

Expression of anti-neurodegeneration genes in mutant *Caenorhabditis elegans* using CRISPR-Cas9 improves behavior associated with Alzheimer's Disease

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SUMMARY

Alzheimer's is ranked as the 6th leading cause of death in the United States, and mainly presented as neurodegeneration. In order to begin to understand its physiology, the specific role of proteins in neurodegeneration, LRP1 and AQP4, need to be studied. This study tested the effect of using the CRISPR-Cas9 system to overexpress the LRP1 and AQP4 proteins, associated with transport of materials and waste across the cell membrane, in *Caenorhabditis elegans* to assess effects on neurodegeneration, such as chemosensation, size, and average speed phenotypes. I hypothesized that combinatorial overexpression of AQP4 and LRP1 would have the greatest effect on reducing neurodegeneration. I tested chemosensory behavior using a chemotaxis test, revealing a decrease in neurodegeneration when both LRP1 and AQP4 were overexpressed. The size of the *C. elegans* did not change, but the speed increased in the strain expressing amyloid beta in the muscle, suggesting that a decrease in amyloid beta allowed muscles more room to contract. These results support our hypothesis and show that the overexpression of LRP1 and AQP4 proteins decrease neurodegeneration and allow *C. elegans* to preserve their olfactory retention. This study will help demonstrate the role of LRP1 and AQP4 in Alzheimer's and determine whether they benefit the system once they are overexpressed.

INTRODUCTION

Approximately 5.7 million people worldwide have Alzheimer's Disease, which eventually causes loss of mental function and memory in those affected. There are a few main hypotheses that try to explain Alzheimer's disease, including the amyloid hypothesis and the Tau hypothesis. The amyloid hypothesis proposes that a protein in the cell membranes of neurons called the amyloid precursor protein (APP) helps transmit signals from the inside of the cell to its environment. APP is eventually broken down by alpha, beta, and gamma secretases. Alpha and gamma secretases break down the protein into a soluble state, but beta secretase activity leads to insoluble APP. Buildup of insoluble APP can generate an amyloid beta plaque that inhibits the connections between neurons (1). Alternatively, the Tau hypothesis suggests that,

in Alzheimer's Disease, the Tau protein, which normally stabilizes microtubules, instead separates from these microtubules, causing them to fall apart. The strands of Tau cause tangles, which disable the transport system and destroy the cell (2).

The LDL receptor family functions to bind ligands for internalization and degradation, as well as for cholesterol metabolism. Previous research suggested that LRP1-APP interactions favor APP processing through the amyloidogenic pathway due to LRP1 binding with sAPP770, an isoform of APP. Recent studies have shown that areas of the brain that have decreased amounts of low-density lipoprotein receptor-related protein 1 (LRP1) have an increased amount of amyloid beta plaques. When the activation of LRP1 is inhibited, there is lethality due to molting defects during the L3-L4 transitions. LRP1 has been suggested to aid in the uptake of cholesterol from the environment in *Caenorhabditis elegans* (*C. elegans*) (3).

Aquaporin 4 (AQP4) is a water channel in the central nervous system and plays a vital role in the balance of water and ions in the brain. AQP4 deficiency in the brain leads to deficits in memory and ability to learn (4). Scientists agree that expression of AQP4 protects the brain from amyloid beta plaques. An association of AQP4 and GLT1, a glutamate transporter, is present in plasma membranes and may function as a dynamic signaling platform. When this signal is disrupted, it can cause neural impairment (5).

LRP1 plays critical roles in amyloid beta metabolism and clearance in neurons. LRP1 knockdown results in decreased uptake and degradation of amyloid beta. Similarly, the deletion of AQP4 exacerbates cognitive defects and induces an increase in amyloid beta accumulation (6). Furthermore, a deficiency in AQP4 results in the decreased upregulation of LRP1 and consequently the decreased uptake of amyloid beta. This suggests that AQP4 is important in the upregulation of LRP1 and clearance of amyloid beta (7).

C. elegans are often used as a model organism for studying Alzheimer's Disease. These animals reproduce quickly, as the period from fertilization to hatching only lasts 12 hours. They mature into adults over the course of 3 days, and then lay about 300 eggs over the course of the next 3 days. Furthermore, amyloid beta plaques in Alzheimer's Disease are caused by the APP gene in humans, which is homologous to the *apl-1* gene in *C. elegans*.

The current treatments of Alzheimer's Disease interfere

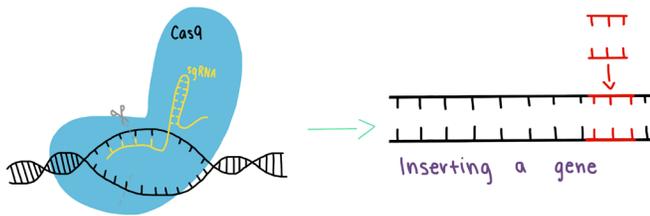


Figure 1: Simplified diagram of CRISPR-Cas9. A double-stranded break is made in the DNA by Cas9, and then the cDNA is inserted.

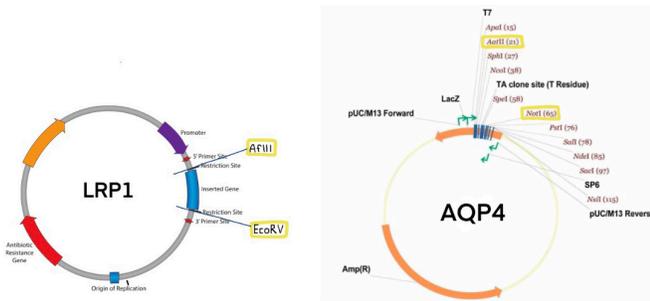


Figure 2: LRP1 and AQP4 plasmid diagrams. Plasmids were commercially obtained. Plasmids were then digested with enzymes depicted to remove the cDNA insert of LRP1(AfIII and EcoRV) or AQP4 (NotI and AatI).

with other associated diseases and do not improve cognitive impairment. For example, neprilysin is utilized to break down amyloid beta plaques, but patients with cardiac conditions take a drug called LCZ696 which inhibits neprilysin. Therefore, new therapeutic options are necessary for those that cannot use neprilysin (9).+

A method called CRISPR-Cas9 has been recently implemented to use for gene editing (**Figure 1**). CRISPR-Cas9 requires a single guide RNA (sgRNA) which can be generated from a CRISPR design program. Additionally, a single-stranded oligonucleotide (ssODN) with 5' and 3' homology arms are designed to contain the gene of interest and restriction sites (10). In addition, the Cas9 recombinant protein is used. In this study, the CRISPR Cas9 system was used for gene knock in of LRP1 and/or AQP4.

Plasmids containing genes of interest were microinjected into the *C. elegans* (**Figure 2**). This is the most widely used form of gene editing in *C. elegans* to create transgenic worms. The DNA is inserted into the distal gonad syncytium or directly into the embryos. Transformation markers are inserted with the gene of interest in order to identify the transgenic worms. In order to see which worms show the phenotype, a GFP vector is also inserted which makes the worm or egg glow green to show the presence of the trait (12).

In the study, primers were added to LRP1 and AQP4 genes matching the insertion site in the *C. elegans* genome. Once this construct was developed, the genes were microinjected into the *C. elegans* with Cas9 and a site-specific guide RNA. The effects of the addition of the genes were then tested

by running chemosensory, size, and speed assays. The negative control hypothesis was that adding no gene to the *C. elegans* would have no effect on the chemotaxis abilities, brood size, or locomotion. The alternative hypothesis was that given only LRP1 or AQP4 was inserted downstream of the *apl-1* promoter, there would be a reduced effect on the chemotaxis abilities, brood size, and locomotion compared to inserting both LRP1 and AQP4 downstream of the *apl-1* promoter in conjunction. When both LRP1 and AQP4 are overexpressed, I predict a significant increase in the uptake of beta amyloid, as evidenced by increases in chemotaxis, brood size, and locomotion. This study could be a major step in the research to develop a better method of curing Alzheimer's at the source, which is a DNA mutation. Once the proteins are overexpressed, the primary presentation of Alzheimer's, neurodegeneration, will be limited.

RESULTS

A GFP plasmid was microinjected in conjunction with the LRP1 and AQP4 genes. The fluorescence of the injected *C. elegans* confirmed that the LRP1 and AQP4 genes were being expressed. To assay the predicted changes in the *C. elegans* after the addition of the AQP4 and LRP1 genes, I measured chemotaxis abilities, size, and locomotion. The chemotaxis assay was run using known attractant of *C. elegans* to measure sensory ability. The chemotaxis index shows an increase in chemosensory ability by moving away or towards certain smells. Because neuronal amyloid beta expression is known to induce defects in chemotaxis, I expected that a chemotaxis assay would be an appropriate measure of the effects of various proteins on amyloid beta levels (13). Additionally, *apl-1* mutations cause defects in movement and brood size, which correlates with the effects of LRP1 and AQP4 on the *apl-1* mutation in Alzheimer's.

To measure the neurological changes after overexpression of the candidate genes, I assayed chemotaxis, brood size, and locomotion. I tested these phenotypes in three different strains of *C. elegans*, including N2 (wildtype), CL2006, a strain that expresses amyloid beta plaques, and VC1246, a strain that expresses mutant *apl-1*. The chemosensory assay showed that when the *C. elegans* were microinjected with LRP1 or AQP4, there was no significant effect on the chemotaxis index. But, when they were microinjected in conjunction, there was a significant increase in the chemotaxis index in all strains. The VC1246 strain showed an increase in the chemotaxis index by 0.5 when both LRP1 and AQP4 were microinjected ($p=0.006785$, p -value < 0.05, student's *t*-test). Whereas, the CL2006 strain showed an increase of 0.12 in the chemotaxis index with both LRP1 and AQP4 overexpressed (**Figure 3**; $p=0.007554$, p -value < 0.05, student's *t*-test). This was expected and supports the hypothesis that together, LRP1 and AQP4 improve chemotaxis abilities. The simultaneous overexpression of LRP1 and AQP4 improved the neurodegenerative phenotype, but did not completely rescue the phenotype to wildtype levels.

The brood size assay showed that there was no significant increase in brood size for any strains, microinjected with LRP1 or AQP4, or both (Figure 4). This was unexpected since the expression of beta amyloid is known to decrease the brood size, and therefore with the addition of LRP1 and AQP4, the brood size should have increased (14). Brood size may have increased growth after a few days and could be tested in the future. In the speed assay, the speed of the *C. elegans* did not increase with the microinjection of LRP1 or AQP4. But when they were microinjected in conjunction, the speed in the VC1246 strain increased significantly by about 20 $\mu\text{m/s}$ overexpressed (Figure 5; $p=0.0269$, $p\text{-value} < 0.05$, student's t-test). This was expected for this strain and suggests that the uptake of the beta amyloid by LRP1 and AQP4 allowed the muscle to contract more efficiently, thereby enabling the animals to move faster. The N2 control did not show a significant increase when microinjected with either of the two genes. Thus, the results support the hypothesis that combined overexpression of LRP1 and AQP4 would increase chemotaxis ability and locomotion more significantly than either protein overexpressed alone.

Overall, the study revealed an increase in the neurological ability of *C. elegans* microinjected with both LRP1 and AQP4 as assessed by an increase in chemosensory ability and speed. These results show that individually, the LRP1 and AQP4 proteins had no significant effect on chemosensation, but when overexpressed in conjunction, they increased the chemosensory capacity of the VC1246, the strain with the *apl-1* mutation, and CL2006, the strain with beta amyloid plaques present.

DISCUSSION

Alzheimer's Disease, a disease with an unclear physiology, is a global health issue of increasing importance. Current treatments focus on mitigating side effects, but do not address the underlying mechanism of disease, which is hypothesized to be caused by accumulation of beta amyloid plaques. The results showed that when LRP1 and AQP4 were given in conjunction, this treatment significantly reduces amyloid beta plaques, as seen through the increase in chemotaxis ability and increase in speed in the strain of *C. elegans* that expresses plaques in their muscle. I conclude that LRP1 and AQP4 function in conjunction to reduce the formation of additional amyloid beta plaques and break down the buildup of these plaques that were previously formed. Since the APP mutation in humans results in the depletion of AQP4 and LRP1, replacing these proteins could contribute to a better sense of environment as seen in the VC1246 worms (15). Our analysis suggests that the *C. elegans* overexpressing both LRP1 and AQP4 had the greatest reduction of neurodegeneration, while overexpression of LRP1 or AQP4 alone only improved it to a lesser degree.

Limits on time, budget, and experience restricted the extent of the experiment. The last steps of a gene editing study are to typically sequence the genome and check mRNA

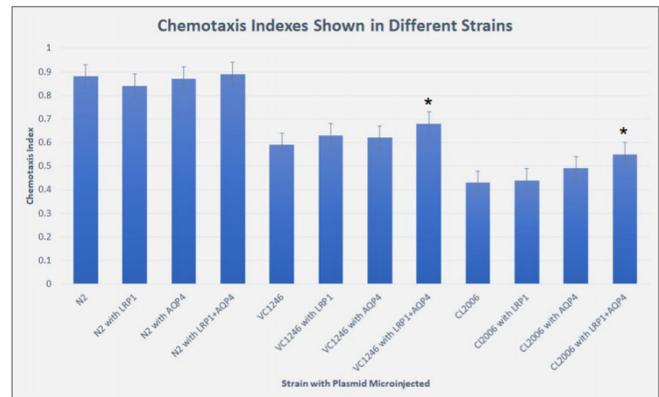


Figure 3: Chemotaxis indexes of different strains after injection. The graph depicts chemotaxis index scores for each strain tested ($n=500$ for each strain). Error bars represent standard error, and asterisks (*) denote statistical significance ($p\text{-value} < 0.05$, student's t-test).

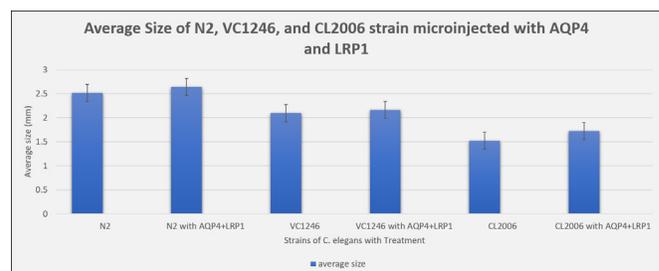


Figure 4: Average size of N2, VC1246, and CL2006 strains microinjected with LRP1 and AQP4. The graph depicts average brood sizes for each strain tests ($n=100$ for each strain). Error bars represent standard error, and asterisks (*) denote statistical significance ($p\text{-value} < 0.05$, student's t-test).

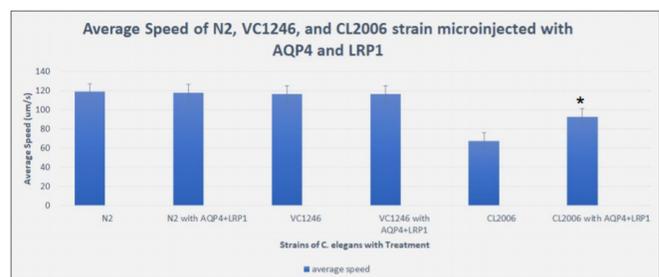


Figure 5: Average speed of N2, VC1246, and CL2006 strains microinjected with LRP1 and AQP4. The graph depicts average speed for each strain tests ($n=100$ for each strain). Error bars represent standard error, and asterisks (*) denote statistical significance ($p\text{-value} < 0.05$, student's t-test).

and protein expression. These methods were not available due to time and monetary constraints. The expression of the GFP protein, however, suggests a successful edit. Testing of the CRISPR-Cas9 system demonstrated up to an 86% precise genome editing efficiency; while this is high for the current methods of genome editing that exist, it is not perfect (16). Furthermore, genome editing has potential side effects.

The additional copies of LRP1 and AQP4 could potentially interact with other proteins in unknown manners.

Although the data suggests an increase in sensory ability and movement under our experimental manipulations, future research can assess the long-term effects as the worms age, as well as effects on their progeny. The CRISPR-Cas9 system can also be applied to a plethora of other diseases by allowing the correction of many missense mutations. The effect of correcting these mutations can then be studied on a larger scale in other model organisms, such as mice, to assess the potential for reversal of the disease (17). In addition, other genes such as CR1 that control the flow of proteins and waste across cellular membranes can be microinjected to test other possible proteins that could decrease neurodegeneration (18). RNAi feeding strains can also be used to knock out genes to show if the removal of the *apl-1* gene influences neurodegeneration. Other strains of *C. elegans* expressing different phenotypes associated with Alzheimer's Disease can be further tested to account for the many different hypotheses currently being debated. Additionally, other properties can be assayed in the future, such as serotonin sensitivity, which can further establish the phenotypic effects of neurodegeneration.

METHODS

C. elegans strains

All strains of *C. elegans* were obtained from the Caenorhabditis Genetics Center at the University of Minnesota. The N2 strain of *C. elegans* is the wildtype strain. The VC1246 strain has the *apl-1* gene mutated, which is homologous to an APP gene mutation in humans. The CL2006 strain is categorized with Alzheimer's Disease with the addition of the UNC-54 gene, which codes for amyloid beta in humans. This strain presents amyloid beta in their muscles. The CL2006 strain is temperature-sensitive, meaning that paralysis and egg-laying deficiencies arise when organisms are raised at 20°C.

Transformation of AQP4 and LRP1 plasmids into *E. coli* with ampicillin selection

Highly Competent DH5alpha *E. coli* (New England Biolabs, NEB) was thawed on ice and 50 µl were added to LRP1/AQP4 DNA (genomics-online). The reaction was mixed gently by pipetting and flicking the tube 4-5 times. The mixture was placed on ice for 30 min, then heat shocked at 42°C for 30 sec. 950 µl of LB media (NEB) was added, and the tube was placed in a shaking incubator at 37°C for 1 hr. From each culture, 50-100 µl of bacteria were spread onto plates and incubated overnight. The transformed cells were grown on ampicillin plates to select for successfully transformed, ampicillin-resistant bacteria.

DNA extraction

Plasmid DNA was extracted from the transformed bacteria using the Monarch plasmid miniprep kit according to the manufacturer's protocol (NEB).

AQP4 and LRP1 double digests

Using the restriction enzymes around the insertion site, an overnight double digest was run to cut out the cDNA from the cloning vector. For the AQP4 plasmid, the restriction enzymes NotI and AatII (NEB) were used. For the LRP1 plasmid, the restriction enzymes AflIII and EcoRV (NEB) were used. Digests were composed of 4 µl Multi Core Buffer, 0.2 µl BSA, 10 µl DNA, 1 µl of each enzyme, and 4.8 µl of sterile water and kept in the fridge overnight. To confirm digestion, ethidium bromide (Biolabs) gels were made with 1X TAE Buffer (Biolabs) and then placed into 1X TAE Buffer to run. 1 µl of loading dye and 5 µl of DNA were added per lane. Gels were run at 150 V for an hour. UV light was then used to visualize bands.

PCR and Gibson Assembly to attach primers and DNA fragments

A 25 µl mixture was made with 2.5 µl 10X standard TaqReaction Buffer (NEB), 0.5 µl 10mM dNTPs (NEB), 0.5 µl 10 µM Forward Primers, 0.5 µl 10 µM Reverse Primer, brought up to 25 µl with Template DNA. The reaction was gently mixed and put in the PCR machine. The cycle was 95°C for 30 seconds, 30 cycles of 95°C for 20 seconds, then 60°C for 30 seconds, then 68°C for 1 minute, then 68°C for 5 minutes and finally held at 4°C.

For the Gibson Assembly mix, 1 pmol of DNA fragment, 10 µl of Gibson Assembly Master Mix (NEB), and 10 µl of deionized water were added to a tube and incubated in the thermocycler at 50°C for 60 minutes.

Microinjection

Microinjection was done with the nanoliter (World Precision Instruments) and paraffin oil was used to ensure worms stayed still. A single guide target RNA sequence was created by SYNTHGO to place the DNA inserts downstream from the *apl-1* promoter. The Cas9 protein was obtained from Sigma Aldrich. The final injection mixture was: 5 µmol of single guide RNA, 5 µmol of Cas9 nuclease, 50 ng/µl of DNA insert and GFP plasmid. These components were homogeneously mixed by gentle pipetting. The mixture was loaded into needles and *C. elegans* were injected at the gonad syncytium and then transferred to new NGM plates after recovery.

Chemotaxis Assay

Chemotaxis plates were first labeled with the center in the middle, control on one side, and attractant on the other side. Approximately 100 worms were washed using M9 Buffer three times and then placed in the center, with one drop of sterile water on one end of the plate and a known attractant on the other side of the plate at the marked areas. A disposable plastic pipette was used as the dropper. 2 µl of 0.5M sodium azide (Biolabs) were put on both sides to paralyze the worms. After 1 hour, the plates were chilled at 4°C for 15 minutes to stop worms from moving and then worms on both sides

of the plate were counted and recorded. The data was then analyzed, and the chemotaxis index was determined. The chemotaxis index is calculated by (number of worms at attractant – number of worms at the control)/ (total number of worms).

Locomotion Assay

Thirty second videos of worms crawling on NGM plates were taken using a microscope. The speed of the worms was quantified by the worm tracker extension in ImageJ.

Measuring Brood Size

Brood size was measured using an application called ImageJ. Images taken of the *C. elegans* were traced using a tool on ImageJ, and the brood size was measured. The sizes were then downscaled based on the amount of zoom in the pictures.

Statistical Testing

T-tests were performed comparing the data to the wildtype and the p values were analyzed in Excel to determine which values were under 5% and therefore statistically significant.

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