Individuals with 27 to 35 repeats do not develop HD, but they can pass on the mutation to their offspring, especially if the mutation is inherited from the father. Individuals with 36 to 39 repeats may or may not develop HD, depending on other factors. The more CAG repeats, the earlier the symptoms appear. HD is the most extensively studied neurodegenerative disorder with a genetic cause. There are genetic tests available to diagnose HD and to predict the risk of developing HD in asymptomatic individuals. There are also prenatal and preimplantation tests to prevent the transmission of HD to the next generation. HD is characterized by involuntary movements called chorea, which affect all muscles and impair all psychomotor functions. HD patients also suffer from cognitive decline and psychiatric symptoms, such as mood disorders and social changes. These symptoms are chronic and progressive, leading to complete dependence and death. Chorea can also be caused by other conditions, such as metabolic disorders or drug-induced side effects. Neuroimaging techniques, such as MR imaging, fluorodeoxyglucose positron emission tomography (FDG-PET), MR spectroscopy, and diffusion tensor imaging, can help to diagnose HD and monitor its progression. The pathophysiology of HD involves the loss of neurons and the dysfunction of neurotransmitter systems, especially the dopaminergic system. There is no cure for HD, but there are treatments to manage the symptoms and to improve the quality of life of HD patients. These include pharmacological interventions, such as dopamine receptor antagonists or depleters, and non-pharmacological interventions, such as psychological and social support. HD is a devastating disease that poses many challenges for patients, families, and healthcare providers. There is hope that gene-targeted therapies will be developed in the near future to stop or slow down the disease process.

INTRODUCTION

Huntington disease (HD) is a fatal neurodegenerative disorder that affects the CNS and causes motor, cognitive, and behavioural problems in the patients [1]. The disease is inherited in an autosomal dominant manner and is caused by a mutation in the Htt gene that results in an expansion of the polyQ domain of the Htt protein beyond 36 glutamines [3]. The brain cells of HD patients have misfolded polyQ Htt proteins that form

clumps, unlike the normal individuals who have diffuse localization of Htt [12]. The length of the polyQ expansion determines how fast the aggregation occurs [13]. The normal function of the cell to recycle and degrade proteins and produce energy is disrupted by the misfolded polyQ Htt, which also binds to other proteins such as CREB binding protein and depletes them from the cell [14]. This leads to a toxic effect of the mutant Htt, but it also affects the normal function of the wildtype Htt, which does not have the polyQ expansion [2, 15]. Therefore, any treatment for HD must consider both the gain-of-function and the loss-of-function effects of the mutant Htt. The age of onset of HD depends on the length of the polyQ expansion, with longer expansions causing earlier and more severe symptoms [4]. The polyQ length explains 60-70% of the variation in the age of onset, while the rest is influenced by environmental and genetic factors [5].

The main feature of HD pathology is the death of the GABAergic MSN in the striatum, which is a part of the brain that controls movement and cognition [2, 6]. The death of the MSN is accompanied by inflammation and activation of glial cells, which are the support cells of the CNS [2]. Microglia, which are the immune cells of the CNS, are activated in both early and late stages of HD and cause damage to the neurons in the striatum and cortex [7]. Astrocytes and oligodendroglia, which are the cells that provide nutrients and insulation to the neurons, are also increased in HD brains, especially in the globus pallidus and the white matter surrounding it [8]. HD patients also show a significant loss of brain volume in different regions, such as the cerebral cortex, the telencephalic white matter, the putamen, and the caudate nucleus [2, 9, 11]. The loss of brain volume can be detected even before the symptoms of HD appear, indicating an early

degeneration of the brain [10]. HD is a disease that affects the whole brain and causes progressive deterioration of the patients' functions and quality of life. There is no cure for HD, and the current treatments are only palliative and symptomatic.

Structure of the Huntingtin Protein

The huntingtin (Htt) protein is a molecule that is found in humans and other vertebrates, and it has a very similar structure and function among them [2,16]. It is a big protein, with a molecular weight of about 350 kDa, and it has a shape that is long and flexible [17,18]. The protein is made up of many repeated units called HEAT (Huntingtin, Elongator factor3, PR65/A regulatory subunits of PP2A, and Tor1), which help the protein to interact with other proteins and to form complex structures [2,24]. One of the special features of the Htt protein is that it has a region near the beginning of the protein that contains many glutamine residues, which are amino acids that have a nitrogen atom in their side chain [2]. This region is called the polyQ tract, and it starts from the 18th amino acid in the protein [2]. The number of glutamines in the polyQ tract can vary a lot, and this can affect how the protein works and how easily it dissolves in water [27,30]. In most people, the polyQ tract has around 20 glutamines, but in some people, it can have more than 40 glutamines, and this can cause a brain disease called Huntington's disease (HD) [20,21,22]. The polyQ tract is surrounded by another region that has many proline residues, which are amino acids that have a ring-shaped side chain [31]. This region is called the polyP tract, and it helps the protein to stay dissolved in water and to interact with proteins that are involved in moving things inside the cell [31,32]. The polyQ tract is not present in all animals that have a similar protein to Htt, but it is very important for the brain functions of animals that have it,

as shown by experiments with mice that do not have the polyQ tract [2,29]. The polyQ tract can also form structures that look like zippers, and these structures can bind to other parts of the protein or to other proteins that have similar structures [2,28]. The Htt protein is therefore a very complex and versatile protein that is involved in many different processes in the cell. The way that HD is inherited and how it affects different people is related to the way that the polyQ tract can change in size and cause problems, which is caused by an increase of the number of repeats of three nucleic acids (C, A, and G) in the first exon of the HD gene, which is located on chromosome 4p16.32 [19].

Htt has various features that allow it to move between the nucleus and the cytoplasm of the cell [2]. It has a nuclear export signal (NES) at the end of its C-terminal domain, which enables it to exit the nucleus [33]. It also has a domain at the beginning of its N-terminal domain, which consists of 18 amino acids and interacts with TPR, a protein that is part of the nuclear pore complex and facilitates nuclear import [33]. This domain also forms a membrane-binding domain that has an amphipathic alpha helical structure and can reversibly bind to different types of vesicles, such as those derived from the endoplasmic reticulum (ER), endosomes, and autophagosomes [34]. This domain is essential for the normal function of Htt, as mutations or deletions in this region cause Htt to accumulate in the nucleus and induce cellular toxicity [33]. Htt is also subject to proteolytic cleavage by various enzymes, such as caspases and calpains, which are conserved among higher vertebrates [35]. These enzymes generate fragments of Htt that are found in the nucleus, but their role is unclear. The proteolysis of Htt is influenced by the cellular context, as it is increased in diseased brains and more selective for the fragments that have the N- and C-terminal

domains, especially in the striatum [2,36]. Another enzyme that can produce N-terminal fragments of Htt is cathepsin, which belongs to the lysosomal degradation pathway [37]. Htt can also undergo different types of post-translational modifications in its N-terminal region, such as ubiquitination, sumoylation, and phosphorylation by kinases like Akt, ERK1, and Cdk5 [2,38]. The phosphorylation level of Htt is regulated by S/T phosphatases PP1 and PP2A [39]. Furthermore, Htt can be palmitoylated in its N-terminal region through the interaction with Huntingtin-Interacting Protein 14 (HIP 14) [40]. Palmitoylation is a mechanism that is used by several proteins that are involved in vesicle trafficking to maintain their proximity to the plasma membrane [2]. Htt is highly expressed in the human brain and testes [41]. In the brain, it is present in both neurons and glial cells [42]. Htt has a complex subcellular localization pattern, which may depend on its conformational state, as different antibodies that recognize different epitopes within the protein show different subcellular staining profiles [43]. Htt is not only localized in the nucleus, ER, and Golgi complex, but also in the axons and synapses of neurons [41], where it is associated with microtubules, caveolae, and synaptosomes [41].

Significance and Function of the Huntingtin Protein

The function and role of the huntingtin protein is still unclear, despite the fact that its genetic location was identified a long time ago. Some studies have proposed that huntingtin is involved in regulating gene expression and transporting molecules inside the cell, based on its presence in both the cytoplasm and the nucleus and its frequent interactions with other proteins [44]. The huntingtin protein also plays a role in neuronal development, synaptic transmission, axonal transport, and autophagy [51]. The huntingtin

gene has a segment of DNA that repeats the sequence CAG, which codes for the amino acid glutamine. In normal huntingtin proteins, this segment has less than 27 repeats, and people with 27 to 35 repeats (called transitional alleles) [45] do not develop the disease, but they may pass on longer repeats to their offspring. People with 36 or more repeats will develop the disease [46]. The number of CAG repeats tends to increase in each generation, which means that the disease becomes more severe and appears earlier in life. This phenomenon is called "anticipation" [47]. The age of onset of the disease is inversely related to the number of CAG repeats. Therefore, people with juvenile- and infantile-onset HD have more repeats than their parents and show symptoms earlier [48].

The huntingtin protein is expressed in many different types of cells, but it is especially abundant in the brain and the testes, and to a lesser extent, in the liver and the lungs [49]. It has a protective role against cell death, but when it is mutated or underexpressed, it causes early apoptosis and dysfunction. The mutation that causes HD is an expansion of the CAG repeats, which results in a longer stretch of glutamine in the protein [46]. This leads to the formation of abnormal aggregates of the protein in the nucleus and the cytoplasm of the cell, which disrupts the normal balance of the cell and triggers apoptosis [50]. The aggregates also interfere with the normal function of huntingtin and its interacting partners, leading to neuronal degeneration, inflammation, oxidative stress, mitochondrial dysfunction, and impaired autophagy[52]. The most affected brain region is the striatum, which is involved in motor control and cognition, followed by the cortex, which is responsible for higher cognitive functions [53]. The symptoms of HD include chorea, dystonia, rigidity, bradykinesia, cognitive impairment, and psychiatric disturb-

ances [54]. There is no cure for HD, but some treatments can help to manage the symptoms and improve the quality of life of the patients [55].

Clinical Indicators of Huntington Disease

HD affects the different aspects of behaviour, cognition, and motor function of humans [51]. The disease has three subtypes, depending on the age of onset: adult-onset, juvenile-onset, and infantile-onset. The most common subtype is adult-onset HD, which usually manifests in the fourth or fifth decade of life. Patients with adult-onset HD experience a range of behavioural symptoms, such as irritability, agitation, loss of inhibition, and aggression, which often precede the motor symptoms [46,52]. The motor symptoms include involuntary movements (chorea), which become less prominent as the disease progresses and are replaced by rigidity and abnormal muscle contractions (dyskinesia). Patients also lose their ability to maintain a sustained voluntary muscle contraction and their fine and gross motor skills, leading to severe disability and dependence [46,52]. The cognitive symptoms include impairment of memory, executive function, language, and visuospatial skills, which worsen over time and result in dementia [51]. The disease course of adult-onset HD is typically 15–20 years from the onset of symptoms to death [51]. The other two subtypes, juvenile-onset and infantile-onset HD, are much rarer and account for about 10% of HD cases. They are characterized by an earlier onset of symptoms, usually before the age of 20, and a more rapid progression of the disease [46,53]. The main features of these subtypes are rigidity, dyskinesia, and cognitive decline, with little or no chorea [46,53]. These patients often show signs of motor deterioration and poor academic performance before they are diagnosed [46,53].

Genetic Modifiers of Huntington's disease

The discovery of the HD defect [54] prompted many researchers to investigate the role of HD genes that were chosen based on their functional relevance. However, a new approach emerged in the early 21st century, which was based on two major advances in human genetics: firstly, the identification of common genetic variations across the genome, known as single nucleotide polymorphisms (SNPs), and secondly, the development of oligonucleotide array technology, which enabled the simultaneous genotyping of hundreds of thousands to millions of SNPs for unbiased genetic studies [55]. This approach, called genome-wide association analysis (GWA), allowed the researchers to scan the entire genome for genetic factors that influence the HD phenotype, without relying on prior knowledge of gene function. To apply this approach to HD with sufficient statistical power, three additional requirements had to be met: firstly, the availability of genomic DNA from a large number of HD subjects for genotyping; secondly, the definition of a robust phenotype that accounted for the effects of the CAG repeat size, which is the main determinant of the HD phenotype; and thirdly, the exclusion of any potential modifier factors that are linked to HTT and act in cis to modify the effect of the mutation, as these factors would confound the genome-wide search [55].

Statistically assessing the relationship between the length of inherited CAG repeats and the age of motor onset provided a vigorous HD phenotype that accounted for the effect of CAG repeats. Due to the danger of including inexplicably influential outliers, the analysis was limited to CAG repeat lengths typical of adult-onset (40 to 53–55) and sufficient representation of subjects to guarantee consistent results [56]. Based on their inherited CAG repeat lengths, >90% of HD subjects met these criteria, allowing a standard curve to be produced that relates CAG repeat size to average age-at-onset. In the absence of the effects of the CAG repeat size, an evaluation of this expected age-at-onset with the individual's observed age-at-onset provided the phenotype for analysis of genetic effects on onset [57]. It was fundamentally a matter of subtracting the expected age-at-onset from the observed age-at-onset to obtain the test phenotype, or residual age-at-onset, which was either a positive or negative number of years based on whether the subject's onset was later or earlier than anticipated. It was possible to test whether genetic variations at the HTT locus other than the CAG repeat size affect age-at-onset by using residual age-at-onset as a relevant HD phenotype and several thousand unrelated HD subjects [55]. In order to examine this, common single nucleotide polymorphisms (SNPs) were compiled across the gene and defined as haplotypes (i.e. the linear array of alleles at multiple SNPs along the chromosome, conducted as a physically linked set to progeny—basically a digital fingerprint for the HTT region) [55]. In addition, expanded CAG alleles associated with HD were found in multiple haplotypes, indicating that multiple independent ancestral HD CAG expansion mutations contributed to the contemporary population of HD individuals [58]. The most common haplotypes, representing more than 83% of HD subjects, were not related with differences in onset age, which suggests that genetic factors usually act in transfer through genes detached from HTT [58]. Accordingly, HD is viewed as a prototypical autosomal dominant genetic disorder based on whether it is passed on to progeny (or not), but the timing of disease onset is actually polygenic, determined by the combination of CAG repeats and other genetic factors.

Factors that play a role in the development of HD

1. Mitochondrial disorder

In early studies, functional abnormalities in mitochondria were discovered, indicating that mitochondrial dysfunction plays an essential role in the pathogenesis of HD and this is usually seen in the early disease process. The caudate and to a lesser extent the cortex of post-mortem HD brains have succinate dehydrogenase deficiency, a component of both the Krebs cycle and the electron transport chain's complex II. As compared to levels in matched control brains, HD brains demonstrated a significant decrease in complex II activity in the caudate nucleus (roughly 50%) [67]. In addition to reductions in complex II activity, complex III activity in the caudate and putamen, as well as complex IV activity in the putamen, have decreased as well [67]. In spite of this, since most of these patients suffered from advanced neuropathy, including severe striatal atrophy (pathological grades 3 and 4 of HD), mutations in mitochondrial sources (i.e., glial, neuronal, etc.) may have occurred [59].

2. Oxidative stress due to ROS

Reactive oxygen species (ROS) are produced in excess in the body, which results in oxidative stress when the body is unable to detoxify them and repair the damage they cause. Animal models with HD showed increased levels of malondialdehyde, 8-hydroxydeoxyguanosine, 3-nitrotyrosine, and heme oxygenase oxidative damage products, and free radicals in the areas of degeneration in the brain of HD affected individuals which propose that oxidative stress is connected with the disease, either as a primary event or a secondary component of the cascade processes of cell death [67]. There is ample evidence that oxidative damage subsidizes significantly to the pathogenesis of neurodegenerative diseases such as HD [60].

3. Apoptosis

There is a link between the pathogenic mechanism of apoptosis and chronic neurodegenerative diseases like HD [61]. Caspases are cysteine-dependent, aspartate-specific proteases that initiate and execute apoptosis. A transcriptional up regulation of caspase-1, caspase-3, and caspase-9 in HD patients as well as in animal models of HD has been reported [62]. Amyloidogenic Mutant Huntingtin (MHtt) has been confirmed to induce apoptosis in HD patients [63].

4. Neuroinflammation

By discharging cell mediators that combat foreign substances and prevent infections, the inflammatory process safeguards our bodies from harm and disease. Neuroinflammation does not directly correlate with HD progression, despite inflammatory processes being evidently confirmed in its pathophysiology. Post-mortem studies of degenerating neurons in HD have discovered high levels of activated microglia and macrophages as well as elevated levels of IL-6, IL-1, and TNF- in the plasma and striatum of HD patients [67]. Microglial cells may identify the pathogenic mHTT aggregates as foreign substances, resulting in neuro-inflammation [64].

5. Neurotoxicity

Disproportionate glutamate neurotransmission leads to excitotoxic neuronal death, which is supplemented by insistent intracellular calcium level elevation [65]. As NMDA (N-Methyl-D-aspartic acid) receptors are over activated by excited amino acids, free radicals are formed and mitochondrial per-

meability transition pores are unlocked, both of which are lethal. The role of neurotoxicity has been documented as significant since the undeviating injection of acids such as QA and kainic acid causes neuro-degeneration of GABAergic MSN in HD [66].

Advances in Huntington's Disease Therapy

The HTT gene mutations that cause HD are still not fully understood, but research on the molecular mechanisms behind them is very promising and could lead to a cure. Currently, there are no neuroprotective therapies that can prevent or slow down the disease, and the only treatments available are symptomatic [67]. One of the main causes of HD is the toxicity of mHTT, the mutant form of the HTT protein, which is produced by the mutated gene. Therefore, reducing the expression of mHTT, either by lowering the levels of HTT mRNA or the protein itself, is a potential strategy to treat HD [68]. Some studies have suggested that gene-silencing techniques that target the CAG repeats in the HTT gene, which are responsible for the mutation, could improve the functional, motor and cognitive outcomes of HD patients, but not their weight loss [67, 69]. These techniques involve using different types of DNA-binding elements, such as zinc-finger proteins, nucleases, epigenetic modulators, or transcription factors, to either block, disrupt, or correct the mutant gene. For example, zinc-finger transcriptional repressors can bind to the DNA and prevent its transcription, while zinc-finger nucleases can cut and edit the DNA [67,70]. Another example of a genome editing technique is CRISPR/Cas9, which can also target and modify the HTT gene. These approaches have the advantage of permanently correcting the CAG expansion that causes HD. Another way of reducing mHTT expression is by using antisense oligonucleotides (ASOs), which are synthetic molecules that bind to the HTT mRNA and trigger its degradation by an enzyme called RNase H1 [71]. ASOs can reach the central nervous system without needing a viral or lipid carrier, and they are easy to develop [67]. A clinical trial by Tabrizi et al [72] used an ASO called IONIS-HTTRx to lower the levels of mHTT in the cerebrospinal fluid of 34 HD patients, who received doses ranging from 10 to 120 mg, and compared them with a placebo group. The results showed that the mHTT reduction was dose-dependent [72]. A similar method of post-transcriptional gene suppression is RNA interference (RNAi), which uses non-coding double-stranded RNA sequences to silence specific genes. RNAi can be achieved by using different types of RNA molecules, such as siRNAs, shRNAs, or artificial miRNAs, which have been shown to reduce the HD symptoms [67]. RNAi is also a promising technique for many other diseases.

One of the main approaches for treating HD is to reduce the synthesis of the mutant HTT (mHTT) protein, which forms toxic aggregates in the brain. This can be achieved by using RNA interference (RNAi) techniques, which involve the use of small RNA molecules that bind to the mHTT mRNA and prevent its translation. Several types of RNA molecules have been used for RNAi, such as short hairpin RNA (shRNA), small interfering RNA (siRNA), and microRNA (miRNA) [67]. These molecules have been delivered to the brain of HD animal models using viral vectors, such as adeno-associated virus (AAV), which contain enhancers and promoters to drive the expression of the RNA molecules. The first trials of RNAi for HD were performed in rodents two decades ago. It is seen that shRNA targeting mHTT reduced its synthesis and prevented the formation of inclusions, gait deficits, and rotarod dysfunction in mice, in recent studies [67]. Similarly,

siRNA injected into the mouse striatum prolonged the survival of striatal neurons, reduced mHTT aggregates, and prevented motor dysfunction [73]. These results were replicated in multiple animal systems, such as rats, monkeys, and sheep [74]. A recent study used a single-stranded siRNA (ss-siRNA) for RNAi and achieved a selective decrease of CAG-expanded HTT protein in various regions of the mouse brain [75]. Another type of RNA molecule that has been used for the suppression of mHTT is miRNA, which is a natural regulator of gene expression. MiRNAs have been shown to have promising effects in genetically modified mice with HD; for example, one study reported that miRNA-mediated knockdown of mHTT prevented regional cortical and striatal atrophy and reduced weight loss [76].

Most of the RNAi techniques do not completely eliminate the production of mHTT, but only reduce it to a certain extent. Therefore, another possible therapeutic approach is to overexpress the wild-type HTT, which may have a protective role against the toxic effects of mHTT. Early trials of this strategy showed that inserting the wild-type HTT into mammalian cells that expressed mHTT reduced cell death [77]. Several natural and synthetic compounds have been suggested as potential candidates for the treatment of HD and other neurodegenerative disorders. One of the most widely studied compounds for HD is tetrabenazine (TBZ), which is an inhibitor of the vesicular monoamine transporter 2 (VMAT2) that blocks the uptake of dopamine into vesicles [67]. TBZ has been shown to exert antichorea effects in patients with HD and was the first approved drug for the disease [78]. However, TBZ has some limitations, such as low bioavailability and adverse effects. Therefore, studies have been conducted to optimize the drug delivery and bioavailability of TBZ using nanotechnology techniques, such as nanoparticles

and nanocapsules [79]. Another class of compounds that may have beneficial effects for HD are flavonoids, which are natural polyphenolic compounds found in plants. Flavonoids have been shown to reduce cellular stress and exert anti-inflammatory and anti-apoptotic effects in the cell [80]. Some examples of flavonoids that have been tested for HD are resveratrol, curcumin, and quercetin.

Marine compounds have been proposed as potential sources of novel drugs for HD and other neurodegenerative diseases. Marine compounds have diverse chemical structures and biological activities, such as anti-oxidant, anti-inflammatory, and anti-apoptotic properties. Some examples of marine compounds that have been investigated for HD are fucoidan, xyloketal B, fucoxanthin, and cerebrosides [67, 81]. A recent study suggested that pridopidine, a dopamine stabilizer, may be a promising drug for HD symptoms. Pridopidine acts on the sigma-1 receptor, which is involved in the regulation of calcium homeostasis, mitochondrial function, and neuroprotection. Pridopidine has been shown to improve motor and cognitive functions in HD animal models and patients [82]. The stage of Huntington's disease (HD) is related to the amount of dopamine in the central nervous system, since dopaminergic conduction disorders are the main cause of HD. Pridopidine, a drug that protects nerve cells from degeneration, has shown promising results in animal models [83]. Drug treatment for HD has the advantage of being based on well-studied active compounds that are effective and tolerable for other similar neurodegenerative diseases [67]. This makes it easier to personalize the medication according to the patient's diagnostics. Another potential treatment for HD is cell replacement therapy using stem cells, which could reduce the symptoms of the disease [84]. Moreover, exercise and physical activity

have been reported to have positive effects on the motor function, gait speed, balance, and social well-being of HD patients [67, 85]. Therefore, exercise could be a complementary therapy for HD. A novel approach to target the underlying cause of HD is the use of monoclonal antibodies that bind to the mutant huntingtin (mHTT) protein and lower its concentration in the cell. This could prevent the mHTT from spreading and causing damage to the brain [84].

Gene therapy: A Possible Treatment of HD in the Future

Research into gene therapy has led to the most exhilarating and favorable advances in HD research. A potential therapy for dominant genetic disorders, silencing mutant genes can offer major benefits. It is generally believed that gene therapy could have a two-fold effect: (i) restoring function to non-dead, but dysfunctional neuronal circuits, and (ii) protecting against disease progression [86]. As a matter of fact, gene therapy will not be used to treat disease, but to prevent it - to eliminate symptoms entirely. Molecular gene therapy targets the transcription and translation processes of DNA into mRNA (messenger RNA) by a process called transcription and the synthesis of proteins using the information in mRNA (a process called translation) [87]. Antisense oligonucleotides (ASOs), zinc finger proteins, and RNA interference techniques are three common methods for gene-silencing [88]. Zinc finger proteins suppress transcription, antisense oligonucleotides suppress translation of mHTT, and RNA interference blocks protein translation [89] In large neuroimaging studies performed during preHD, measurable measures of brain regions such as the striatum have been found to be excellent biomarkers of disease progression and will be useful in future gene therapy trials [90].

CONCLUSION

Research indicates that the pathology of Huntington's disease (HD) may be significantly influenced by the loss of function in the normal Htt protein, despite the disease primarily being attributed to a toxic gain of function caused by the expansion of polyglutamine (polyQ) sequences. The Htt protein is known to interact with various effector proteins and is involved in critical cellular processes such as transcription and intracellular trafficking. These interactions and processes are vital for the proper processing and localization of numerous proteins. Consequently, a deficiency in Htt function could potentially have a more extensive impact on cellular physiology than previously comprehended. Given the variety of cellular disruptions caused by this deficiency, it appears that certain neurons may be more resilient than others. Identifying which altered physiological processes most significantly contribute to the progression of HD will be imperative for the development of effective therapeutic interventions. As current therapeutic strategies under development aim to reduce Htt levels, it is also essential to ascertain the degree to which cells can tolerate a reduction in normal Htt expression.

In the field of human disease research, utilizing human subjects is considered the benchmark for validating experimental findings related to disease pathogenesis. This approach is instrumental in elucidating the mechanisms underlying disease onset and in exploring viable treatment modalities for genetic disorders. Take HD, for instance, a genetic condition precipitated by the incessant elongation of the CAG trinucleotide repeat within the Htt gene. The extent of this repeat sequence is a determinant of the age at which HD symptoms manifest, as the accumulation of toxicity from the expanded repeat sequence reaches a critical threshold, instigating cellular damage processes that cul-

minate in neuronal impairment and the onset of the disease. However, the length of the CAG repeat is not static; it is subject to somatic expansion influenced by both genetic and environmental factors. This variability leads to differential susceptibility, damage, and toxicity across various cell types, resulting in diverse phenotypic expressions among individuals. Thus, the DNA repair mechanisms that govern the length of the CAG repeat are pivotal targets for therapeutic interventions aimed at delaying or averting the onset of HD and similar trinucleotide repeat disorders. Nonetheless, these DNA repair mechanisms are modulated by a plethora of genes that serve as modifiers of HD onset, complicating the prediction of disease trajectory for individual patients. Furthermore, these modifier genes may influence not only DNA repair pathways but also other processes that initiate cellular damage and toxicity in HD. Another dimension of HD that remains elusive is the normal function of the Htt protein, which is implicated in synaptic vesicle trafficking and endosomal signaling—processes that are crucial for neuronal development and functionality. The precise mechanisms by which Htt facilitates these synaptic functions, and how they are disrupted by the mutant form of Htt in HD-afflicted neurons, are areas that warrant further investigation. A more thorough characterization of the normal roles of Htt could unveil new therapeutic avenues and foster a more holistic understanding of HD.
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Inside the Nucleon: Tomographic Interpretations and Universality of GPDs with DDVCS

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ABSTRACT: The goal of Double Deeply Virtual Compton Scattering (DDVCS) experiments is to better understand the internal structure of the nucleon. Previous attempts to resolve the internal structure of nucleons have resulted in electromagnetic form factors and parton distribution functions for elastic scattering and deep inelastic scattering processes, respectively. Generalized Parton Distributions (GPDs) are the latest attempt to unify these models of nucleon structure. The GPDs of DDVCS give us ability to investigate off of the diagonal where $x \neq \pm\xi$. The main goal of our analysis is to determine the best experimental setup in order to deduce the kinematic variables on which GPDs depend from the lab observables. The effectiveness of our data collection in the laboratory is by determined the physical kinematics, Q^2, Q'^2, t, x_i, ϕ_{LM}, ϕ_{CMV}, and θ_{CMV}. We can then run DDVCS experiments and collect data on observables to improve upon the current models for GPDs of the nucleon.

INTRODUCTION

The fundamental aim of particle physics is to discover and understand matter down to its smallest possible constituents. The discovery of the quark in 1964 revolutionized this process, breaking apart nucleons, which were previously believed to be elementary particles[6]. These subatomic particles were detected using scattering, a process in which a high energy particle beam is directed at a stationary object, resulting in collisions between the particles in the beam and object. Data is collected from these collisions, such as cross sections or scattering byproducts, which is then used to infer information about the internal structure of the object. The model that we are attempting to explore is Generalized Parton Distributions, currently the most detailed model of nucleon structure to exist. GPDs are a hybrid of its predecessors, form factors (produced through elastic scattering) and parton distributions (produced through deep inelastic scattering)[8]. The particular scattering process that we are investigating is Double Deeply Virtual Compton Scattering (DDVCS), in which an electron beam is scattered off a proton, exchanging a virtual photon in the process. The outgoing virtual photon will then decay into a detectable muon-antimuon pair as seen in Figure 1[7].
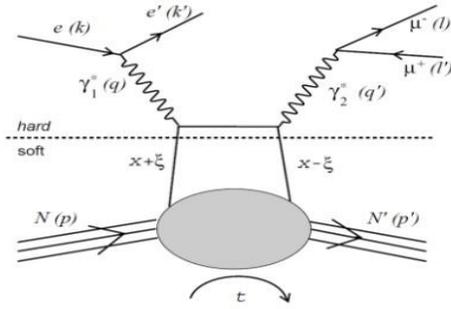
Figure 1: DDVCS $e- + e- \longrightarrow \mu+ + \mu-$ [2]

The current reactions studied are Timelike Compton Scattering (TCS) and Deeply Virtual Compton Scattering (DVCS). Both of these collisions include one virtual photon, with TCS containing an outgoing virtual photon and DVCS an incoming virtual photon. In DDVCS experiments, both the initial and final photons are virtual. The virtuality of the photons impacts the matrix elements that describe the reaction. The matrix element refers to the probability amplitude of finding a quark at a space-time point in a nucleon, then finding the same quark at another space-time point in the nucleon which has now changed its momentum [5]. The involvement of 2 space-time points means that the matrix element is non-local, and the differing momenta of the initial and final nucleon makes the matrix element non-forward. GPDs depend on the following kinematic variables: Q^2, the virtuality of the exchanged photon, t, the momentum transfer to the nucleon, Bjorken x (x_{Bj}), the fraction of the total nucleon momentum, and three reaction angles. These kinematics be used to calculate ξ and ξ', components of light cone frame momentum, using the following equations [3].

$$\xi = \frac{Q^2 + Q'^2}{2s + Q^2 + Q'^2 - 2M_p^2 + t} \qquad (1)$$

$$\xi' = \frac{Q^2 - Q'^2 + t}{2s + Q^2 + Q'^2 - 2M_p^2 + t} \qquad (2)$$

Using ξ and ξ', the longitudinal momentum transfer fraction of incoming spacelike photon, $-2(\xi-\xi')$, and the longitudinal momentum transfer fraction of outgoing timelike photon, $(2\xi'-\xi)$, can be calculated. These momentum transfers make DDVCS unique because in a DDVCS reaction, $\xi \neq \xi'$ unlike TCS and DVCS reactions where $\xi = \pm\xi'$ [3]. When $\xi \neq \xi'$, xBj and ξ dependence can be decoupled, allowing access to 'off-diagonal elements' of GPD regions. Currently, with TCS and DVCS, only elements on the diagonal xBj = $\pm\xi$ are accessible due to the dependence of x and ξ on each other. However, x and ξ decoupling enables newly accessible regions of GPDs, which will allow for a more detailed picture of the distribution of nuclear forces inside a nucleon as well as the determination of parton transverse densities. Such investigation will add to the current model of GPDs and facilitate more detailed tomographies of the nucleon.

Another focus of the DDVCS experiments is the question about the universality of GPDs. Under current theories, GPDs are presumed to be universal, which means that the calculations of GPDs will be the same regardless the experiment used to measure them. However, there is no concrete experimental proof to support this conclusion. DDVCS reactions have the unique ability to simultaneously study spacelike (defined as Q2 > Q'2) and timelike regions (defined as Q'2 < Q2) in order to determine the two regions result in the same leading order and twist. The agreement of results between the spacelike and timelike regions would then in turn provide support of the universality of GPDs
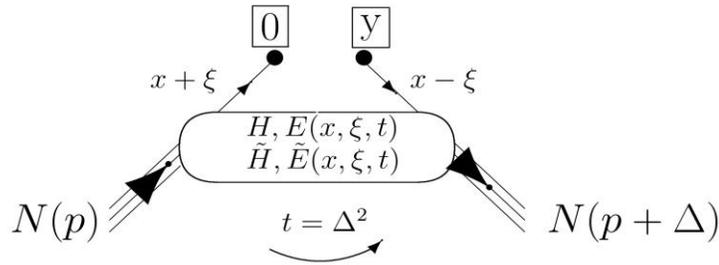
Figure 2: Illustration of non-local non-forward matrix element[5]

METHODS

In order to simulate DDVCS collisions, we used the event generator DEEPGen, developed by Dr. Marie Boër. These event generators, written in C++ and run through ROOT, generate simulated particle collisions events given a certain set of parameters, such as luminosity, beam energy, and phase space. It can be altered depending on the type of collision it is replicating. The version that we used, DEEPGen 5.0, simulates deep exclusive photo- and electro- production of lepton pairs and photons, including DDVCS as well as DVCS and TCS. The parameters of the event generator were set to match experiments at Hall C of Jefferson Lab with an 11 GeV electron beam. The generator creates equal weighted events and uses a Monte-Carlo simulation technique to weight the events with an n-differential cross section[4]. The weights are multiplied by a normalization factor, where L is luminosity and $\Delta\Omega$ is the dimension of the phase space.

There are five weighting options; total unpolarized, DDVCS, Bethe-Heitler (BH), DDVCS/BH, and beam spin asymmetry. The beam spin asymmetry weighting provides insight into how difficult it will be to measure the asymmetric polarization of the electron beam. Simulated events with the total unpolarized weight are proportional to the number of measured events in the physical experiment. The total unpolarized weight is calculated with the DDVCS weight and BH weight, Wtot = |WDDVCS + WBH|2. Analyzing the data with DDVCS and BH weighting allows their contributions to be distinguished. The BH contribution is precisely known due to it's dependence on QED calculations and proton form factors[1]. It describes the hard (known) region of DDVCS reactions. The DDVCS contribution, on the other hand, describes the soft region of the reactions and can not be calculated.

The ratio of DDVCS and BH weighting allows for insights into where the so-called 'new physics' can be found. Areas where DDVCS/BH is large are promising in terms of information about the GPDs in the previously inaccessible regions.
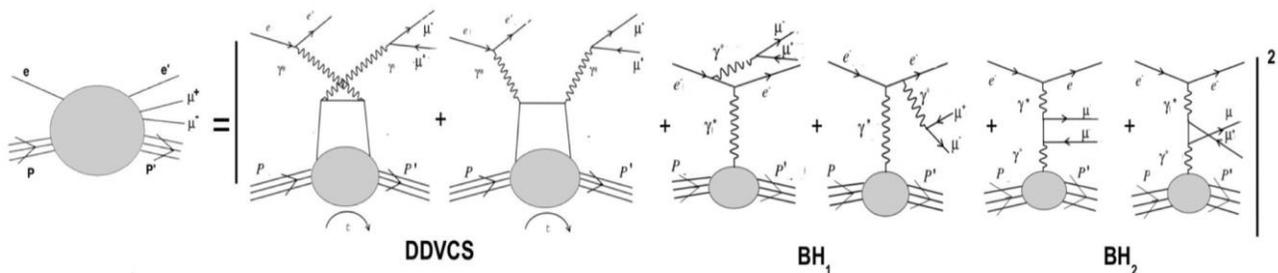

Figure 3:The DDVCS and BH contributions to the total unpolarized weight[2]

After generating the simulated and weighted DDVCS events, we analyzed the data to better understand how the experimental kinematics variables relate to each other. In addition to the variables of t, ξ, ξ′ displayed in Figure 1 above, the relationship between the various angles in the particle scattering, shown below, were explored. The experiment takes place on three planes, with the far left plane being created by the initial and final electrons from the electron beam, the middle plane by the photons and the nucleon, and the plane on the far right by the muons. The notation CM indicates the measurements are taken from the center of mass frame. The angles of interest are θCM, the angle between the incoming and outgoing electrons, ϕ_L, the angle between the plane of the electrons and the plane of the photons, and ϕCM, is the angle between the plane of the muons and the plane of the photons. The last angle, θCM, is the angle the scattered muon makes from parallel.
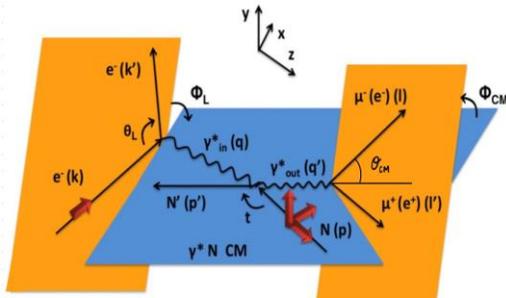

Figure 4:Collision vectors and angles

DISCUSSION

While the earlier graphs were created to better understand the relevant kinematic relationships, the later graphs explored how events can be interpreted. The first set of graphs (Figure 5) traces out the amount of events that exists within a certain angle range (corresponding to the angles in Figure 4). The ϕ angles have a range of π and the θ graph measures entries between 0 and π.
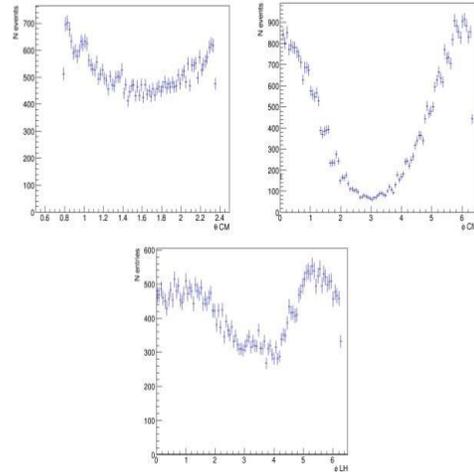

Figure 5: θ_{CM} (upper left), ϕ_{CM} (upper right), and ϕ_L (lower)
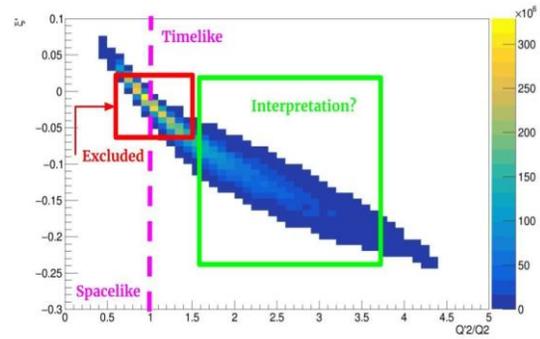

Figure 6: $\dfrac{Q^2}{Q'^2}$ vs ξ,

Both of the center of mass angles (ϕ_{CM} and θ_{CM} appear largely symmetric without many irregularities. However, the ϕ_L graph displays unexpected asymmetries, which may arise due to relations between all three angles and warrants more exploration.

The next figure, which compares $\dfrac{Q'^2}{Q^2}$ to ξ, provides important information about events that need to be excluded from the data set and events that might be useful for physical interpretation. The events along the timelike and spacelike cut line are events that must be excluded. The line corresponds with $\dfrac{Q'^2}{Q^2} =$ 1 which makes the data difficult to interpret.
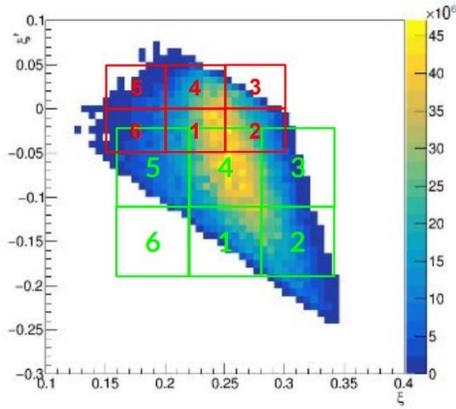
Figure 7: ξ vs ξ'

Figure 7 is a comparison of ξ and ξ' with boundaries that define later data cuts in Q^2 vs Q'^2. By comparing the ξ and ξ', we are granted the ability to determine how far 'off the diagonal' we can measure our events. The diagonal, in this case, is defined as $x = \pm\xi$ and has previously restricted the investigation of GPDs in the ERBL and DGLAP regions. The cuts that are made focus on areas where measured events can be physically interpreted.

The cuts on the ξ vs ξ' graph correspond to selected Q^2 and Q'^2 bands (Figure 8). The graphs are also restricted in terms t, between −0.15 GeV and −0.55 GeV. t must be constrained because while there are more events measured between −0.55 GeV and

−1.05 GeV, when the momentum transfer becomes too large, approximations are rendered invalid and a physical interpretation loses meaning. Thus, the graphs must be constrained in t.

The value of these graphs are derived from the fact that Q^2 and Q'^2 cannot be measured outright. The transfer of the virtual photon's momentum occurs in the soft region, which is not physically accessible with our current technology, but can still be calculated from the measured kinematics. Using the Q^2 vs Q'^2 selected bands and the Bjorken x hypothesis, the structure of the proton can be determined. The Bjorken x hypothesis states that, for point-like particles, as the limit $Q^2 \rightarrow \infty$, GPDs lose dependence on Q^2. Hence, if the GPD demonstrates dependence on Q^2, it's structure cannot be classified as point-like.[9]

SUMMARY AND OUTLOOK

Double Deeply Virtual Compton Scattering is the next step when it comes to accessing and analyzing GPDs. The incoming and outgoing virtual photons make DDVCS unique from previous experiments, allowing access to regions of Generalized Parton Distributions that were previously unreachable. This is done through taking advantage of the fact that in DDVCS, $\xi \neq \pm\xi'$ which allows access
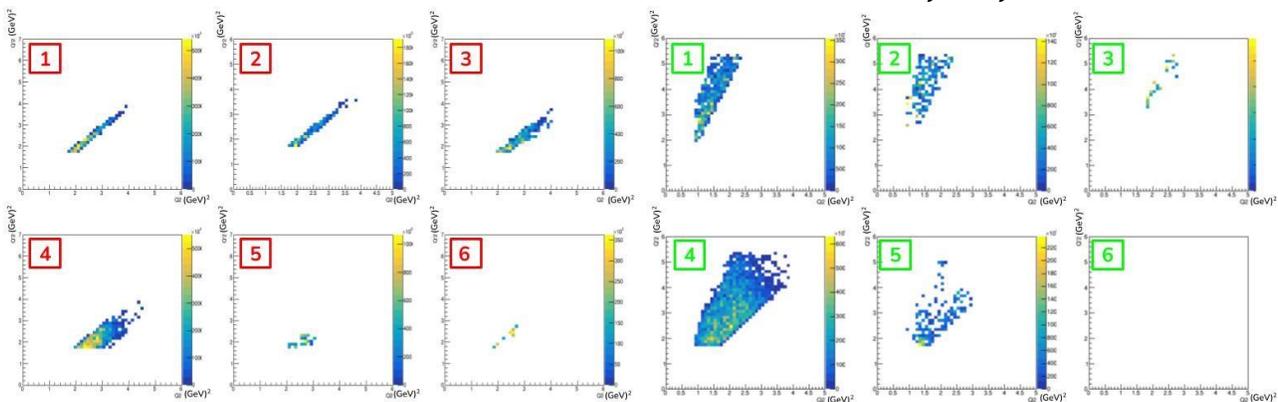

Figure 8: Q^2 vs Q'^2 Selected Bands, Spacelike (left) and Timelike (right)

beyond the x = ±ξ diagonal GPDs are currently restricted to.

DDVCS also provides opportunity to compare measurements of GPDs in spacelike and timelike regions simultaneously, which can be used to evaluate the validity of GPD universality. The DEEPGen Event Generator provided events that could be applied to different weighting systems and analyzed. Boundaries were created for Q2 vs Q'2 using ξ vs ξ', allowing for greater insight into proton GPDs as well as an understanding of the extent to which measurements can be taken 'off of the diagonal.'

The future of this project requires more data analysis and planning for the Jefferson Lab proposal. The kinematics regarding the Bjorken x Hypothesis contains a vast domain of interpretation that has potential to be explored further. Additionally, more detailed data analysis can be done by with regards to the relationship between the experimental angles and the other kinematics variables, as this study lacks the proper angular corrections and acceptance cuts from Q2 and other values. Looking forward, there exists a great deal more investigation into DDVCS to be done, as it appears to be an untapped realm when it comes to uncovering more physics regarding proton GPDs.

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ABBREVIATIONS

DDVCS: Double Deeply Virtual Compton Scattering
GPDs: Generalized Parton Distributions
TCS: Timelike Compton Scattering
BH: Bethe-Heitler

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