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The Role of CSN-5 in the Migration of Neurons in C. elegans

Rebekah Anne Cocks  
Worcester Polytechnic Institute

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The Role of CSN-5 in The Migration of Neurons in *C. elegans*

A Major Qualifying Project Report
Submitted to the Faculty
of the
WORCESTER POLYTECHNIC INSTITUTE
in partial fulfillment of the requirements for the
Degree of Bachelor of Science
by

Rebekah Cocks
May 1, 2014

Project Advisor
Professor Elizabeth Ryder

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Abstract
During the development of *C. elegans*, neurons migrate to specific locations in the worm through the actions of various proteins; one protein known to be involved in neuronal migration is MIG-10. As part of an ongoing effort to better understand other proteins involved in the process of neuronal migration, CSN-5, a protein involved in proteosome activity and found to associate with MIG-10, was knocked down using both feeding RNAi and through the creation of a transgenic *C. elegans* line that produced dsRNA. No effect was measured in the migration of the neurons; however, a conclusion was not reached on the effect of CSN-5 on neuronal migration, because no effect was seen in the MIG-10 controls. The lack of effect in the control may be due to the promoter used for expression, and thus future experiments should use a different promoter that turns on earlier in development.
Acknowledgements

I would like to extend my greatest gratitude to Professor Ryder for her continued support and guidance throughout this entire project and to Erin Flaherty for her ever present help in lab and in being so patient and willing to help me to learn the techniques used in the lab.
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Introduction

The nervous system is comprised of a network of neurons that connect to one another in circuits. These circuits send signals throughout the organism to interpret sensations from stimuli, regulate bodily functions to maintain homeostasis, process thought, react to internal and external stimuli, and carry out all other biological functions. Neuronal migration during development plays a key role in the successful connections of these circuits (Quinn and Wadsworth, 2008). During development, neurons migrate distances many times greater than the lengths of their cell bodies to their final place in the organism. When neuronal migration is either interrupted or fails in some way, the correct connectivity in the nervous system is not achieved, which leads to dysfunction. This dysfunction may be linked to various nervous symptom disorders such as Down’s syndrome, autism, and many others. The study of neuronal migration and how this process works and how it is controlled genetically, has the potential to lead to finding treatments for neuronal connectivity disorders and for nervous system injuries (Quinn and Wadsworth, 2008). Research has shown that extracellular cues signal changes in the neuron’s intracellular messenger systems, which then signal changes in the cytoskeleton, thus showing that neuronal migration is rooted in changes in the cytoskeleton (Heng et al., 2012). These studies have revealed some of the intracellular and extracellular molecules that signal neuronal migration, along with the genes that transcribe these molecules. This project specifically looked at the role of the gene, csn-5, on the migration of neurons in C. elegans. CSN-5 has been found to associate with MIG-10, a known signaling molecule in neuronal migration.
Neuronal Migration via Axon Guidance

Axon growth is one of the early steps in neuronal migration. A specialized structure called the growth cone, positioned at the tip of the growing axon leads the axon in the direction guided by extracellular guidance cues. The growth cone is constantly examining the extracellular environment for attractive or repulsive cues, by extending and retracting the membrane projections on the growth cone. These protrusions give the appearance of finger-like projections on the axonal growth cone (Dent et al., 2011). As the membrane is extending and retracting, the membrane is coming in contact with extracellular signals. These extracellular guidance cues include slits, netrins, ephrins, semaphorins, wnts, sonic hedgehog, and bone morphogenic proteins. As the signals interact with the guidance receptors on the surface of the axon growth cone (Figure 1), Filamentous actin (F-actin) and microtubules asymmetrically accumulate in the growth cone (Quinn and Wadsworth, 2008). The first stage of axon growth, protrusion, occurs after receiving the external cues which is when the F-actin polymerization drives the extension of the cell membrane at the growth cone. The microtubule driven transport of the organelles and vesicles into the peripheral regions of the cell happen in the second step of axon growth, engorgement. The third and final step of axon growth, consolidation, occurs when the extended membrane and the rest of the growth cone forms into an axonal shaft and the organelles and vesicles move into place (Dent et al., 2011).
Figure 1: Model of Cytoskeletal Dynamics in Growth Cone (Dent et al., 2011). The growth cone is exposed to a positive growth signal in panel A. Panel B shows the protrusion of the growth cone led by filamentous actin. Panel C shows the integration of the microtubules and actin, and panel D shows the retraction of the actin and microtubules of the growth cone on the side opposite from the guidance cues.

Other processes, such as exocytosis, calcium signaling and protein translation, may also play a role in growth cone signaling because they have been found to also show asymmetry in response to guidance cues. The question that remains to be fully answered is how the cells synthesize the
information gathered from the external gradient into the response shown by asymmetry within the cell and growth cone. Recent studies have found proteins that are also asymmetrically concentrated in the growth cone in response to guidance cues and these proteins and the genes that control them are being studied to further determine the role each of these proteins in neuronal migration (Quinn and Wadsworth, 2008).

C. elegans as a Model Organism

Due to the complexity of the human body, along with the ethical implications and impracticality of doing extensive genetic research on humans, model organisms are used extensively in biological, and specifically genetic research. The use of model organisms is made possible by the conservation of the genetic code in many organisms and the homologous nature of genes in humans and some organisms. One model organism commonly used in research is the worm, *Caenorhabditis elegans*, also known as *C. elegans*. *C. elegans* is a frequently used model organism for a variety of reasons. It was the first multicellular organism and the second eukaryote to have its complete genomic sequence obtained, which enabled scientists to study specific genes, their sequences, the effects of any specific changes in the sequences, and the specific sequences that encode proteins (Hodgkin, 2005). *C. elegans* is also an ideal model organism due to its small size, which makes storage simpler; its transparency, which makes studying the phenotypic effects of mutations and genetic knockdowns more feasible in live specimens; its non-parasitic nature, which makes working with the species safer; and its rapid life-cycle of only three days until full development, which allows for shorter times in between generations and faster result acquisition.
Specifically, *C. elegans* has been used extensively in neurobiological research. *C. elegans* may appear to be a simple organism, but in reality, its neurons have been shown to be sophisticated information processors and are involved in complex circuits with other neurons (Hobert, 2010). The nervous system is the most complex tissue in *C. elegans*, with 302 neurons and 56 glial cells, which is equivalent to 37% of the somatic cells in a hermaphrodite worm. *C. elegans*, much like humans, has both sensory and motor circuits, exhibits behavior such as modulation and learning, and has many of the same transmitter and receptor systems as humans. The complexity shown in both cell type and cell function of the neurons, coupled with the fact that comparatively, *C. elegans* is a simple organism, makes it ideal for neurobiological research. The complexity makes its nervous system and the function more comparable to a human nervous system, but the simplicity of the organism as a whole renders *C. elegans* a useful organism for research. Another reason *C. elegans* is useful in neurobiological research is the fact that *C. elegans* is transparent, and that there are fluorescent reporter genes for almost all of the neurons, making the phenotypic effects of genetic manipulation easily detectible and easily measured in the live worms (Hobert, 2010).

**MIG-10**

MIG-10, one of the proteins that has been found to localize asymmetrically in the axon growth cone of the AVM and PVM neurons (Chang et al., 2006), may be a key in understanding how very shallow extracellular signal gradients can be magnified within the cell to produce a growth response. MIG-10 responds to UNC-6 (netrin), an attractive guidance cue (shown in Figure 2), as well as SLT-1 (slits), which are repulsive guidance cues, by localizing in the direction of the netrins and away from the slits (Xu and Quinn, 2012). When MIG-10 was overexpressed in the absence of netrins and slits, the axons showed a multi-polar phenotype with undirected
outgrowths (Quinn et al., 2006). When SLT-1 and UNC-6 were introduced into the environment of the axon with the overexpressed MIG-10, the MIG-10 became mono-polarized and the axon growth was enhanced by the overexpression. How the localization of MIG-10 triggers a cascade of intercellular signaling, that ultimately results in the extension of the axon, is still being determined. Evidence has pointed to the possibility that the localization of MIG-10 stimulates the localization of other molecules involved in actin polymerization pathways.

![Image](image.png)

**Figure 2: MIG-10 Localization in Response to Netrin:** In response to the UNC-6 signal, the UNC-40 receptor is bound which catalyzes the change of Rac-GDP to Rac-GTP and the localization of PtdIns(3,4)P₂. MIG-10 then localizes in response to these changes. (Quinn and Wadsworth, 2008).

MIG-10 is a member of the group of proteins called the MRL molecules. The molecules in this family of adaptor molecules participate in the regulation of cell adhesion, migration, cell growth, and actin dynamics. RIAM and Lamellipodin (Lpd), found in humans, are also members of the MRL family and are orthologous to the MIG-10 protein (Colo et al., 2012). MIG-10 has been studied in *C. elegans* and research has shown that MIG-10 accomplishes its role in axon outgrowth by localizing and linking other proteins involved in signal pathways that promote actin localization. MIG-10 is localized in response to the attractive guidance cue, UNC-6. The
binding of UNC-6 activates the receptor, UNC-40, which in turn localizes MIG-10 (Figures 2, 3). MIG-10 then links other proteins that signal actin polymerization (Xu and Quinn, 2012). The linkage of these proteins has been found to amplify small gradients in the extracellular region into significant intracellular signals that promote axonal growth via the localization of actin and microtubules (Quinn and Wadsworth, 2008).

**Figure 3: Schematic of MIG-10 Pathway:** The UNC-6 signaling molecule binds to the UNC-40 receptor which stimulates a signaling cascade. Phosphatidylinositol Phosphate (PIP) is phosphorylated to Phosphatidylinositol Bisphosphate (PIP$_2$) by Phosphoinositide 3-Kinase (PI3K). RAC is also activated in response to the receptor activation. RAC and PIP$_2$ then interact with MIG-10 at the RAS-Association (RA) Domain and the Pleckstrin Homology (PH) domain, respectively. MIG-10 then signals other proteins such as UNC-34 and ABI-1. UNC-34 is the *C. elegans* member of the Ena/VASP family of proteins involved in actin polymerization (Tymon, 2013).

There are three different isoforms of MIG-10, which differ only in the lengths of their N-terminal domains. The three isoforms are MIG-10A, MIG-10B, and MIG-10C. MIG-10A and MIG-10B have been researched more extensively than MIG-10C. Figure 4 shows diagrams and the differences between the isoforms.
Figure 4: MIG-10 Isoforms: Difference in the length of the N-Terminal Domain are seen in the three isoforms. Each of the isoforms have two FP4 domains, one at the N-terminal domain and one at the C-terminal domain. The RAS association (RA) domain and the Pleckstrin Homology (PH) Domain are both shown in each of the isoforms in the internal sequences (Tymon, 2013).

MIG-10 Function in Different Cells

There has also been some evidence that MIG-10 functions in cells other than just neurons. MIG-10 functions in the excretory cell, and may function in the epidermal cells that underlie the outgrowth of the excretory processes, called canals (McShea et al., 2013). The multiple locations of function may indicate that excretory cell outgrowth is in part controlled by the expression of mig-10 and that neuronal and excretory migration may be aided by the function of MIG-10 in the underlying cells. There are many available promoters in C. elegans with known locations of expressions, which makes the study and manipulation of mig-10 expression in different cell feasible. While MIG-10 has been studied extensively, there is still much that is unknown about MIG-10, including the regulation of MIG-10 expression.

CSN-5 and MIG-10

MIG-10 and CSN-5 have been found to associate in a yeast-two hybrid screen in vitro (O’Toole and Gosselin, 2008), indicating that there is a potential for their interaction during neuronal migration and development.
The COP9 Signalosome (CSN)

The COP9 signalosome (CSN) is an eight subunit, highly conserved protein complex found in both plants and animals. The functions of CSN have been found to include the regulation of protein degradation via deubiquitination – which influences protein stability, and the regulation of transcription (Chamovitz, 2009).

CSN-associated kinases were found to regulate transcription via the regulation of the phosphorylation of the substrates of the ubiquitin-proteasome pathway. Many vital transcription regulators are acted on by the CSN-associated kinases and thus much of CSN’s regulation – both activation and repression – of transcription can be associated with the activity of these kinases. (Chamovitz, 2009).

CSN has been found to function in the regulation of the degradation of proteins. A one to one correspondence between the subunits of CSN and the subunits of the lid complex on the 26S proteasome indicates the association of CSN with the proteasome (Cope and Deshaies, 2003). Further research determined that CSN, when associated with the 26S proteasome, is involved with deneddylation. Deneddylation is the removal of Nedd8 from cullin-based E3 ubiquitin ligases. Nedd8 is a ubiquitin-like modifier. Ubiquitin molecules signal the degradation of the protein via relocation to the proteasome. Thus, deneddylation of the E3 ubiquitin ligases regulates ligase activity (Chamovitz, 2009).
CSN-5: Subunit of Interest

CSN-5 (also known as Jab1) is one of the highly studied eight subunits found in the CSN protein complex. CSN-5 has been found to be one of the few subunits to work, not only when associated with the rest of the protein complex, but also independently of the subunit in both the cytoplasm and in the nucleus. CSN-5 has been implicated in a variety of cellular functions. It positively regulates cell proliferation by inactivating several negative regulatory proteins. CSN-5 inactivates these proteins via subcellular localization, degradation, and deneddylation. CSN-5 also stabilizes certain proteins, is a transcriptional cofactor, and may be involved in promoting gene expression during the development of the nervous system (Shackleford and Claret, 2010).

The Mpr1-Pad1-N (MPN) terminal domain on the CSN-5 subunit contains a Jab1/CSN5 MPN domain Metalloenzyme (JAMM) motif, which appears to be key in the deneddylation activity of CSN (Figure 5). The MPN domain is a protein-protein platform, while the JAMM motif is a cofactor of enzyme activity. The JAMM motif is the same domain present in the lid of the 26S proteasome, RPN11, which is involved in the proteasome’s cleavage of the ubiquitin. While the rest of, or at least some of the other CSN subunits are necessary for the deneddylation of proteins, CSN-5 appears to have a significant role in this activity (Shackelford and Claret, 2010).
Figure 5: Domains of the CSN-5 Protein: Mpr1-Pad1-N (MPN) domain contains the Jab1/CSN5 MPN domain Metalloenzyme (JAMM) Motif, which is key in the deneddylation activity of CSN. JAMM motif is the same domain that is present in the lid of the 26S proteasome (Shackelford and Claret, 2010).

RNAi

The interference of gene expression via interfering RNA (RNAi) was first discovered in C. elegans when double stranded RNA was introduced into the organism and was found to silence gene expression. Since its discovery, RNAi has been used extensively as a method for studying expression of individual genes (Kuttenkeuler and Boutros, 2004). The mechanism of regulation of gene expression occurs when dsRNA is introduced into the cell and a complex called the Dicer recognizes the dsRNA and cleaves it into RNA fragments of approximately 20 base pairs in length. These smaller segments of RNA are called small interfering RNA (siRNA). The siRNA is then unwound by the RNA-Induced Silencing Complex (RISC). The single stranded siRNA in the RISC complex can bind to complementary sequences of RNA and regulate its expression. RISC can regulate gene expression on many levels but the more common mechanisms are through RNA degradation or translational inhibition. The mechanism of RNAi is diagramed in Figure 6 (Hannan, 2002).
**Figure 6: Mechanism of RNAi:** dsRNA enters the cell and is chewed up by the Dicer. The RISC then recognizes the small fragments of dsRNA and uses the sequence to recognize complementary sequences and either amplify, degrade, inhibit translation, or remodel chromatin (Hannan, 2002).

**RNAi in *C. elegans***

The method of gene silencing via RNAi has been used extensively in *C. elegans* due to its reliability in results, the ease with which phenotypic effects can be determined, as well as the ease with which the dsRNA is introduced into the organism. dsRNA can be introduced into the worm via injection into the adult animal, feeding of the worm with bacteria that have been transformed to express the dsRNA, or soaking the worms in solution that contains dsRNA (Figure 7). These three mechanisms have found to be effective in the introduction of dsRNA into the worms and can be utilized to study the phenotypic roles of genes via their knockdown. One caveat when designing dsRNA to be introduced into the worm is that care must be taken to ensure the sequence is specific to only the target gene. Due to conserved sequences, some genes share similar sequences and thus a poorly chosen sequence could down-regulate the target gene along with a homologous gene (Kuttenkeuler and Boutros, 2004).
Three methods for RNAi knockdown can be used in C. elegans. One method is feeding the worms bacteria that express the dsRNA and the other two are soaking or injecting the worms with dsRNA to create transgenic lines that produce dsRNA. The dsRNA then acts through RNAi to knockdown the target gene (Kuttenkeuler and Boutros, 2004).

**Microinjection**

Microinjection is one method that can be used to introduce DNA into the worms for a variety of different uses, including creating transgenic animals, selectively introducing dsRNA for RNAi knockdown, and introducing other molecules directly into the cells. DNA is injected into the distal arm of the gonad, and then is delivered to the progeny. Some of the progeny may produce a transgenic line of C. elegans.

To create a transgenic line that produces dsRNA, two plasmids are injected into the worms, one that directs the sense and one that directs the antisense sequences of the target gene. These would then form dsRNA and result in knockdown. When using the technique of microinjection of plasmids for dsRNA production, specific promoters can be used to confer transcription in certain cells and at certain times in development. This makes microinjection valuable because it enables the knockdown of the target gene in specific cells (Evans, 2006).
**Feeding RNAi**

Another method to introduce dsRNA into the *C. elegans* is through the use of feeding RNAi. This is done by expressing dsRNA specific to the gene of interest in the bacteria that the worms feed on. The dsRNA is taken up by the worms and the offspring of the worms placed on the plate are quantitated for the effect of the RNAi knockdown. This technique has been found to not be very effective in RNAi expression in the neurons of the *C. elegans*, however the TU3595 strain has been engineered to increase the effect in the neurons. The transmembrane protein, SID-1 is required for the uptake of the RNAi and the TU3595 strain is systemically *sid-1* deficient, except for the neuronal cells where *sid-1* is expressed, thus allowing the increased uptake of RNAi in the neurons (Calixto et al., 2010a).

**Project Hypothesis and Goals**

Based on the information that CNS-5 is associated with MIG-10 and the role that CSN-5 plays in the function of the proteasome, it was hypothesized that CSN-5 plays a role in the regulation of MIG-10 through MIG-10’s proteosomal degradation. To begin to test this hypothesis, this project began with the goal of working to determine the role of CSN-5 in the migration of neurons in *C. elegans*. To determine this role, a knockdown of the *csn-5* gene in the neurons of the *C. elegans* was attempted through two different methods. The first method was through cloning of a segment of *csn-5* to be used for microinjections to create a transgenic worm strain that would produce dsRNA specifically in the neurons. The second method was to feed the worms bacteria producing *csn-5* dsRNA. Both of these methods would knockdown the *csn-5* gene in the *C. elegans*. However, due to difficulties, conclusive results were not obtained to determine the role of CSN-5 in neuronal migration. Results from the attempted control knockdown of *mig-10* led to a change in the methodology.
Methods

Molecular Biology

Primer Selection

To amplify a sequence of the genes of interest for microinjection, a primer was created for PCR amplification. The primer was created by first selecting a sequence to amplify. This was done by determining a 100-200 base pair sequence on either the 5’ or 3’ end of the gene, trying to avoid highly conserved sequences. The sequences chosen were compared against the rest of the C. elegans genome to ensure the chosen segment did not have another matching segment of more than 17 base pairs. This helped to ensure that other genes aside from the gene of interest would not be knocked down. Forward and reverse primers were then created for this segment. These primers consisted of the sequence “GCGATA” as a starting sequence and then the desired restriction sites and finally approximately 25 base pairs of the sequence. Both forward and reverse primers for both the sense and the antisense strands were created in order to create dsDNA segments of the sense and the antisense strands.

PCR

Once the primers were created, they were used to amplify the segment of the gene of interest through Polymerase Chain Reaction (PCR). The Takara Ex Taq polymerase was used to amplify the DNA as this polymerase adds an Adenine on to the 5’ end of the DNA sequence, which makes the ligation into the T-tailed vector possible. The reaction components in Table 1 were reacted together and cycled through the temperature cycle found in Table 2. One reaction was done for the sense strand primers, and one for the antisense strand primers. The template that was
used was the undiluted cDNA of the gene of interest obtained from a mini-prep and the dNTPs were 10mM in each NTP (ATP, GTP, CTP, TTP).

Table 1: PCR Components per 50 µl reaction

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Takara Ex Taq</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>10X Ex Taq Buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>Template</td>
<td>2 µl</td>
</tr>
<tr>
<td>5µM Forward Primer</td>
<td>5 µl</td>
</tr>
<tr>
<td>5µM Reverse Primer</td>
<td>5 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>28.75 µl</td>
</tr>
<tr>
<td>dNTPs</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

Table 2: Temperature Cycle of the PCR Reaction

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td>2</td>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>3</td>
<td>72°C</td>
<td>1 min/kb</td>
</tr>
<tr>
<td>4</td>
<td>Cycle to step 1 30 times</td>
<td></td>
</tr>
</tbody>
</table>

After the sequences underwent PCR, the PCR product was run through a 1.3% agarose gel and extracted using the QIAquick Gel Extraction Kit.

Ligation and Transformation

The purified DNA from each PCR reaction was ligated into the pGEM T-Easy vector at a 4:1 molar ratio of insert to vector. The amounts necessary were determined by first measuring the DNA concentration and then calculating the volume necessary to have a 4:1 molar ratio. The insert and the vector then underwent a ligation reaction at 16°C overnight. The components of the reaction and the amounts are shown in Table 3.
Table 3: Components of Ligation Reactions

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA Ligase Buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>pGEM Vector DNA</td>
<td>Calculated based on concentration of insert</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>Calculated based on concentration of insert</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Calculated to bring reaction volume to 20 µl</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Five microliters of each of the ligated DNA constructs were transformed into 50µl High Efficiency Competent Cells (NEB) each. The DNA was added to the competent cells, which were then incubated on ice for 30 minutes. After 30 minutes, the cells were heat shocked at 42°C for 30 seconds and then 450µl of SOC media were added to the cells. The cells were then incubated in a shaker at 37°C for three hours. After incubation, 50µl and 150µl aliquots of cells from each construct were plated on LB+ 50µg/ml AMP plates that had been treated with 80µl of 50 µg/ml X-gal and 100µl of 1mM IPTG. The plates were incubated overnight at 37°C.

Isolating Target DNA

Five white colonies from each of the different DNA constructs were selected using the blue/white selection and placed in 5mL of liquid LB+ 50 µg/ml Amp. These cultures were incubated overnight at 37°C. The DNA from the cultures that grew was isolated using the QIA Spin Prep Mini-Prep Kit. To determine if the constructs contained the correct insert, a restriction digest of the DNA was performed (Table 4) and the constructs that showed bands consistent with the expected lengths of DNA containing the inserts were sequenced and compared with the expected DNA sequence.
Table 4: Components of restriction digests.

<table>
<thead>
<tr>
<th></th>
<th>Non-HF Enzymes</th>
<th></th>
<th>HF Enzymes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
<td>Volume</td>
<td>Components</td>
<td>Volume</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>2 µl</td>
<td>Cut Smart Buffer</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>1:10 BSA</td>
<td>2 µl</td>
<td>1:10 BSA</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>Enzyme 1</td>
<td>1 µl</td>
<td>HF Enzyme 1</td>
<td>0.3 µl</td>
<td></td>
</tr>
<tr>
<td>Enzyme 2</td>
<td>1 µl</td>
<td>HF Enzyme 2</td>
<td>0.3 µl</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>2 µl</td>
<td>DNA</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>12 µl</td>
<td>dH₂O</td>
<td>13.3 µl</td>
<td></td>
</tr>
</tbody>
</table>

RNAi Feeding

The RNAi plates used consisted of NGM agar and 1mM IPTG and 25 µg/ml carbenicillin; these plates were poured and allowed to set. The RNAi strains of bacteria that were used were streaked onto LB+50 µg/ml Amp+12.5 µg/ml Tet plates. The bacteria was grown up over night and then a clump of the bacteria was taken from the plate with a sterile pipet-tip and was placed into a sterile 15ml conical tube which contained 5ml LB+50 µg/mL Amp. The cultures were placed into a rotating drum and incubated at 37ºC overnight.

After the incubation period, the cultures were spun down in a table top centrifuge in order to concentrate the bacteria. After the cultures had been spun, all but approximately 1mL of the supernatant was removed by decanting. The pellet was then resuspended in the remaining liquid with a sterile pipet. For each RNAi strain, two plates were steriley seeded with 5 drops of the culture RNAi bacteria using a sterile Pasteur pipet. Plates were allowed to dry overnight in the hood.

After the plates were dry, four L4 C. elegans were transferred from non-starved plates to the plates seeded with the RNAi bacteria. The strains used were transferred onto two plates of each type of RNAi. The plates were then placed at 20ºC for approximately one day. After the
incubation period, the four worms, which had become adults were transferred to secondary plates that had been prepared in the same way as the primary plates and the plates were labeled in such a way that the transfer from the primary to the secondary was easily completed. Both the primary and the secondary plates were incubated at 20ºC for approximately 72 hours. After the incubation period, four L4s from each secondary plate were transferred to a tertiary plate that was seeded and prepared in the same way as the primary and the secondary plates. The worms left on the secondary plates were washed off and the migration of the neurons in the L4s were measured. The tertiary plates were stored at 20ºC for approximately 72 hours, at which time they were washed off and the migration of the neurons in the L4s was measured.

**Quantitative Analysis**

**Placing Worms onSlides**

In order to measure the migration of the neurons in the worms, several drops of M9 were placed onto the plate by using a sterile Pasteur pipette. The plate was then swirled to remove the worms from the plate and into the liquid. The liquid solution was then removed from the plate using a Pasteur pipette and was placed into a 1.5mL Eppendorf tube using a Pasteur Pipette.

A premade 2mL aliquot of 2% agarose gel was heated to liquid in the microwave and 20µl of 1M sodium azide was added to the agarose. Two drops of the resulting solution was placed in the center of a microscope slide and another blank microscope slide was placed on top of the slide to form an agarose pad. Using a 20µl micropipette, 5µl of the worm pellet that formed on the bottom of the Eppendorf tube was placed on the agarose pad after removal of the top microscope slide and a blank cover slip was immediately placed on top.
Measuring Neuronal Migration

Using a Zeiss compound microscope equipped with epifluorescence, the L4 progeny were examined using DIC and fluorescent imaging. The DIC was used to view the bodies of the worms and the fluorescent imaging was used to view the ALM and the AVM neurons which were marked with GFP. Pictures of the bodies of the worms were taken along with pictures showing the ALM and the AVM neurons, then the fluorescent and the DIC pictures were overlaid in order to measure the migration of the neurons. The program ImageJ was used to measure the neuronal migration based on the pictures of the worms. The measurements followed the midline of the body of the worms and were made from the posterior bulb of the pharynx to the middle of the cell body of the ALM neuron and from the middle of the vulva to the middle of the cell body of the AVM. The distance from the posterior bulb of the pharynx to the middle of the vulva was also measured and was used to normalize the data. Figure 8 shows a worm with the neurons, pharynx, and vulva labeled and the method of measuring the migration distance.

![Migration Distance Measuring Method](image)

Figure 8: Migration Distance Measuring Method: The migration of the ALM was measured from the back of the pharynx to the center of the ALM cell body and the migration of the AVM was measured from the center of the vulva to the center of the cell body of the AVM. Both migration distances were normalized to the distance between the back of the pharynx and the center of the vulva. All measurements were traced along the midline of the worm.
Results

MIG-10 is a protein known to be involved in the migration of neurons and it was recently suggested that the CSN-5 protein interacts with MIG-10 (O’Toole and Gosselin, 2008). The interaction of these proteins led to the hypothesis that CSN-5 is also involved in the migration of neurons. To test this hypothesis, RNAi knockdown of csn-5 was done in C. elegans to determine the effect of a knockdown in neuronal cells. The two methods that were used to knockdown csn-5 were feeding RNAi and cloning for microinjection to create a strain expressing dsRNA in mechanosensory neurons (Figure 9).

Cloning of short mig-10 pieces for Microinjection

To test the hypothesis that the utilization of short segment RNAi is an effective knockdown method, short mig-10 sequences were cloned into vectors containing the mec-4 promoter region, which directs expression to the mechanosensory neurons, including AVM and ALM. Previous work in the lab has shown that mig-10 acts cell autonomously to affect cell migration in these neurons; thus, it was expected that the knockdown of mig-10 in these neurons would result in a truncated phenotype. Based on this expectation, mig-10 knockdown was used as the control for the experiment to determine if the methodology was resulting in expected results.
Figure 9: Schematic of cloning strategy: Primers were created to clone a segment of the target gene which was then amplified via PCR using Taq polymerase. The resulting amplified sequence was then ligated into the pGEM T-Easy T-tailed vector (Promega). The resulting construct was transformed into competent *E. coli* cells which were grown on LB+Amp+X-gal+IPTG plates (see Methods for concentrations), white colonies were picked, and cultures were grown. From the cultures, the DNA was extracted and tested to see if it was the correct sequence via restriction digest and sequencing. The sequence was then extracted from the T-tail vector and ligated into a vector with the desired promoter. After this step is complete, the resulting vector with target gene and promoter can be injected into the worms.
To create these short \textit{mig-10} sequences, primers were created using the methodology outlined above (See Methods); the sequences are shown in Table 5. These primers were used to PCR two segments of \textit{mig-10} that were outside the conserved sequence encoding the RA and PH domains, and had minimal homology to other \textit{C. elegans} genes (Figure 10). A 100 base pair segment and a 200 base pair segment from both the sense and anti-sense strand were amplified using PCR, ligated into the T-tailed vector and then transformed into the \textit{E. coli} cells. The 200bp segment did not show expected sequencing results and thus was not cloned into the \textit{mec-4} promoter. The 100bp segment was cloned into the vector containing the \textit{mec-4} promoter and then injected into the worms. Erin Flaherty carried out the injections using a 50ng/µl injection of the vector with 100 ng/µl of unc-122:GFP as the co-injection marker. The results of the injection were quantitated by Erin Flaherty, as well, and did not show any significant change in the migration of the ALM or AVM neurons, which may have been indicative of a flaw in the methodology or in the technique over all. It was determined to try a different promoter with the \textit{csn-5} clones, \textit{unc-86}, which is expressed earlier in the development of the worms.

\textbf{Figure 10: Location of \textit{mig-10a} sequences amplified by PCR for cloning.} The 200bp segment was located close to the 3’ end of the gene and the 100bp segment was located close to the 5’ end.
Table 5: Primer Sequences for mig-10 100 and 200bp Sense and Antisense Segments: Sequence name: First letter, “M” indicates mig-10 sequence, second character indicates location of segment (5’ end versus 3’ end). Third character indicates whether the sequence is the sense or the antisense sequence, and the fourth character is just the identifying primer number.

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5S1</td>
<td>GCG ATA GCT AGC GAA GCT CTT GAA ACT CAA CTC AAC TC</td>
</tr>
<tr>
<td>M5S2</td>
<td>GCG ATA GGT ACC ATT GAC TTC ACA TTT TCC CGG CTA G</td>
</tr>
<tr>
<td>M5A3</td>
<td>GCG ATA GCT AGC ATT GAC TTC ACA TTT TCC CGG CTA G</td>
</tr>
<tr>
<td>M5A4</td>
<td>GCG ATA GGT ACC GAA GCT CTT GAA ACT CAA CTC AAC TC</td>
</tr>
<tr>
<td>M3S1</td>
<td>GCG ATA GCT AGC AAT TCA ATT GAG TTC TCA TAT GAT GAA T</td>
</tr>
<tr>
<td>M3S2</td>
<td>GCG ATA GGT ACC CTC GAC GGA GCA CAT CTA GAG G</td>
</tr>
<tr>
<td>M3A3</td>
<td>GCG ATA GCT AGC CTC GAC GGA GCA CAT CTA GAG G</td>
</tr>
<tr>
<td>M3A4</td>
<td>GCG ATA GGT ACC AAT TCA ATT GAG TTC TCA TAT GAT GAA T</td>
</tr>
</tbody>
</table>

Cloning of short, 160bp csn-5 pieces for RNAi knockdown

To test the hypothesis that csn-5 is involved in the migration of neurons, short sequences of csn-5 were cloned to be used to create a transgenic C. elegans line that would produce dsRNA to act as RNAi to knockdown csn-5 in the C. elegans. The location of the segment of csn-5 is shown in Figure 11. The primers used to create the approximately 160bp segment of the csn-5 are shown in Table 6. Again, primers were chosen to create a PCR product that had little homology to other C. elegans genes.

![Figure 11: Location of csn-5 sequence PCRRed for cloning](image)
The segments were cloned using the strategy outlined in Figure 9. The segments were amplified using PCR and then ligated into the T-tailed vector, transformed into the bacteria, cultured, and the resulting DNA was extracted and tested for accuracy of the sequence. The cloning into the T-tailed vector was successfully completed with the correct csn-5 target sequence, but due to time constraints, the segments of csn-5 were not cloned into the mec-4 promoter.

**Preliminary Results of Feeding CSN-5 RNAi Knockdown Suggest No Effect on Neuronal Migration**

To further test the hypothesis that CSN-5 is involved in the migration of neurons, *C. elegans* were fed bacteria that were producing csn-5 dsRNA. One round of experimentation was done under various conditions. The two worm strains that were used were: NY2054, which is considered the ‘wild type’; and TU3595, which is a strain that only takes up the RNAi in the neurons (Table 7).

**Table 6: Primer Sequences for csn-5 160bp Sense and Antisense Segments.**

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csn5_S1</td>
<td>GCG ATA GCT AGC TGT CGG CCG TCG ATA AGA AGC</td>
</tr>
<tr>
<td>Csn5_S2</td>
<td>GCG ATA GGT ACC CCA GCT TGG CGA GCA AAC AAT AG</td>
</tr>
<tr>
<td>Csn5_A3</td>
<td>GCG ATA GCT AGC CCA GCT TGG CGA GCA AAC AAT AG</td>
</tr>
<tr>
<td>Csn5_A4</td>
<td>GCG ATA GGT ACC TGT CGG CCG TCG ATA AGA AGC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY2054</td>
<td>ynl54 [pPilp-20::gfp]; him-59(e1490)</td>
</tr>
<tr>
<td>TU3595</td>
<td>uIs72 [pCFJ90 (Pmyo-2&gt;mCherry), Pmec-18sid-1, Pmec-18mec-18::gfp]; sid-1(pk3321) him-5(e1490); lin-15b(n744)</td>
</tr>
</tbody>
</table>
Worms were exposed to the *csn*-5 dsRNA and the control empty vector dsRNA for one or two generations (secondary or tertiary plates, respectively; see Methods) and then the migration of the ALM and AVM neurons were measured. Figure 12 depicts the data from the tertiary plates for all of the conditions except for the ‘wild type’ *csn*-5 feeding condition, which was the secondary plate because no worms survived on the tertiary plate. Because the *csn*-5 fed wild type worms died, the RNAi technique was working, as expected in the worm overall, since null mutations in this gene are lethal. Any differences seen in the graphs were not statistically significant. Only a few worms were measured for each set of conditions (as indicated on the graph in Figure 12), and thus a conclusion cannot be reached on the effect of CSN-5 knockdown on neuronal migration. The results of the migration of the ALM neurons are shown in figure 12a and the results of the migration of the AVM neuron are shown in Figure 12b.

![ALM Migration](image1)

![AVM Migration](image2)

**Figure 12:** Normalized Migration Distance for RNAi Feeding Experiments: a. ALM migration distance in TU3595 and NY2054 C. elegans when fed empty vector and *csn*-5 RNAi. No statistical difference in migration. b. AVM migration distance under same conditions, no statistical difference.
In addition, *mig-10* RNAi feeding experiments done simultaneously by Erin Flaherty with the *csn-5* feeding experiments also showed no significant difference in neuronal migration in either the NY2054 strain or the TU3595 strain. This is indicative of a problem with the method of feeding RNAi for knockdown in neurons, since *mig-10* acts cell autonomously in neuronal migration in ALM.
Discussion

CSN-5, the ortholog of the fifth subunit of the COP9 signalosome, is involved in the process of protein degradation as it is associated with the de neddylation activity of the proteasome. Through the information obtained from previous experiments that CSN-5 associates with MIG-10 and the knowledge of the function of CSN-5, it was hypothesized that CSN-5 functions in the regulation of MIG-10 through MIG-10 degradation. This project aimed to determine the role of CSN-5 in neuronal migration in *C. elegans in vivo* to begin to determine if CSN-5 does function in the regulation of MIG-10.

Two approaches were taken to determine the role of CSN-5 in neuronal migration, both involving the knockdown of *csn-5* through RNA interference. The first approach, cloning of short segments of *csn-5* for microinjection did not yield results in the time allowed for this project. Thus, it is recommended that this experiment is continued. However, short *mig-10* segments were successfully cloned and injected to be used as the control. MIG-10 is known to be involved cell autonomously in the migration of neurons (McShea et al., 2013) and thus it was expected that knockdown of *mig-10* in the neurons would have a similar impact on the migration of the neurons as the *mig-10(ct41)* mutants. The *mig-10* knockdown showed no effect on the neuronal migration, and thus this is indicative of a potential flaw in the methodology. One possible reason for this result could be that the promoter (*mec-4*) that was used in the creation of the transgenic *C. elegans* is not expressed early enough in development to successfully knockdown *mig-10*. If *mig-10* is expressed before the transgenic promoter is turned on, then *mig-10* transcripts and MIG-10 protein will arise, and even if knockdown of translation does occur once the *mec-4* promoter is activated, there could be enough protein present to carry out the
migration function. *Mec-4* could be turning on after the gene of interest is expressed. Neuronal migration begins very early in development and thus the associated proteins are translated early in development. Thus, it is recommended that a different promoter, *unc-86*, which is expressed in the cells that give rise to the migrating neurons as well as the neurons themselves, be used in the continuation of this experiment.

During experimentation, difficulties were run into while cloning the target genes for microinjection, particularly when cloning the *csn-5* gene. The difficulties encountered were during the cloning of the gene into the pGEM T-Easy vector. After the transformation and plating of the bacterial constructs, white colonies from the plates were picked, cultured, and the DNA extracted. Despite using AMP, X-gal, and IPTG as screens on these plates, there were still colonies that did not have the desired construct. The gel that resulted from a digest of the extracted DNA from the grown cultures showed a band around the 10kb marker. This was not expected as the total construct size was expected to only be around 3kb and the segment that was expected to be digested out was only 160bp. This result is indicative of a contaminate. After many times of ligating the PCR product into the vector, transforming the constructs, plating the bacteria, picking colonies, extracting the DNA, and digesting the resulting DNA, the construct was successfully cloned and isolated. The difficulties in cloning was likely due to a contaminate in the lab, specifically on the micropipettes. When pipette tips with filters were used, the cloning was successful and the large contaminant seen on the gel in previous experiments was not observed.
Feeding RNAi was also used as a technique to determine the effect of knockdown. When problems were encountered with cloning the short segments for microinjection, feeding RNAi experiments were done to determine if knockdown of csn-5 could be achieved through a different method. Time only allowed for one round of feeding RNAi experiments and thus there were not enough data points to be able to determine anything conclusively. The results that were obtained showed no statistical difference between the empty vector fed and the csn-5 RNAi fed C. elegans. However, as with the microinjection experiment, mig-10 RNAi was performed on the worms as a control, and showed no effect on the neuronal migration. This is again indicative of a flaw in the methodology. In past experiments, it has been difficult to get any results in feeding RNAi knockdown in the neurons of C. elegans in the wild type background (Calixto, et al, 2010a). One reason for the resistance of neurons to RNAi is that neurons do not take up the dsRNA like other cells do. Thus, the strain, TU3595, was engineered to only take up RNAi in the neuronal cells (Calixto et al., 2010b). However, our lab has been unable to achieve knockdown in this strain with any genes attempted. Until a control gene shows an effect on the neuronal migration, no conclusions should be drawn from other feeding experiments.

While no conclusions about the role of CSN-5 in neuronal migration were drawn from this project, the work completed has set up future experimentation. The microinjection experiments should be continued, with the first step being to clone the mig-10 segments into a vector containing the unc-86 promoter. If using the unc-86 promoter shows results in the mig-10 injections, then the csn-5 pieces should be cloned into the unc-86 promoter and injected.
By working to determine which genes are responsible for the migration of neurons in *C. elegans*, the migration of neurons in human can be better understood. As the migration of neurons in humans is better understood, treatments and therapies can be created to better help individuals who have neurological disorders that are due to mistakes or incomplete neuronal migration.
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