Exploration of Neurodegeneration Through Backwards Locomotion in *Caenorhabditis elegans*

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Abstract

This study aimed to investigate the effects of RNA interference on the *dop-1*, *dop-3*, and *glr-1* receptor genes in *Caenorhabditis elegans* (C. elegans), which are crucial to dopaminergic and glutamatergic signaling pathway regulation. Disregulation of these pathways contributes to the pathogenesis of neurodegenerative diseases such as Parkinson's disease. Two behavioral assays were used to assess the genes' roles in C. elegans locomotion and their involvement in chemosensation, via drop-test assay, and mechanosensation, via nose touch assay. However, DNA sequencing revealed that instead of the intended receptor genes, the transformed bacteria contained *cest-26* and *vps-41* genes. Thus, the results were inconsistent with the hypotheses and previous studies. Nevertheless, the assays were completed and avoidance index values that measure backwards locomotion were calculated. In the drop-test, all experimental strains had very low avoidance index values, while in the nose touch, all strains showed avoidance index values of approximately 0.85. These results indicated that the *cest-26* gene, a lipid content regulator, had no observable effect on locomotion, chemosensation, or mechanosensation. *vps-41* has been proven to contribute to dopamine neuron protection, and the drop-test assay results suggest some gene involvement in chemosensation, but the direct causal relationship between the gene and the behavior requires further study.

Keywords: C. elegans, chemosensation, mechanosensation, backwards locomotion, Parkinson's disease

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The nematode C. elegans is commonly used as a model organism when studying neurodegeneration at the intersection of fields such as molecular and cellular biology as well as neurobiology. Its defined connectome and relatively small number of neurons, 302 to be exact, allow for targeted studies and clearer understanding of the roles of neuromodulators in various behaviors than when more complex organisms are utilized (Chase et al., 2004). Despite the reduced complexity of the C. elegans neural system compared to mammals such as humans, information gleaned from research on the neuromodulators and signaling pathways that make up this system can further our understanding of the human nervous system because of the similarities in structure and function between both animal systems. These similarities are apparent even at the genomic level, as many C. elegans genes involved in neurotransmitter signaling pathways have human orthologs that serve parallel roles (Hart, 2006).

Various biological modification techniques have been used to identify the roles that certain genes and their expression play in regulating C. elegans behavior. A common technique, used to ascertain gene function without modifying the DNA of the organism, is RNA interference (RNAi). This process targets the mRNA of the chosen gene(s) for degradation and prevents it from being expressed, and subsequent observations through behavioral assays or fluorescence can be used to determine the functions of the gene(s).

In this manner, RNAi of genes involved in the dopaminergic and glutamatergic pathways in C. elegans have allowed researchers to gain a better understanding of these signaling pathways and their contribution to various C. elegans behaviors, such as feeding and sensory responses (Hart, 2006). In this study, double-stranded RNA was targeted to perform RNAi on three

separate receptor genes to explore the aforementioned pathways: *glr-1*, *dop-1*, and *dop-3*. *glr-1* is a glutamate receptor that enables enzyme binding activity in C. elegans. As well as being expressed in interneurons that initiate forwards and backwards locomotion, *glr-1* is vitally important to the ASH neuron that detects mechanical stimuli of the nose, osmotic levels of the environment, and some repellants (Maricq et al., 1995). The ASH neuron contacts interneurons on the glutamatergic signaling pathway, allowing the worms to respond to mechanical stimuli. Deletions of *glr-1* have shown no effect on osmotic avoidance or chemical stimuli, but evidently have a significant effect on backwards locomotion in response to mechanosensory stimulation assays (Zheng et al., 1999). The human ortholog to *glr-1* is the AMPA type glutamate receptor subunit GluR1 (Aguilera, 2020). *dop-1* also plays a role in C. elegans's response to mechanosensory stimuli, as it is expressed in mechanosensory neurons ALM and PLM. *dop-1* is an excitatory D1-like dopamine receptor that regulates locomotion and is also involved in memory and learning (*Biogenic Amine Neurotransmitters in C. Elegans*, n.d.-b). Its human ortholog, DRD1, is the most abundant dopamine receptor in the central nervous system (*Dop-1 (Gene) - WormBase : Nematode Information Resource*, n.d.). *dop-3*, a dopamine receptor that falls within the g-protein-coupled receptor family alongside *dop-1*, inhibits locomotion rate and affects olfactory sensation. It is expressed in the same neurons as *dop-1*, and the two dopamine receptors have been shown to interact antagonistically. The human ortholog of *dop-3*, DRD3, has been hypothesized to be involved in numerous neurological diseases (*Dop-3 (Gene) - WormBase : Nematode Information Resource*, n.d.).

The dopaminergic and glutamatergic pathways that the above genes contribute to are not only involved in normal C. elegans behaviors such as area-restricted search, but are also crucial to various neurodegenerative diseases when disrupted (Hills et al., 2004). Abnormalities in the

dopaminergic pathway, which includes the receptors, in particular, can be found at the root of Parkinson's disease (PD), among others (Rangel-Barajas et al., 2015). Similarly, disregulation of the glutamatergic signaling pathway results in pathogenesis of PD, often through accumulation of extracellular glutamate and the resulting toxicity (Wang et al., 2020). Therefore, it is crucial to understand the role that receptors of both pathways play in regulating normal behavior, to better understand the mechanisms of the receptor and signaling disregulation that can be seen in PD and other neurodegenerative diseases.

Methods

Design

A quantitative approach was used for this experiment. In order to knock down the *dop-1*, *dop-3*, and *glr-1* genes in the C. elegans, stage L4 *rrf-3* C. elegans were fed E. coli that were transformed with the RNAi knockdown genes. L4 C. elegans were used as these worms are developing vulvas and not quite ready to reproduce. *rrf-3* strain C. elegans were used as these worms allow for more foreign RNA, consequently creating a stronger RNAi phenotype (Simmer et al., 2003). The offspring of the nematodes that consumed the E. coli were used for the assays, since they had the bacterial plasmid integrated into their genetic material. This left the worms without the function of the selected genes. The *rrf-3* strain C. elegans were also tested using the behavioral assays, serving as a negative control for the other experimental strains.

Behavioral Assays

Because RNAi of the three genes have been proven to have an effect on chemosensation and mechanosensation in C. elegans, two types of behavioral assays were used to assess the functions of these genes in both locomotion regulation and the aforementioned sensory responses. Chemosensation is the C. elegans's ability to perceive and react to a certain chemical

stimulus, while mechanosensation refers to their ability to react to mechanical stimuli, such as touch and vibrations. The former response was tested through the drop test assay, and the latter was tested through the nose touch response.

Drop test

The drop test was developed to study the avoidance response of worms to repellants (Hart, 2006). In this study, the repellant chemical was a 30% nonanol solution diluted in 100% ethanol (Ezak & Ferkey, 2010). This solution was dropped near the tail of the worm while it was moving forwards. 10µl glass capillaries pulled by hand on a flame were used to administer a small dosage (~5 nanoliters) of the solution, as a volume exceeding a few nanoliters would cause the worm to swim in the solution (Hart, 2006). Once in contact with the tail, the repellant reaches the worm's anterior sensory organs via capillary action. If the chemical is sensed as a repellent, the worm will exhibit a backwards movement, while if the chemical is not sensed as a repellent, the worm will continue its forwards movement. To ensure the worms do not become conditioned to a specific behavior, an inter stimuli interval (ISI) of at least 2 minutes was maintained between successive repellant drops to one worm. Additionally, no single worm was tested for more than 20 consecutive drops (Hillard et al., 2004).

The movement of worms were measured using an avoidance index (AI). The AI follows this formula: $\frac{\#\ positive\ responses}{total\ \#\ of\ trials}$. Thus, the AI will range between the values 0 and 1, where higher values indicate stronger responses and lower values indicate weaker responses (Hillard et al., 2002). The worms' response to the repellent drop was scored as positive if the worm reacted within 4 seconds of contact with the repellent (Hillard et al., 2002). The worms' reaction times were also tracked, as different strains of C. elegans with different RNAi knockdowns were expected to demonstrate different response times.

Nose touch

The nose touch assay was used to measure backwards locomotive response to mechanosensory in the C. elegans. An eyebrow hair was used, due to its ideal thickness. The hair could not be wet or slippery as the worms would not respond to the hair and consequently disrupt the results of the assay. A petri dish with a bacterial lawn composed of an OP50 and LB dilution (1:9 ratio) was required, so the worm was kept in close to normal living conditions. Worms' response to the nose touch assay are more accurate when in bacteria, but using a thin lawn also stopped the nematode from digging into the bacteria to avoid the hair. A worm was placed on the agar plate, and the hair was placed in front of the nematode at a 90° angle to the worm's head, in the direction it was moving (Park et al., 2021). When the worm was touched by the hair on its nose, its ASH sensory neurons that control withdrawal responses were activated (Maricq et al., 1995). The time it took for the nematode to initiate backwards locomotion was tracked and recorded. The expected positive result was an initiation of backwards locomotion within four seconds after contact with the eyebrow hair. The assay was completed 5 times for every worm with 10 worms of every strain. Moreover, the worms were not hit with the hair on any other part of their body, as this would lead to habituation and inaccurate results. Both of these measures were taken to ensure accurate and efficient results.

The worm's response to the hair was recorded with a visual observation of the nematode's movement along with a timed reaction rate, the number of seconds it took for the nematode to move backwards after it made contact with the hair. Then, the percentage of worms that exhibited backwards movement was calculated. Calculating the percentage of backwards movement for the drop-test is identical to the formula for calculating the AI.

Results

Sequencing Results

Prior to testing the behavior of the different RNAi knockdown strains of C. elegans, a DNA miniprep was conducted, which allowed us to isolate the plasmid DNA from bacterial cultures. The resulting plasmid DNA was sent to a sequencing center, to ensure that the E. coli had been transformed with the genes we intended to transform: *dop-1*, *dop-3*, and *glr-1*. Unfortunately, due to the time constraints of this project, the behavioral assays were conducted before we received the sequencing results. The sequencing results were received after the assays were completed. From the sequencing results, we discovered that the *dop-1* gene was actually a *cest-26* gene, which is predicted to code for a carboxylesterase and, while being relevant to the production of lipids and implicated in certain cancers, is not relevant to the study of neural signaling pathways. However, the *dop-3* gene turned out to be the C. elegans *vps-41* gene, which is involved in protecting the worms against the degeneration of dopamine neurons. We did not receive the sequencing results for the *glr-1* gene, but based on the inaccuracy of the *dop-1* and *dop-3* genes, it was reasonable to conclude that the *glr-1* gene was also incorrect.

During the nose-touch assay, the *"glr-1"* worm RNAi strain exhibited close to normal response, moving backwards just as the control *rrf-3* worms did. The *"glr-1"* worms did not respond to the drop test assay in the same way as the control, continuing to move forwards instead of initiating backwards locomotion as was expected, indicating that the gene that was knocked-down did have some impact on C. elegans behavior. However, because it is impossible to know which exact gene was knocked down, it cannot be determined whether the gene was directly or indirectly related to locomotion, chemosensation, or some other system that altered the drop test response.

Drop-test

The avoidance index for the drop-test assays are very low. Apart from the *rrf-3* strain, which was the negative control, all six other experimental strains had a low avoidance index. This indicates that the genes that were knocked down affected the worms' chemosensation. The table below shows the avoidance index of each RNAi strain C. elegans tested with the drop-test assay.

Table 1

Avoidance index ($\frac{\#\ positive\ responses}{total\ \#\ of\ trials}$) of each RNAi strain for the drop-test assay

C. elegans Strain:	Avoidance Index:
rrf-3 strain	0.86
RNAi knockdown of *cest-26*	0.28
RNAi knockdown of *vps-41*	0.10
RNAi knockdown of *"glr-1"*	0.06
RNAi knockdown of *cest-26* & *vps-41*	0.02
RNAi knockdown of *cest-26* & *"glr-1"*	0.14
RNAi knockdown of *vps-41* & *"glr-1"*	0.02

Nose Touch

The avoidance index for the nose touch assays seem to hover around the value of approximately 0.85. This indicates that the worms exhibited backwards movement about 85% of the time, no matter which genes were knocked down. The table below shows the avoidance index of each RNAi strain C. elegans tested with the nose touch assay.

Table 2

Avoidance index of each RNAi strain for the nose-touch assay

C. elegans Strain:	Avoidance Index:
rrf-3 strain	0.82
RNAi knockdown of *cest-26*	0.88
RNAi knockdown of *vps-41*	0.84
RNAi knockdown of *"glr-1"*	0.86
RNAi knockdown of *cest-26* & *vps-41*	0.80
RNAi knockdown of *cest-26* & *"glr-1"*	0.84
RNAi knockdown of *vps-41* & *"glr-1"*	0.90

Discussion

Limitations

Most limitations for this project stems from how the genes that were knocked down were not the genes that we intended to knock down. We were unable to clearly examine the relationship between the intended genes (*dop-1*, *dop-3*, *glr-1*) and the dopaminergic/glutaminergic pathways, but instead unintentionally observed the impact of *cest-26*, *vps-41*, and *"glr-1"* on chemosensation and mechanosensation.

It is also important to note that the assays themselves have limitations. For example, both the drop-test and the nose touch assays involve manual observation. Thus, both assays were very time-consuming and induced fatigue in the researchers performing them. Additionally, the drop test especially is at the risk of inconsistencies in drop delivery between trials. It was critical to deliver very small drops to avoid killing the worm in the nonanol dilution solution, but delivering these small drops at the right location was a challenge. Administering repellent drops that were

too large and immediately or eventually killed the worm was not a rare occasion. These factors most probably affected the final results of the assays.

Future Directions

Needless to say, to properly investigate the glutamatergic and dopaminergic signaling pathways using the RNAi methods proposed in this study, it is critical to obtain the correct genes.

Interestingly, despite the unintended change in RNAi genes, the C. elegans vps-41 gene is related to the original purpose of this experiment, as it has been mechanistically linked with the prevention of the pathogenesis of PD. This gene is thought to be involved in lysosomal trafficking, and assists with neuroprotection by preventing build-up of compounds such as alpha-synuclein (α-syn) (Gaeta et al. 2022). Aggregates of α-syn lead to neurodegeneration due to neurotoxicity and contribute to pathogenesis of PD. In particular, it has been proven that expression of the human *vps-41* gene prevents dopamine neuron loss, thereby protecting dopaminergic signaling pathways from disregulation (Ruan et al., 2010).

However, the *rrf-3* strain of worms that underwent RNAi in this study are not transgenic PD models, and thus, the function of *vps-41* as it relates to α-syn is not relevant. In non-transgenic worms, the *vps-41* gene is primarily involved in lysosomal fusion events in the endomembrane system and "for transport of lysosomal membrane proteins and regulated secretion [of neuropeptides]" (van der Welle et al., 2021). Lysosomes play a leading role in ensuring that unwanted substrates do not aggregate inside cells and even extracellularly, and degrade toxic compounds such as α-syn (Lie & Nixon, 2019). Because efficient lysosome trafficking is crucial for all cellular and neural functioning, it is entirely possible that the RNAi of the *vps-41* gene in this study resulted in disruption of normal neural functioning for various neurons, potentially including the neurons that control chemosensation. This may explain the

difference in behavioral results not only between the *vps-41* RNAi and control worms during the drop test assay, but also between the *vps-41* results of both assays. This is because the *vps-41* (*"dop-3"*) worms were able to move backwards in response to mechanosensory stimulus, indicating that the neurons that regulate mechanosensation and backwards locomotion were sufficiently functional, while the lack of backwards movement in response to the drop test assay suggests that chemosensation in the worms was affected (Sengupta, 2007).

Conclusion

The high nose touch avoidance index results and significantly lower drop-test avoidance index results, although unexpected, highlight a potential effect of the *cest-26* and, in particular, the *vps-41* gene on abnormal chemosensation and following response behaviors in .C elegans. Because these genes are not directly related to chemosensation or locomotion, these results present potential new directions of RNAi research for further exploration of the role that *cest-26* and *vps-41* genes play in regulating C. elegans behavior that is central to the study of neurodegeneration.

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