Specific patterns of motor neuron loss in cnd-1 mutants affect backward locomotion in Caenorhabditis elegans

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Georgia State University

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SPECIFIC PATTERNS OF MOTOR NEURON LOSS IN CND-1 MUTANTS AFFECT BACKWARD LOCOMOTION IN CAENORHABDITIS ELEGANS

by

ANELA BISCEVIC

Under the Direction of Dr W. W. Walthall

ABSTRACT

In complex organisms, genes determine cellular fates and functions. By studying gene networks during development, we can learn how cellular networks emerge. With only 302 neurons, the C. elegans nervous system is ideal to study these two types of networks. A mutation in the gene cnd-1 was previously found to cause a variable loss of embryonic ventral nerve cord (VNC) motor neurons. cnd-1 is homologous to the mammalian neuroD1 gene that is necessary in establishing neuronal cell fates. Our goal was to understand the role of cnd-1 by focusing on the cell lineages of the embryonic VNC motor neurons. By using transcriptional reporter genes, we found that motor neuron loss occurred in specific cell lineages in cnd-1 mutants. Furthermore, we observed ectopic expression in additional embryonic VNC motor neurons in the posterior, suggesting that cnd-1 may be necessary in establishing the distinct cell fates of the embryonic VNC motor neurons.

INDEX WORDS: Caenorhabditis elegans, flp-13, unc-4, unc-47, acr-5, Neuronal development
SPECIFIC PATTERNS OF MOTOR NEURON LOSS IN CND-1 MUTANTS AFFECT BACKWARD LOCOMOTION IN CAENORHABDITIS ELEGANS

by

ANELA BISCEVIC

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Georgia State University

2014
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by

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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2014
DEDICATION

In memory of my dear aunt Hikmeta Alic, who despite having lived a difficult life, taught me very early on that life is to be lived with lots of smiles, jokes and most importantly, a head held high.
ACKNOWLEDGEMENTS

First and foremost, I am thankful to my mentor Dr. Bill Walthall for all your guidance during this project and helping me to complete this research expeditiously and efficiently. Thank you for taking time out of your busy days and personally making sure that I knew every concept that I needed to know. Your care for teaching is far above average and I will forever be indebted. I also sincerely thank my committee members, Dr. Casyona Johnson and Dr. Kavita Oommen, for being available to serve on my committee and for helping me along this process in a timely manner. Thank you for working with me, despite scheduling conflicts and time constraints.

Thank you to the wonderful staff at the Biology department, especially Moneka Jones for your open-door policy and helping me to plan my coursework accordingly. I owe a well-deserved thank you to my lab members. Thanks to Richard for all your advice in the lab and a special thanks to Mohammad, Shelley and Aaron for creating the uplifting atmosphere in our lab. Your support got me through each long day and I hope you are aware that it means a lot. I would also like to thank the other lab members, past and present – Crystal, Logan, Christian, Jasmine and Abir. Outside of the lab, I would like to thank Ajla, Sarah, Cindy, Mahathi, Bonny, Shawndra, Vishakh and Josh for your acquaintanceship and support.

My most sincere gratitude I owe to my parents, who always had endless faith in me. Thank you for bringing me here from Bosnia – I hope that I am doing justice to your sacrifice and hard work because you deserve only the best from your only daughter.
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<th>Definition</th>
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<tr>
<td>a</td>
<td>Anterior</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic Helix Loop Helix</td>
</tr>
<tr>
<td>d</td>
<td>Dorsal</td>
</tr>
<tr>
<td>FLP</td>
<td>FMRFamide-Like Neuropeptide</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GRN</td>
<td>Gene Regulatory Network</td>
</tr>
<tr>
<td>l</td>
<td>Left</td>
</tr>
<tr>
<td>L1</td>
<td>First larval stage</td>
</tr>
<tr>
<td>L2</td>
<td>Second larval stage</td>
</tr>
<tr>
<td>L3</td>
<td>Third larval stage</td>
</tr>
<tr>
<td>L4</td>
<td>Fourth larval stage</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser Scanning Microscope</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode Growth Media</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>p</td>
<td>Posterior</td>
</tr>
<tr>
<td>$p$</td>
<td>$p$-value</td>
</tr>
<tr>
<td>r</td>
<td>Right</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor $\beta$</td>
</tr>
<tr>
<td>v</td>
<td>Ventral</td>
</tr>
<tr>
<td>VNC</td>
<td>Ventral nerve cord</td>
</tr>
</tbody>
</table>
INTRODUCTION

1.1 Gene networks establish cellular networks

In the past, molecular studies were focused on single genes, but now research has shifted toward a systems approach. We now know that genes are only one part of larger gene regulatory networks (GRNs). Particularly during development, a large group of biomolecules must interact to specify the fate of a cell. In addition to the DNA that makes up a gene and its RNA product, nearby *cis* regulatory elements and regulators such as transcription factors (TFs) are also examples of the biomolecules that are part of GRNs (reviewed by Barabasi and Oltvai, 2004). Together, these unique interactions create a unique pattern of transcriptional and translational control in a cell lineage. Generally, the underlying GRNs establish functional cellular networks. However, in addition to GRNs, the regional contacts to signaling molecules called paracrine factors can also induce a certain cell fate.

Because of the complex interactions in many GRNs, it is best to study an organism that is simple, yet provides homology to the gene to be studied. *Caenorhabditis elegans*, a small free-living soil nematode, is an ideal organism to study. The *C. elegans* nervous system has been particularly of interest to many researchers. With only 302 neurons, *C. elegans* as a model organism provides researchers with a manageable way to address specific questions (White et al., 1978). In addition, *C. elegans* shares much homology with important developmental genes found in other species. Comparative studies have shown that it is the homologous genes in these GRNs that are essential in executing certain developmental programs and ultimately, establishing cellular networks (reviewed by Wagner, 2007). For example, the *C. elegans* gene *unc-55* encodes a nuclear receptor that has a similar ligand-binding domain to those of *seven-up* in
Drosophila and *coup-TF* in chicken (Zhou and Walthall, 1998; Downes et al., 1996). In *C. elegans*, UNC-55 acts as a downstream repressor of *unc-30* and is necessary for promoting the fate of the GABAergic VD motor neurons in *C. elegans* (Shan et al., 2005). The protein products of some of these genes potentially also could be used to rescue the mutation of their respective homologous gene. The protein product of *unc-30*, which is homologous to the mammalian *pitx2* gene, was previously shown to successfully rescue a GABAergic developmental defect in *pitx2* mutant mice (Westmoreland et al., 2001). Hox, or homeodomain, genes, oftentimes also are conserved across different species. *mab-5* in *C. elegans* has been found to be involved in anteroposterior patterning during development similarly to the homologous *Antennapedia* class of genes in insects (Wang et al., 1993).

### 1.2 *cnd-1* mutants lack motor neurons in the VNC

In this thesis, we focus on one member of the conserved family of *neuroD* genes. In mammals, *neuroD1* encodes a bHLH protein that acts as a transcriptional regulator when it forms a heterodimer with E47/Daughterless (DA). This interaction within the GRN ultimately establishes specific neuronal cell fates in the mammalian brain (Breslin et al., 2003). The *C. elegans* *neuroD1* homolog (*cnd-1*) encodes a bHLH protein, which can heterodimerize with HLH-2 in order to bind DNA and regulate gene expression. *hlh-2* is the *C. elegans* homolog to E47 (Grove et al., 2009; Murre et al., 1989).

One of our aims is to test whether a mutation in *cnd-1* has an effect on locomotory behavior and the cellular network required for locomotion. *cnd-1* is normally expressed in the embryonically born DA, DB and DD motor neurons of the ventral nerve cord (figure 1.1), but in *cnd-1* loss-of-function (*ju29*) mutants, some of these VNC motor neurons do not form during
embryogenesis (Hallam et al., 2000). In this thesis, we will carefully examine motor neuron loss in conjunction with \textit{cnd-1 (jd19)} mutants.

**Figure 1.1.** The embryonically born VNC motor neurons (modified from Sulston et al., 1983).

Hallam \textit{et al.} found that the loss of motor neurons in \textit{cnd-1} is more severe in the anterior than posterior of the body (2000). Despite this result, there still is no clear explanation as to why some motor neurons are present in some animals, while other motor neurons never appear in others. In other words, as of now we do not know how or why individual motor neurons are lost in \textit{cnd-1}. One reason this conundrum has persisted is because the reporter genes that would allow the DA motor neurons to be distinguished from the DB motor neurons were unavailable at the time of the Hallam \textit{et al.} publication. \textit{pacr-2::gfp} and \textit{punc-129::gfp}, the reporter genes commonly used, are expressed in both the DA and DB motor neurons. Also compounding the dilemma is the observation that in \textit{cnd-1} mutants neighboring motor neurons frequently invert positions (Hallam et al., 2000). Thus, the \textit{C. elegans} cell lineage that is often touted to be invariant and highly predictable suddenly becomes difficult to trace in \textit{cnd-1} mutants (Sulston and Horvitz, 1977; Sulston et al., 1983).
Therefore, in order to effectively identify motor neurons present and absent in \textit{cnd-1}, different reporter genes must be used. \textit{acr-5}, which encodes a subunit of an acetylcholine receptor, is expressed in the VB and DB motor neurons (Winnier et al., 1999; Esmaeili et al., 2002). \textit{unc-4}, which encodes a homeoprotein, is expressed in many cells, including VA and DA motor neurons (Miller and Niemeyer, 1995). \textit{unc-47} encodes a transmembrane protein that aids in the packaging and vesicular transport of gamma-aminobutyric acid (GABA) across synapses. It is expressed in the DD motor neurons and other GABAergic neurons such as the VD motor neurons (McIntire et al., 1997). These reporter constructs each are distinctively expressed in the DA, DB or DD motor neurons during the first larval stage. The VA, VB and VD motor neurons are only born later and thus will not be seen in L1 animals (Sulston and Horvitz, 1977). \textit{flp-13}, a gene encoding an FMRFamide-like neuropeptide that may modulate synaptic transmission, is expressed specifically in the DD motor neurons (Cinar et al., 2005, Li et al., 1999).

1.3 The cellular network of \textit{C. elegans} locomotion

In \textit{Caenorhabditis elegans}, locomotion occurs in a sinusoidal pattern that is mediated by specific interactions within a neural and muscular cellular circuit. The nematode is able to move forward or backward, which can be induced by gentle touch with a thin hair at the tail or head of the body. The touch receptor cells are first to respond during such a test for touch sensitivity, activating different groups of interneurons and neurons depending on the touch stimulus.

When an animal is gently touched on the tail, the circuitry for forward locomotion is activated. The PVC interneurons are activated via gap junctions with the touch receptors. Next, the PVC interneurons activate the B-type motor neurons, which include the DB and VB motor neurons. These in turn release acetylcholine (Ach) across their chemical synapses to cause
muscle excitation during forward locomotion. However, in order to create undulatory forward movement, a balance must exist between muscle contraction and relaxation on the opposing ventral and dorsal sides of the body. For example, when the DB motor neurons are active, their neuromuscular junctions (NMJs) release Ach in order to cause the dorsal muscles to contract. The opposing ventral muscles, however, are relaxed because the DB motor neurons are also activating the VD motor neurons. The D-type motor neurons, which include the DD and VD motor neurons, release GABA as a neurotransmitter, causing muscles to relax rather than contract (White et al., 1976, McIntire et al., 1993). The VB and DD motor neurons also interact by the same mechanism, although in this case, the ventral muscles contract and the dorsal muscles relax. By dynamically alternating these two variations of the circuitry across the entire body of the animal, the sinusoidal forward motion is created.

Backward locomotion operates by the same mechanism, with a few exceptions. When the animal is gently touched on the head, the touch receptors activate the AVA, AVB and AVE interneurons. Next, these interneurons synapse onto the A-type motor neurons, which include the DA and VA motor neurons. The process continues identically as that of the DB and VB motor neurons, except that here, backward locomotion is the outcome rather than forward locomotion. Overall, this process is visually represented in figure 1.2.
As one can predict, any imbalance in the cellular network of locomotion will offset this intricate pattern of muscle contraction and relaxation that is needed for proper sinusoidal locomotion. In one of the early studies of the *C. elegans* nervous system, when the AVA or AVD cells were ablated with a laser, animals were unable to move backward. In the same study, when the PVC cells were ablated, animals were unable to move forward. The laser ablations in the study also showed that the DB and VB motor neurons are needed for forward locomotion, while the DA and VA motor neurons are needed for backward locomotion (Chalfie et al., 1985). More recently, scientists have made new observations which help to explain the underlying genetic
network and cellular interactions that together establish this neural circuit. When *unc-4*, a gene that encodes a homeoprotein, is mutated, VA motor neurons adopt the synaptic configuration of the VB motor neurons, blocking backward motion by removing excitatory input onto the ventral muscle. This mutation creates a dorsal bias during backward locomotion (Miller and Niemeyer, 1995). Comparably, when the nuclear receptor *unc-55* is absent, the VD motor neurons adopt the fate of the DD motor neurons, increasing dorsal muscle inhibition at the expense of ventral muscle inhibition. As a result, the animal coils ventrally (Zhou and Walthall, 1998). In summary, these experiments show that changing the cellular networks, whether by laser ablations or genetic mutations in GRNs, can affect the locomotory behavior in the animal.

1.4 **Backward locomotion is severely affected in *cnd-1***

In our initial observations for forward and backward locomotion of *cnd-1* mutants, we observed a severe phenotype during backward locomotion, but not forward locomotion. While the animal showed a dorsal bias while moving in both directions, we observed a pronounced dorsal coil during backward locomotion, but only a slight bias during forward locomotion. Furthermore, this dorsal coil was most pronounced in the posterior of the body. Interestingly, L1 animals exhibited a ventral coil instead of a dorsal coil (figure 1.3). The observation that L1 animals exhibit a ventral coil while L2 animals (and older) exhibit a dorsal coil has been made by Oommen (1999). This suggests that the DD motor neurons may be affected. Immediately after hatching, the animals have DD motor neurons with NMJs to the ventral muscles. However, at the end of L1, these NMJs break down and the neuron establishes a synaptic connection to the dorsal muscles instead, which remains throughout the life of the worm (White et al., 1978; Park et al.,
The switch in locomotory behavior between L1 and L2 in *cnd-1* suggests that there is a lack of DD motor neuron function.

**Figure 1.3.** Backward locomotion in response to touch in L1 *cnd-1 (jd19)* mutants. Response shown for a 3-second period. Note the severe ventral coil in the posterior part of the body.

### 1.5 *cnd-1* may be needed for establishing cell lineages

First and foremost, we wish to specifically identify the motor neurons that are absent in *cnd-1* mutants. Even though previous studies have suggested that motor neuron loss in *cnd-1* mutants may be region-specific, the question remains whether this loss may be because certain cell lineages of *cnd-1* mutants are affected. By successfully identifying each motor neuron lost, we will be able to understand when and where during development *cnd-1* causes a defect.

In *C. elegans*, cells that adopt the same fates generally do not come from the same lineages, with the exception of some repeating sublineages. Even if the intrinsic molecular profiles, the type of neurotransmitter, synaptic specificity, or any anatomical feature of each neuronal cell are taken into account, one simply cannot find any specific cell lineages that uniformly share a common identifiable feature (Hobert, 2010).
One reason for this is the complexity of development. Each progenitor cell could potentially autonomously specify its own cell fate based on the regulatory proteins that are already present early in the egg, or it could be conditionally specified based on its location and interactions with neighboring molecules. Oftentimes, cells depend on both autonomous and conditional specification. One of our questions is whether \textit{cnd-1} plays a broad role early in development, helping the DA, DD and DB motor neurons in autonomous specification, or whether \textit{cnd-1} has a role in facilitating the asymmetric cell divisions based on downstream molecules. If the former is true, we should observe that \textit{cnd-1} mutations cause equal loss of motor neurons in all three types of motor neurons. However, if the latter is true, we expect to observe distinct patterns across the different lineages that may indicate conditional interactions with neighboring molecules. In other words, as the cell divisions progress, neighboring molecules may provide guidance for each cell to adopt its unique cell fate.

CND-1::GFP expression begins at the 14-cell stage and persists through L1 (Hallam et al., 2000). The three types of embryonic motor neurons of the VNC arise from the AB cell lineage. After three subsequent cell divisions in the anterior (a) and posterior (p) or left (l) and right (r) directions, two of the produced daughter cells include the ABplp and ABprp. These progenitor cells are at first situated on the left and right axis of the embryo, respectively. At about 230 minutes post fertilization, they cave inward and migrate to what is to become the ventral nerve cord (Sulston et al., 1977). These migrating cells are the same cells where CND-1::GFP expression is observed.

Immediately after CND-1::GFP expression begins, it segregates to the ABprpp and ABplpp cell lineages. At 230 minutes post fertilization, CND-1::GFP expression is confined to the ABplppap and ABprppap lineages (Hallam et al., 2000). The ABprpp and ABplpp lineages

\textit{...}
are represented in figures 1.4 and 1.5, respectively. The figures depict the majority of the VNC neurons. Not shown are DB2 (arising from the ABarappappa lineage), DB1 and DB5 (arising from the ABplpa lineage) and DB3 and DB4 (arising from the ABprpa lineage) motor neurons (Sulston et al., 1983).

**Figure 1.4.** The ABprpp cell lineage. Highlighted box represents the lineages where CND-1::GFP is reported to be expressed 230 minutes post fertilization by Hallam et al., 2000 (lineage information from Sulston et al., 1983).

**Figure 1.5.** The ABplpp cell lineage. Highlighted box represents the lineages where CND-1::GFP is reported to be expressed 230 minutes post fertilization by Hallam et al., 2000 (lineage information from Sulston et al., 1983).
1.6 Purpose of this study

The major aim of our study is to understand the specific pattern of motor neuron loss and to propose how this pattern may impact locomotion. Based on the severe coiling in the posterior that we have observed, we hypothesize that the DD motor neurons must also be severely affected in cnd-1 animals in the posterior. In addition our hypothesis, we suspect that DA motor neurons must be intact in the posterior. The severe dorsal coil that we observe must exist not only because of a lack of dorsal muscle inhibition, but also because of a mechanism that allows the dorsal muscles to contract during backward locomotion. This mechanism is provided by the cholinergic input from the DA motor neurons. Alternatively, motor neuron loss may not be directly attributable to the regions of the body where a severe coil is observed in cnd-1 animals. It is possible that motor neurons must function broadly as a whole, rather than having region-specificity. It is also possible that the axons of nearby neurons are able to compensate for the lack of other neurons because they span across vast distances and thus may overlap with regions where the loss occurred.

Secondly, this work attempts to elucidate the role of cnd-1 in cell lineages. As shown, CND-1::GFP persists during embryogenesis but is confined to the ABplppap and ABprppap lineages at 230 minutes post fertilization (Hallam et al., 2000, figures 1.4, 1.5). If cnd-1 has a lineage-specific role, then motor neuron loss must be most severe in the cell lineages where CND-1::GFP is expressed for a prolonged period. Thus, we hypothesize that motor neuron loss is most severe in the ABplppap and ABprppap lineages, whereas, we reason, motor neuron loss must be less severe in the cell lineages where cnd-1 is not expressed or ceases to be expressed sooner. Lineages that encompass the latter example include progenitor cells that give rise to all DB motor neurons (DB1 – DB7) and also DA6 and DA7 motor neurons. Two feasible
hypotheses exist as alternatives. Both are possible mainly because $cnd-1$ begins to be expressed at the 14-cell stage and persists during L1. First, $cnd-1$ function may be involved in VNC development early in the embryo, immediately at the onset of expression. In such a case, a wider array of VNC cell lineages could be affected than proposed. By contrast, $cnd-1$ function may gain importance later in development and thus may play a role in only a subset of the motor neurons that we propose are affected.
2 MATERIALS AND METHODS

2.1 Worm maintenance, matings and transgenics

Worms were grown and maintained at 22 °C on NGM agar with seeded OP50 *E. coli* as their food source, as described by Brenner (1974). Alleles used were *cnd-1 (jd19), punc-129 (er80)* and *pflp-13 (NY2037)*, which were available in our laboratory. The latter two were introduced as GFP-expressing transcriptional reporter fusions as described by Chalfie *et al.* (1994). *pacr-5::gfp, punc-4::gfp* and *punc-47::mCherry* reporters were obtained from the *Caenorhabditis* Genetics Center.

Reporter genes were introduced into the *cnd-1* or other transgenic background by creating a mating plate of five N2 Bristol strain males with two L4 virgin hermaphrodites of a mutant gene or transgene of interest. The subsequent heterozygous male generation was then crossed with hermaphrodites of the next mutant gene or transgene of interest. After two generations, hermaphrodites were then screened and selected for the expected phenotype. A summary of reporter genes that were used is listed in table 2.1.
Table 2.1. List of reporter genes used, their corresponding protein products and VNC neurons where expressed in L1.

<table>
<thead>
<tr>
<th>Reporter gene</th>
<th>Protein product</th>
<th>VNC neurons where expressed in L1</th>
</tr>
</thead>
<tbody>
<tr>
<td>punc-129::gfp</td>
<td>TGF-β signaling molecule</td>
<td>DA, DB</td>
</tr>
<tr>
<td>pflp-13::gfp</td>
<td>FLP neuropeptide</td>
<td>DD</td>
</tr>
<tr>
<td>punc-47::mCherry</td>
<td>GABA vesicular transporter</td>
<td>DD</td>
</tr>
<tr>
<td>punc-4::gfp</td>
<td>Homeodomain transcription factor</td>
<td>DA</td>
</tr>
<tr>
<td>punc-4::gfp; punc47::mCherry</td>
<td>Homeodomain transcription factor + GABA vesicular transporter</td>
<td>DA, DD</td>
</tr>
<tr>
<td>pacr5::gfp</td>
<td>ACh receptor subunit</td>
<td>DB</td>
</tr>
</tbody>
</table>

2.2 Microscopy

To ensure that data were collected only from early- to mid-L1 animals, eggs were individually transferred onto new plates, allowed to hatch, and animals collected for imaging were within 8 hours of the initial transfer. Animals were pipetted into a solution of M9 buffer, then mounted on 3% agar pads containing 1 mM sodium azide, which helped to immobilize the animals during imaging.

2.2.1 Confocal microscopy

To verify GFP or mCherry expression, a Zeiss LSM500 confocal microscope was used with its accompanying LSM510 software. Images were compiled from the LSM Z stacks and processed in Adobe Photoshop to obtain the clearest and most informative images. Most animals were imaged with the 40x oil immersion objective. Some were imaged with the 100x oil immersion objective where detail was needed.
2.2.2 DIC optics

Differential interference contrast (DIC) microscopy was used to check for the presence or absence of neuronal cell bodies in the VNC independent of the expression of fluorescence. DIC provided the resolution necessary to optically section preparations and identify the cell bodies of individual motor neurons in the VNC based upon position.

We used the 100x oil immersion objective of a Zeiss Axioplan microscope to view the VNC. The ocular image was relayed to a Dell monitor via the Scion Corporation TWAIN 1392 camera input and then shown on the monitor by using the Scion Visicapture software.

Because neighboring VNC cell bodies often invert positions in \textit{cnd-1}, all the motor neurons of the VNC that were part of our study, with the exception of DA5 and DB5 motor neurons, were grouped into clusters during data collection (Hallam et al., 2000).

2.3 Behavioral assay

Animals were checked for cell bodies expressing \textit{punc-4::gfp} on the LSM confocal microscope during L1 as described above, except the concentration of sodium azide was only 0.4 mM. This lower concentration ensured that there were minimal adverse effects from the sodium azide. Additionally, the animals were kept on the agar pad no longer than 15 minutes during the mounting and imaging process and were afterwards immediately transferred to a healthy NGM plate with a fresh lawn of \textit{E. coli}.

Next, the animals were allowed to grow to early adulthood and their locomotion was recorded by using a Canon camcorder while the animals were viewed through a Zeiss Stemi SV II Apo dissecting microscope. The animals were gently touched on the head with an eyelash to test their backward locomotion. A still image was taken 2 seconds after the touch stimulus and
posterior body curvature, defined as the angle $\kappa$, was measured based on the lines shown in figure 2.1. If the animal displayed a dorsal curvature, $\kappa$ was a positive value. If the animal displayed a ventral curvature, $\kappa$ was a negative value. The smaller the absolute value of $\kappa$, the more severe the dorsal or ventral curvature.

In order to calculate $\kappa$, first a line was drawn between the tail and the head region (line a). Next, a perpendicular line was drawn through the middle of the vulva (line b). The intercept of these two lines was labelled point A. A hypotenuse (line c) was drawn between the tail and the most ventral or most dorsal point on line c, depending on whether the animal was a dorsal or ventral coiler (figure 2.1). Lastly, a perpendicular line was drawn from point A to line c and the degree of curvature, $\kappa$, was measured, as shown.

A scatter plot was prepared that placed a value termed the A/P difference on the x axis, while the $\kappa$ that was measured during early adulthood was plotted on the y-axis. A/P difference was calculated by subtracting the total number of cell bodies expressing $punc-4::gfp$ in the anterior from the total number of cell bodies expressing $punc-4::gfp$ in the posterior. Anterior cell bodies included DA1, DA2, DA3, DA4 and DA5 motor neurons and any additional cells nearby that we observed to be ectopically expressing $punc-4::gfp$, while posterior cell bodies included DA6, DA7 motor neurons and any additional cells nearby that we observed to be ectopically expressing $punc-4::gfp$. Unfortunately, we were unable to also include the DD motor neurons in our behavioral analysis. $punc-47::mCherry$ reporter requires meticulous analysis under the 100x objective in order to identify the DD motor neurons, which we were unable to do, considering that we needed to work swiftly so that the animals could be recovered after imaging without any harm.
Figure 2.1. Posterior body curvature, as defined by κ.

2.4 Statistical analysis

For the fluorescent and DIC optics data, a Mann Whitney test was used to compare wild type animals to *cnd-1* mutants. Significance is presented as follows: $p < 0.05$ denoted as *, $p < 0.01$ denoted as **, $p < 0.001$ denoted as *** and $p < 0.0001$ denoted as ****.

The behavioral assay was plotted on a scatter plot, with A/P difference on the x-axis and posterior body curvature, κ, on the y-axis, as described above.
3 RESULTS

3.1 Expression of fluorescent reporter genes

3.1.1 Comparison of DD motor neurons based on pflp-13::gfp and punc-47::mCherry reporter gene expression in wild-type and cnd-1 (jd19)

In both pflp-13::gfp and punc-47::mCherry reporters, we observed an anterior to posterior gradient of decreasing fluorescence in the animals (figures 3.1, 3.2). The most significant decline was in the posterior, which includes the DD4, DD5 and DD6 motor neurons. Out of 57 animals imaged in both reporter backgrounds, 46 animals failed to express fluorescence in all of the three posterior DD motor neurons and 11 animals showed fluorescent expression in only one of the three posterior DD motor neurons. We did not observe any animals that had two or more of the three posterior DD motor neurons showing fluorescence. By contrast, the two anterior-most DD motor neurons (DD1 and DD2) mostly retained fluorescent expression in cnd-1. Out of the 57 total animals, fluorescence was expressed in the DD1 motor neuron of 48 animals, while fluorescence was expressed in the DD2 motor neuron of 45 animals.
Figure 3.1. Fluorescent expression of pflp-13::gfp. A) The frequencies of motor neurons present in wild type (n=15) and cnd-1(jd19) (n=28) background. $p < 0.05$ denoted as *, $p < 0.0001$ denoted as ****. B) Image of wild-type animal expressing pflp-13::gfp. C) Image of cnd-1 (jd19) animal expressing pflp-13::gfp.
Figure 3.2. Fluorescent expression of *punc-47::mCherry*. A) The frequencies of motor neurons present in wild type (n=15) and *cnd-1(jd19)* (n=29) background. *p* < 0.0001 denoted as ****. B) Image of wild-type animal expressing *punc-47::mCherry*. C) Image of *cnd-1 (jd19)* animal expressing *punc-47::mCherry*. 
3.1.2 Comparison of DA motor neurons based on punc-4::gfp reporter gene expression in wild-type and cnd-1 (jd19)

By using punc-4::gfp as a reporter, we observed a gradual decline in GFP expression from DA1 to DA5 (figure 3.3). Unlike the results seen in pflp-13::gfp and punc-47::mCherry, this decline does not continue in the posterior. Rather, in the punc-4::gfp background, the gradual decline in GFP expression continued only up to DA5. However, the DA motor neurons in the posterior, which include DA6 and DA7, were mostly unaffected in cnd-1 (jd19) mutants. In all samples, DA6 expressed punc-4::gfp. We only observed two animals (out of 105) that did not express punc-4::gfp in DA7.

We also observed additional cells expressing GFP in the VNC (figure 3.4). Usually, these additional cells were positioned next to DA6 or DA7 (table 3.1). Out of the 105 animals sampled, 14 displayed two additional cells expressing GFP. These cells were next to DA6 and DA7 (figure 3.4B). Because punc-4::gfp is expressed in both DA and VA motor neurons, we next wanted to test whether the additional cells could be VA motor neurons that differentiated precociously. VA motor neurons are postembryonic cells that differentiate about 13 to 16 hours post hatching, with the anterior-most cells differentiating first (Sulston and Horvitz, 1977). We imaged a group of L1 larvae at 0 to 4 hours post hatching and another group at 4 to 8 hours post hatching. There was no observable increase in additional cells expressing GFP in the older animals (figure 3.5). This suggested that the cells were present at birth and thus were embryonic, rather than postembryonic, cells.
**Figure 3.3.** Fluorescent expression of *punc-4::gfp*. A) The frequencies of motor neurons present in wild type (n=15) and *cnd-1*(*jd19*) (n=105) background. $p < 0.05$ denoted as *, $p < 0.001$ denoted as ***, $p < 0.0001$ denoted as ****. B) Image of wild-type animal expressing *punc-4::gfp*. C) Image of *cnd-1* (*jd19*) animal expressing *punc-4::gfp*. Animal is mounted with ventral side up.
Table 3.1. *punc-4::gfp* was ectopically expressed in the VNC of *cnd-1 (jd19)* mutants. Table shows number of animals (N) in which an additional *punc-4::gfp*-expressing cell was observed next to a VNC neuron (DA1 to DA7).

<table>
<thead>
<tr>
<th>Additional GFP expression, N</th>
<th>DA1</th>
<th>DA2</th>
<th>DA3</th>
<th>DA4</th>
<th>DA5</th>
<th>DA6</th>
<th>DA7</th>
<th>Total sample size</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>43</td>
<td>36</td>
<td>105</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4. *punc-4::gfp* expression in posterior. A) Wild type animal shows DA6 and DA7 expressing *punc-4::gfp*. B) Two additional cells express *punc-4::gfp* in the posterior of a *cnd-1* (*jd19*) mutant.
Figure 3.5. Number of cells expressing punc-4::gfp in posterior at different post-hatch times. N=14 for wild type, N=10 for cnd-1 (jd19) 0-4 hours post hatching, N=12 for cnd-1 (jd19) 4-8 hours post hatching.

3.1.3 Comparison of DB motor neurons based on pacr-5::gfp reporter gene expression in wild-type and cnd-1 (jd19)

When we viewed the animals using the pacr-5::gfp reporter, we observed that there was no significant difference in GFP expression between the wild-type and cnd-1 (jd19) animals. In pacr-5::gfp, we also observed cells that express GFP in the head and tail, as shown in figure 3.6. These cells have not been identified yet, but they are not part of the VNC neurons (Winnier et al., 1999). Based on this data, we concluded that DB motor neurons are unaffected, and therefore not lost, in cnd-1 (jd19) mutants.
Figure 3.6. Fluorescent expression of *pacr-5::gfp*. A) The frequencies of motor neurons present in wild type (n=12) and *cnd-1(jd19)* (n=31) background. Animal is mounted with ventral side up. B) Image of wild-type animal expressing *pacr-5::gfp*. C) Image of *cnd-1 (jd19)* animal expressing *pacr-5::gfp*. 
3.1.4 Comparison of DA and DB motor neurons based on \textit{punc-129::gfp} reporter gene expression in wild-type and \textit{cnd-1 (jd19)}

Our \textit{punc-129::gfp} data suggested that the anterior motor neurons and the DB6/DA7 pair were most affected (figure 3.7). However, the fallacy of using \textit{punc-129::gfp} as a reporter is that it does not distinguish between the DA and DB motor neurons because it is a general marker for cholinergic neurons. Furthermore, \textit{unc-129} expression varies in several reporter genes. For example, we observed in the wild type that \textit{punc-129::gfp} was rather faint in DB3 and in majority of cases, was not expressed in DA7. Recently, a study of \textit{punc-129::mCherry} expression in DA and DB motor neurons has found that visible fluorescence was only observed in DA1 through DA6 and DB3 through DB7. No fluorescent expression was observed in DA7, DB1 and DB2. The study also noted that \textit{punc-129::mCherry} expression is faint in DB3, which is also our observation in our wild type animals that are expressing \textit{punc-129::gfp} (Goodwin et al., 2012).

From our observations in the \textit{pacr-5::gfp} background, we found that the DB motor neurons are not affected in \textit{cnd-1}. With this newfound information, we analyzed the \textit{punc-129::gfp} data again. As expected, there was no significant decrease in GFP expression in DB5, nor was there a decrease in DB7. A slight, gradual decline of GFP expression in the anterior was also observed in \textit{punc-129::gfp}. This included the DB3, DA2, DB4, DA3 and DA4 motor neurons. Knowing that DB3 expression in \textit{punc-129::gfp} tends to be faint, we wondered whether the DB3/DA2 pair of neurons could be better preserved in \textit{cnd-1} than what we were able to observe under the \textit{punc-129::gfp} reporter. Based on our separate \textit{pacr-5::gfp} and \textit{punc-4::gfp} data, we found that the DB3/DA2 pair of neurons are mostly present in \textit{cnd-1} animals.
Despite these confirmatory findings, there were also some unexpected results in the *punc-129::gfp* background. For example, in the *punc-4::gfp* background, GFP expression was lowest in DA5. In *punc-129::gfp*, there was no significant decline in GFP expression in DA5 between wild-type and *cnd-1* animals. Even though *punc-129::gfp* as a reporter gene can be helpful in some cases, our inability to distinguish between the DA and DB motor neurons, and the variability in the intensity of fluorescence expression required that we also use other reporter genes in our experiment. Thus, our *punc-129::gfp* results were interpreted in context with the *punc-4::gfp* and *pacr-5::gfp* data.
Figure 3.7. Fluorescent expression of punc-129::gfp. A) The frequencies of motor neurons present in wild type (n=6) and cnd-1(jd19) (n=23) background. p < 0.01 denoted as **, p < 0.001 denoted as ***. B) Image of wild-type animal expressing punc-129::gfp. C) Image of cnd-1 (jd19) animal expressing punc-129::gfp.
3.2 Lineage analysis

By combining \textit{punc-4::gfp} and \textit{punc-47::mCherry} into the \textit{cnd-1 (jd19)} mutant background, we tested whether the loss of fluorescence can be attributed to specific lineages (figure 3.8). While we did not find any observable relationship between the lack of fluorescence in DA and DD motor neurons, a pattern emerged that could be attributable to cell lineage. We observed that the lineages in which CND-1::GFP continued to be expressed 230 minutes post fertilization as shown by Hallam \textit{et al.} were most affected (2000). These included the ABprppap and ABplppap lineages (figures 1.4, 1.5). The cells that did not belong to these lineages (all DB motor neurons, DA6, DA7, DA8 and DA9) were not significantly affected in \textit{cnd-1 (jd19)} mutants. Also, within the ABprppap and ABplppap lineages, the posterior daughter cells were more affected than the anterior daughter cells. The posteriorly dividing progenitor cells are known to migrate closer towards the posterior, which was consistent with the gradual decline in fluorescence expression that we observed in the \textit{pflp-13, punc-47} and \textit{punc-4} backgrounds (Sulston \textit{et al.}, 1977).
Figure 3.8. \textit{punc-4::gfp; punc-47::mCherry} expression. Expression shown in A) wild type and B) \textit{cnd-1 (jd19)} mutants. Green carrot shows lack of \textit{punc-4::gfp} expression where DA4 is expected; red carrots show lack of \textit{punc-47::mCherry} expression where DD2, DD3, DD4 and DD5 are expected.
3.3 Behavioral analysis

To test whether the additional cells in the posterior that are expressing \textit{punc-4::gfp} have an effect on the dorsal muscle excitation, we focused on the behavior of the animal during backward locomotion. We tested 20 early adult animals and found that 13 animals had a dorsal curvature in the posterior of the body, while 7 animals had a ventral curvature in the posterior of the body (table 3.2). This result was unexpected because it was previously thought that adult \textit{cnd-1} mutants had a dorsal bias only.

However, the variability in the ventral or dorsal bias seems to be explained by the A/P difference. The A/P difference is calculated by subtracting the number of cells expressing \textit{punc-4::gfp} in the anterior from the number of cells expressing \textit{punc-4::gfp} in the posterior. For the 7 animals with the ventral bias, we found that the A/P difference ranged from 0 to 3. For the 13 animals with the dorsal bias, we found that the A/P difference ranged from -2 to 0. Thus, it appears that the animals that had fewer cells expressing GFP in the anterior compared to the cells expressing GFP in the posterior were more likely to be ventral coilers in the posterior. The cell bodies that ectopically expressed \textit{punc-4::gfp} in the posterior of \textit{cnd-1} mutants were also included in our calculation of A/P difference. Interestingly, when we observed this ectopic expression, we observed that the animals were either ventral or dorsal coilers. Of the two animals that had an ectopically expressing cell next to both DA6 and DA7, the animals were ventral coilers. This was surprising because we initially hypothesized that a strong presence of DA motor neurons in a region would cause dorsal bias if the DD motor neurons are not functioning. However, we found that a strong presence of DA motor neurons in the anterior was able to cause the animals to become a ventral coiler in the posterior.
Table 3.2. *punc-4::gfp* expression in the VNC establishes dorsal or ventral bias. 1: cell is expressing GFP; 0: no GFP expression was observed; +1: an additional cell expressing GFP was present next to the neuron.

<table>
<thead>
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<th>Sample</th>
<th>DA1</th>
<th>DA2</th>
<th>DA3</th>
<th>DA4</th>
<th>DA5</th>
<th>DA6</th>
<th>DA7</th>
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<th>Bias</th>
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<td>0</td>
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<td>1 +1</td>
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<td>-2</td>
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</tr>
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</table>
3.4 DIC optics

To confirm that motor neurons were not present, rather than \textit{cnd-1} affecting the expression levels of the reporter genes that were used in this study, we opted to image the VNC by differential interference contrast microscopy, or DIC optics. This technique is ideal to image transparent samples that would otherwise be difficult to see in the case of \textit{C. elegans} (figure 3.10A). Because \textit{cnd-1} frequently causes neighboring motor neurons to invert positions, some of the motor neurons were assigned to their respective clusters for data collection purposes.

Our results (figure 3.10B) indicated that motor neuron loss was most frequent anterior to the gonadal primordium, with the DA4/DD3 cluster, as well as DA5, being the most affected. This was expected because the \textit{punc-4::gfp}, \textit{pflp-13::gfp} and \textit{punc-47::mCherry} reporters showed a similar gradual decline in fluorescence in our results. By contrast, DB5 was present in most of our images, confirming our \textit{pacr-5::gfp} results.
Posterior to the gonadal primordium, the majority of motor neurons were present. We observed that out of the 87 cnd-1 animals viewed by DIC optics, 41 animals had one or more of the full set of triplet cell bodies in the posterior. This suggested that despite the severe loss of pflp-13::gfp and punc-47::mCherry expression in the posterior DD motor neurons (DD4, DD5, DD6), overall, the posterior motor neurons of the VNC were mostly present. However, we were unable to explain whether the DD motor neurons were present, but simply lost their GABAergic signals, or whether they were lost but replaced by other cell bodies ectopically in the VNC. Our punc-4::gfp data showed additional cells ectopically expressing GFP in the posterior. If they were cells other than DD motor neurons, then out of the 105 animals observed in the punc-4::gfp background, we would have expected to occasionally find more than the six posterior motor neurons to exist in the VNC. However, in these 105 animals, we never observed more than the six posterior motor neurons to be present during DIC imaging. This led us to propose that perhaps many of the posterior DD motor neurons are still present in cnd-1 mutants, but that they possibly adopt the fate of the DA motor neurons at the expense of their GABAergic fate.
Figure 3.10. DIC microscopy confirms loss of motor neurons in *cnd-1*. A) Representative image of an L1 stage *cnd-1* mutant. VNC Cell bodies present are denoted by black arrows. White circles represent areas where cell bodies are expected, but are missing. B) A comparison of cell bodies between wild-type and *cnd-1 (jd19)* show that cell bodies are missing in the VNC during L1 stage. $p < 0.0001$ denoted as ****. N=55 for wild-type, N=87 for *cnd-1(-)*.
4 DISCUSSION

How and at what time during development neuronal cell fates are established are questions that still remain to be answered. We know that bHLH genes play a key role in the process of neuronal cell fate specification. Previous research has shown that when certain bHLH genes are mutated, the cells that normally adopt a neuronal cell fate adopt another cell fate. For example, when the bHLH gene \textit{hlh-14} is lost, cells that normally develop into neurons become hypodermal cells instead (Frank et al., 2003). When another bHLH gene, \textit{lin-22}, is lost, the anterior V cell lineages that normally develop into epidermal cells become postdeirid neuroblasts instead. In males, the posterior V5 cell lineage that normally produces one sensory neuron and an epidermal cell produces two sensory neurons instead. Thus, the V5 daughter cell that is fated to become an epidermal cell assumes the fate of a sensory neuron in \textit{lin-22} mutants (Wrischnik and Kenyon, 1997). When \textit{ngn-1} and \textit{hlh-2}, also proneural bHLH genes, are mutated, asymmetric divisions in pharyngeal cells are abolished and the blastomeres undergo a symmetric left-right cell division instead of producing three cells (Nakano et al., 2009).

These and other experiments suggest that the proneural bHLH genes promote asymmetric cell divisions. In other words, in a normal animal, certain lineages produce neural progenitors and another daughter cell to assume a different cell fate, such as a hypodermal cell progenitor. When proneural bHLH genes are mutated and lose function, steps leading to distinct cell fates in the daughter cells are somehow lost and both cells oftentimes acquire the same cell fate. Our results indicated that in \textit{cnd-1} mutants, the DD motor neurons may have lost their GABAergic fate and expressed the cholinergic \textit{punc-4::gfp} reporter instead (figures 3.1, 3.2, 3.4). The question then is, what roles do the proneural bHLH genes play in the mechanisms that determine
these distinct cell fates? Is it possible that the progenitor cells have within them an intrinsic mechanism to adopt a certain “default,” or cell autonomous fate as established by an early proneural gene? Or perhaps could a bHLH gene such as *cnd-1* play a more active role by being part of a dynamic signaling mechanism in which each motor neuron precursor is subjected to different cell-to-cell interactions, thus purposely leading it to become a specific neuron?

To answer these questions, we must study *cnd-1* in the context of cell lineages. Cell lineages can give us clues to when and where during development *cnd-1* is necessary to create the permissive conditions for a progenitor cell to become cholinergic or GABAergic. *cnd-1* has been shown to be expressed in cell lineages that give rise to the DA, DB and DD motor neurons (Hallam et al., 2000). These neurons maintain their identity based on gene regulatory proteins that bind to a region on a downstream gene, usually the promoter. For example, the protein products of *unc-55* and *unc-4* act as repressors that maintain different neuronal cell fates. *unc-30* is turned on in VD and DD motor neurons. However, *unc-55* is expressed only in the VD motor neurons, where the binding of UNC-55 suppresses the transcriptional activation by UNC-30 in a subset of genes to establish the distinct VD motor neuron fate. If the function of *unc-55* is lost, the cells that usually become the VD motor neurons adopt the DD cell fate (Zhou and Walthall, 1998). In a similar fashion, *unc-4* and *unc-37* are expressed in the DA and VA motor neurons, where their protein products suppress the transcription of *acr-5* (Winnier et al., 1999). One key question to ask is whether *cnd-1* is required in order for these distinct transcriptional programs to be established specifically in the embryonically born motor neurons.

Rather than the VNC neurons being equally or stochastically affected in *cnd-1* mutants, we observed that the DB motor neurons are not significantly affected but the DA and DD motor neurons are (figures 3.1, 3.2, 3.3). Thus, we propose that *cnd-1* may play a role in establishing
the function of UNC-30 and UNC-4. We also observed that some motor neurons within the same motor neuron type mostly develop normally in *cnd-1* mutants while others are never present or potentially adopt a different cell fate. Therefore, we argue, the transcriptional gene programs alone are not sufficient to understand the role of *cnd-1*. We argue that in conjunction with transcriptional regulation, these VNC progenitors must also be affected by cell-to-cell interactions in their vicinities. We observed cells in the posterior that expressed *punc-4::gfp* ectopically (figure 3.4). One possibility is that these cells were DA motor neurons that underwent an additional cell division. Because the DB motor neurons were not significantly affected in the *pacr-5::gfp* reporter, we ruled out the possibility that these ectopically expressing cells could have been DB motor neurons that adopted the DA motor neuron fate. Another potential explanation is that the ectopically expressing neurons in the posterior were DD motor neurons that adopted a cholinergic fate instead of a GABAergic fate and thus expressed *punc-4::gfp*. Out of the 105 animals viewed under the *punc-4::gfp* reporter, only two animals showed a cell body in the posterior that failed to express *punc-4::gfp*. Many of these animals had ectopic expression of *punc-4::gfp* in the posterior, in addition to the normally expressing DA6 and DA7 (figure 3.3). Interestingly, this ectopic expression was usually in the VNC regions where DD motor neurons are expected. *pflp-13::gfp* and *punc-47::mCherry* expression in the DD motor neurons, on the other hand, was almost nonexistent in the posterior (figure 3.1, 3.2). In this case, the lineage would be intact. However, the progenitors of the DD motor neurons could potentially be unable to become differentiated into GABAergic motor neurons.

This finding was supported by our DIC optics data. Even though we did not observe *punc-47::mCherry* or *pflp-13::gfp* expression in the posterior DD motor neurons, many of the
posterior cell bodies were present, as shown in DIC imaging (figure 3.10). Out of 87 animals, we observed that 41 animals had one of more complete triplet of posterior cell bodies.

The ability to change neuronal cell fates has previously been found to be possible in \textit{neuroD1}, the mammalian homolog of \textit{cnd-1}. In the mammalian forebrain, MASH1, an achaete/scute bHLH protein, normally induces the development of GABAergic interneurons (Casarosa et al., 1999). In the cortex, however, \textit{mash1} is repressed by NeuroD1 and thus GABAergic interneurons do not form there. When \textit{neuroD1} was ectopically expressed in the forebrain cells, it was able to repress MASH1 and inhibit the formation of GABAergic neurons (Roybon et al., 2010). Interestingly, another study found that MASH1 binds to PHD1 (paired homeodomain protein 1), a downstream target that is most similar to the \textit{C. elegans} UNC-4 protein (Saito et al., 1996). These gene regulatory networks have been most studied in mice and \textit{Drosophila}. It is plausible that \textit{C. elegans} might share a similar gene network. At the moment, we do not know of a \textit{C. elegans} homolog for MASH1. However, \textit{unc-4, unc-30} and \textit{cnd-1} are some of the \textit{C. elegans} genes that are both known to be homologous to mammalian genes and also have parallel functions to the mammalian gene networks (Hallam et al., 2000; Saito et al., 1996; Westmoreland et al., 2001).

In this supposedly conserved gene network, proneural bHLH genes may be one of the first genes to be expressed. \textit{cnd-1} expression may be required to create a permissive state for the progenitor cells to be specified. This is supported by our findings. The cell lineages in which \textit{cnd-1} is expressed at 230 post fertilization (DA1 – DA5, DD1 – DD6) are most severely affected in \textit{cnd-1} mutants, whereas in cell lineages where \textit{cnd-1} is only briefly expressed (all DB motor neurons, DA6, DA7) we did not observe any severe loss of neurons or lack of fluorescence (figures 1.4, 1.5; Hallam et al., 2000). Interestingly, within the cell lineages where a significant
defect was observed in *cnd-1* mutants, the defect was more severe in the anterior daughter cells than the posterior daughter cells. A clear pattern arose – DD6 was more likely to be lost than DD4, DD4 and DD6 were more likely to be lost than DD2, DA5 was more likely to be lost than DA3, etc. Because the posteriorly dividing cells also assume a more posterior position in the VNC, this finding was restated as a gradual anteroposterior decline in the fluorescent expression of *punc-4::gfp* (excluding DA6 and DA7, where *cnd-1* does not continue to be expressed normally at 230 minutes post fertilization), *pflp-13::gfp* and *punc-47::mCherry* (figures 3.1, 3.2, 3.3; Hallam et al., 2000).

Thus, we conclude, the role of *cnd-1* may be to control the cell interactions between the VNC progenitors in both the anterior and posterior so that the fate of these progenitors can be specified equally, regardless of their position in the VNC. Next, another bHLH such as an achaete/scute may be involved in the cell specification process, while gene regulatory proteins such as UNC-4 may finally act as repressors that maintain the ultimate cell fates of the VNC motor neurons. We propose that *cnd-1* is involved in early neurogenesis and that if mutated, it suppresses the GABAergic fate of the DD motor neurons mostly in the posterior. If *cnd-1* is mutated, it may not be able to control the downstream achaete/scute gene, which in turn may control *unc-4* as its downstream target. At the moment, we do not know of any proteins that are similar to MASH1 (mammalian achaete/scute homolog 1) in *C. elegans* and this thought is merely a speculation. We can currently only suggest that an intermediate achaete/scute gene may exist in *C. elegans*. Nonetheless, three achaete/scute genes are known so far in *C. elegans* that may be potential candidates – *hlh-3*, *hlh-6* and *hlh-14* (Doonan et al., 2008).

Our behavioral analysis supported our findings. In the animals assayed, forward locomotion was barely affected. The animals displayed a slight dorsal bias, however, their
sinusoidal forward movement was intact. This suggested that the DB motor neurons were unaffected and that the lack of DD motor neuron function, which is to cause dorsal muscle relaxation, may have contributed to the slight dorsal bias during forward locomotion. By contrast, during backward locomotion, we observed a severe dorsal coil in the posterior of most of our animals (figure 1.3). The dorsal coil, again, could be attributable to the lack of DD motor neuron function. However, the severity of the coil was most likely caused by the cells that were ectopically expressing punc-4::gfp, and thus, causing the dorsal muscles to hypercontract (figure 3.3, table 3.2, figure 3.9). To our surprise, some of our assayed animals also showed a severe ventral coil (table 3.2, figure 3.9). They did not show any different pattern of fluorescent reporter expression in the posterior from what we had observed in the dorsal coilers. They did, however, have a larger number of cells in the anterior that were expressing punc-4::gfp (table 3.2). This finding suggested that the overall balance between the DA motor neurons in the anterior and posterior seems to play a role in whether an animal is a ventral or dorsal coiler. Of course, DD motor neurons also factor into this balance, but unfortunately they were not part of our behavioral analysis because of previously mentioned reasons.

Based on our fluorescent reporters and DIC optics, we conclude that in cnd-1 mutants, some motor neurons were lost while others adopted a different cell fate. Therefore, the defect appears to be in the cell lineage, as well as the cell specification process of the cells that were present. Because we did not observe a significant effect on the DB motor neurons, we propose that the posterior DD motor neurons lost their GABAergic fate and expressed punc-4::gfp, a cholinergic marker. The cell lineages most affected by cnd-1 were ABprpp and ABplpp, in which CND-1::GFP has previously been shown to be expressed 230 post fertilization (Hallam et al., 2000). Therefore, we reason that cnd-1 is necessary even shortly before the VNC motor
neurons differentiate (approximately 290 to 320 minutes post fertilization) (Sulston et al., 1983). Without \textit{cnd-1}, some of the VNC motor neurons do not adopt their expected cell fates.

The Hallam \textit{et al.} study found that \textit{cnd-1} was affecting the DA, DB and DD motor neurons. These neurons were missing not because of programmed cell death, but rather because the cell lineages failed to divide properly (2000). At the time, the limitation of this study was the unavailability of reporter transgenes that would allow one to distinguish between the DA and DB motor neurons. In our experiment, we used \textit{punc-4} and \textit{pacr-5} as transcriptional reporters, which allowed us to distinguish between the DA and DB motor neurons (figures 3.3, 3.6). This revealed that the DB motor neurons were not affected in \textit{cnd-1} mutants (figure 3.6) and that DA motor neurons were present in the posterior (figure 3.4). The \textit{punc-4::gfp} reporter also revealed ectopic expression in cells in the posterior, in addition to being expressed in DA6 and DA7 (figure 3.4, table 3.1). Furthermore, we were able to find that the GABAergic markers \textit{pflp-13::gfp} and \textit{punc-47::mCherry} showed a gradual decline of expression in the posterior (figures 3.1, 3.2).

Interestingly, many of the cell bodies where the posterior DD motor neurons are expected were present but failed to express the above GABAergic reporters (figures 3.1, 3.2, 3.10). Ultimately, these stark changes in the cellular network in the posterior corresponded to a distinct locomotory behavior. As a result, most animals exhibited a severe dorsal coil in the posterior of the body during backward locomotion (figure 3.9).

By addressing \textit{cnd-1} from a cell lineage perspective, we were able to provide more information on its role in VNC development. Progenitor cells exist as multipotent cells. Then, a unique pattern of gene expression that is controlled by GRNs is established. This leads to developmental programs in which the cell fate of the progenitor cell becomes more defined until the cell becomes committed to a certain cell fate. During this process, the progenitor cell is
increasingly restricted and permissive conditions are created that lead to a specific cell fate. This process requires an array of biomolecules that make up the GRN, paracrine factors and epigenetic control. As embryogenesis continues, *cnd-1* expression is progressively restricted to the ABprppap and ABplppap lineages (Hallam et al., 2000). These lineages include DA1 to DA5 and DD1 to DD6 (Sulston et al., 1983). Interestingly, we found that these lineages are most affected in *cnd-1* mutants. Thus, we conclude that *cnd-1* is actively involved in the developmental programs that establish the different cell fates within these lineages.
REFERENCES


