Molecular and Neuromuscular Mechanisms Underlying Locomotion and Proprioception in *Caenorhabditis elegans*

Victoria Jayne Butler

MRC Laboratory of Molecular Biology
Downing College
University of Cambridge

A dissertation submitted to the University of Cambridge for the degree of Doctor of Philosophy

April 2012
Preface

I certify that this dissertation is the result of my own work carried out between October 2008 and April 2012 at the MRC Laboratory of Molecular Biology, Cambridge, England and at HHMI Janelia Farm Research Campus, Virginia, US. Where information has been derived from other sources or from collaboration, this has been specifically indicated in the text. No part of this dissertation has been submitted for any other qualification at any other university. The total length of this dissertation does not exceed 60,000 words.
Acknowledgements

Firstly, I would like to thank my supervisor, William Schafer, for the guidance he has provided during my PhD. He has given me the freedom to pursue my own scientific ideas and develop my research skills.

I thank the Medical Research Council for my MRC research studentship and the MRC Laboratory of Molecular Biology for supplying facilities and materials that I have needed to do my research.

I am grateful to HHMI Janelia Farm Research Campus, US, for supporting and funding a three-year Visitor’s Project to allow me to conduct a research project abroad. I thank my host at Janelia Farm, Mitya Chklovskii, and the Visitor’s Project Program Manager, Chonnettia Jones. I thank Kevin Moses, Katie Breneman and Elena Rivas for including me within the Janelia Farm Graduate Program events. I especially thank Rex Kerr for including me within his worm lab at Janelia Farm, for the help he has given me developing my setup and imaging software, and for teaching me the basics of calcium imaging.

I thank Wafa Amir for teaching me optics. I thank Tilman Triphan for teaching me computer programming. I thank James Cregg, Robyn Branicky, Lee Henry and Jon Michael-Knapp for teaching me molecular biology. I thank the members of the Instrument Design and Fabrication at Janelia Farm for help building my setup, the Molecular Biology Facility for their help and advice in generating calcium imaging constructs and to Margaret Jefferies for being an amazing lab coordinator and support whilst I worked at Janelia Farm.

I thank all the members of the Schafer lab, the de Bono lab, the Kerr lab and the Chklovskii lab for their inspiring and enlightening conversations. I have shared a bench with Marios Chatzigeorgiou, Denise Walker, Laura Grundy and Jaye-Anne Gallagher, and I thank them for fun times in the lab. I have been lucky to supervise a very talented Physics Masters Student, Virginie Grand-Perret, and I thank her for her work developing analysis code for my calcium imaging recordings.

Finally, I thank my family for their support in all the decisions I have taken throughout my studies, even when it took me away from home.
Summary

Even with its simple nervous system, the nematode worm *Caenorhabditis elegans* can display a range of complex behaviours. Movement can be viewed as the main output of the *C. elegans* nervous system. Locomotion behaviour consists of “crawling” on a solid surface and “swimming” in liquid. These sinusoidal movement patterns result from alternating ventral and dorsal contractions of the longitudinal body wall muscles and are controlled by 113 of the 302 *C. elegans* neurons. Despite the robust neuron wiring diagram, it is still not fully understood how the motor circuit coordinates locomotion. As such, the main goal of my thesis has been to understand how genes act within the locomotory circuit to generate and regulate locomotion behaviour. To do this I built a whole-worm tracking and calcium imaging microscope and generated GCaMP3 transgenic lines to monitor the activity of neurons and muscles within the locomotion circuit.

My results from body wall muscle imaging in wildtype worms provide evidence for the cross-inhibition model of *C. elegans* locomotion: dorsal muscle calcium signal correlates with dorsal bending, ventral muscle calcium signal correlates with ventral bending and dorsal and ventral muscle activity is out-of-phase. These basic relationships also apply to backward crawling and to swimming locomotion. During crawling locomotion, maximal muscle activity and maximal body curvature show a small but significant phase-shift, with maximal muscle activity preceding maximal body curvature. Phase-shifts are comparable between dorsal and ventral muscle but the magnitude increases along the body of the worm and is significantly higher in posterior muscle compared to anterior and mid-body muscle. Such a relationship has also been observed for the longitudinal red muscle fibres of many fish species that display anguilliform locomotion similar to *C. elegans*.

I found that mutants with abnormal locomotion behaviour display altered patterns of body wall muscle activity. Differences in the levels of body wall muscle calcium and contraction observed in these mutants contribute to differences in their body posture and reduce the overall efficiency of locomotion. The fainting body postures observed in *nca-1*(gk9);*nca-2*(gk5), *unc-79*(e1068) and *unc-80*(e1069) mutants result from calcium depletion and body
wall muscle relaxation. In contrast, the fixed body postures frequently adopted by the motor-neuron mis-wiring mutants unc-55(e402) and unc-4(e120) occur actively through higher levels of calcium and muscle contraction.

As a calcium sensor in C. elegans, I found that GCaMP3 displayed greater than 5-fold change in fluorescence during crawling locomotion and it has a sufficiently high baseline fluorescence to allow the continuous tracking of muscle and motor neurons. However, my results suggest that, as a non-ratiometric calcium indicator, GCaMP3 is very sensitive to movement artefacts within the z-axis. Furthermore, I found that there were incompatibilities between several promoters and GCaMP3 expression in selected motor neuron populations, and preliminary GCaMP3 calcium signals from D class motor neurons are inconclusive.
Table of Contents

Preface i
Acknowledgements ii
Summary iii
Table of Contents v
List of Figures x

Chapter I: Introduction 1

1.1 *Caenorhabditis elegans* as a model organism 2
1.2 *C. elegans* neurobiology 3
1.3 *C. elegans* genetics 5
1.4 *In vivo* calcium imaging in *C. elegans* 6
1.5 The *C. elegans* locomotion circuit 8
1.6 Genetic specification of the motor circuit 12
1.7 Current models for how the motor circuit coordinates locomotion 14
1.8 The generation and propagation of sinusoidal bending waves 19
1.9 Putative proprioceptive molecules in *C. elegans* 23
1.10 Thesis aims 26

Chapter II: Quantitative Analysis of Locomotion Behaviour 28

2.1 Abstract 29
2.2 Introduction 30
2.2.1 Machine Vision to Quantitatively Analyse Locomotion Behaviour 30
2.2.2 Genes hypothesized to function in proprioception during locomotion 30
2.2.3 Genes regulating the activation of motor neuron synapses 31
2.2.4 Genes regulating the wiring of the locomotion circuit 31
2.3 Methods 34
2.3.1 Strains 34
2.3.2 Genotyping of strains 34
2.3.2.1 Identification of deletion sites in the putative stretch-receptor mutants *unc-8(e15lb145)* and *del-1(ok150)* 34
2.3.2.2 Identification of mutation sites in the fainter mutants *nca-1(gk9), unc-79(e1068) and unc-80(e1069)* 36
2.3.3 Genetic Manipulations

2.3.3.1 Generation of nca-1(gk9); nca-2(gk5) double mutant

2.3.3.2 Generation of unc-8(e15lb145); del-1(ok150) double mutant

2.3.4 Locomotion Assays

2.3.5 Measurement of Locomotion Features

2.3.6 Statistical Analysis

2.4 Results

2.4.1 Locomotion behaviour of Wildtype N2 worms and the Putative Stretch Receptor Mutants unc-8(e15lb145) and del-1(ok150)

2.4.2 Locomotion behaviour of the fainter mutants nca-1(gk9), nca-2(gk5), unc-79(e1068) and unc-80(e1069)

2.4.3 Locomotion behaviour of the mis-wiring mutants vab-7(e1562), unc-3(e151), unc-4(e120), unc-30(e191), unc-55(e402) and unc-104(e1625)

2.5 Discussion

2.5.1. The role of proprioception in locomotion behaviour

2.5.2 The role of cation leak channels in locomotion behaviour

2.5.3 The roles of the A, B and D motor neuron classes in locomotion

2.5.4 Utility of Machine Vision for quantifying behavioural phenotypes

Chapter III: Construction of GCaMP3 Transgenic Lines and Calcium Imaging Worm Tracker

3.1 Abstract

3.2 Strains

3.3 Molecular Biology

3.3.1 Genotyping

3.4 Primers

3.5 Construction of GCaMP3 and GFP expression vectors

3.5.1 Generation of body-wall muscle lines

ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T]

3.5.2 Generation of D class GABAergic motor neuron lines

ljIs133[unc-47::GCaMP3-SL2-tagRFP-T] and ljIs134[unc-47::GFP-SL2-tagRFP-T]

3.5.3 Generation of B class acetylcholinergic motor neuron lines
**ljIs135[acr-5::GCaMP3-SL2-tag-RFP-T] and ljIs136[acr-5::GFP-SL2-tagRFP-T]**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5.4 Strategies for making A class motor neuron lines</td>
<td>78</td>
</tr>
<tr>
<td>3.6 Integration of extrachromosomal arrays</td>
<td>80</td>
</tr>
<tr>
<td>3.7 Mapping the integration site for body wall muscle GCaMP3 construct</td>
<td>81</td>
</tr>
<tr>
<td>3.7.1 Two point mapping for linkage analysis</td>
<td>81</td>
</tr>
<tr>
<td>3.7.2 Three point mapping</td>
<td>82</td>
</tr>
<tr>
<td>3.8 Crossing of integrated arrays into mutant lines</td>
<td>82</td>
</tr>
<tr>
<td>3.9 Calcium Imaging Setup</td>
<td>83</td>
</tr>
<tr>
<td>3.9.1 Calcium imaging hardware components and configuration</td>
<td>83</td>
</tr>
<tr>
<td>3.9.1.1 Tracking Stage</td>
<td>83</td>
</tr>
<tr>
<td>3.9.1.2 Fluorescence Excitation</td>
<td>83</td>
</tr>
<tr>
<td>3.9.1.3 Fluorescence Emission Detection</td>
<td>85</td>
</tr>
<tr>
<td>3.9.2 Worm Tracking</td>
<td>85</td>
</tr>
<tr>
<td>3.9.3 Software</td>
<td>87</td>
</tr>
<tr>
<td>3.9.3.1 Worm tracking Software</td>
<td>87</td>
</tr>
<tr>
<td>3.9.3.2 Calcium Imaging Software</td>
<td>87</td>
</tr>
<tr>
<td>3.10 Calcium Imaging Assays</td>
<td>88</td>
</tr>
<tr>
<td>3.11 Post-processing of calcium image sequences</td>
<td>89</td>
</tr>
<tr>
<td>3.11.1 Checking stability of laser illumination over time and between powers</td>
<td>89</td>
</tr>
<tr>
<td>3.11.2 Fluorescence bleed-through correction</td>
<td>89</td>
</tr>
<tr>
<td>3.11.3 Checking for saturation of the image</td>
<td>90</td>
</tr>
<tr>
<td>3.11.4 Checking stability of laser illumination on stage movement</td>
<td>90</td>
</tr>
<tr>
<td>3.12 Quantitative Analysis of Calcium Imaging Sequences</td>
<td>93</td>
</tr>
<tr>
<td>3.12.1 Muscle Calcium Imaging Analysis</td>
<td>93</td>
</tr>
<tr>
<td>3.12.2 Neuron Calcium Imaging Analysis</td>
<td>94</td>
</tr>
<tr>
<td>3.13 Preliminary imaging results from neurons</td>
<td>95</td>
</tr>
</tbody>
</table>

**Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Abstract</td>
<td>103</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>105</td>
</tr>
<tr>
<td>4.2.1 Crawling and swimming locomotion display distinct kinematics</td>
<td>105</td>
</tr>
<tr>
<td>4.2.2 Theoretical models relating muscle activity to body curvature</td>
<td>106</td>
</tr>
</tbody>
</table>
4.2.3 Preliminary evidence supporting theoretical models 108
4.3 Methods 110
  4.3.1 Strains 110
  4.3.2 Calcium Imaging Assays and Muscle Calcium Analysis 110
  4.3.3 Statistical Analysis 110
  4.3.4 Phase-shift Measurements 111
4.4 Results 112
  4.4.1 Calcium imaging from the body wall muscle of freely-crawling wildtype worms 112
  4.4.2 Calcium imaging from the body wall muscle of freely forward crawling wildtype worms constrained in the z-axis 132
  4.4.3 Calcium imaging from the body wall muscle of backward crawling wildtype worms 150
  4.4.4 Measuring the phase-shift between muscle activity and body curvature during crawling 156
  4.4.5 Calcium imaging from the body wall muscle of freely-swimming wildtype worms constrained in the z-axis 159
  4.4.6 Measuring the phase-shift between muscle activity and body curvature during swimming 176
4.5 Discussion 179
  4.5.1 GCaMP3 as a non-ratiometric calcium indicator in freely moving worms 179
  4.5.2 Calcium dynamics and muscle activity 180
  4.5.3 Muscle activity in crawling worms 181
  4.5.4 Muscle activity in swimming worms 183
Chapter V: Imaging Muscle Activity in Locomotion Mutants 185
  5.1 Abstract 186
  5.2 Introduction 188
    5.2.1 Imaging muscle activity in locomotion mutants 188
    5.2.2 Imaging muscle activity during crawling and swimming in the fainter mutants 188
    5.2.3 Imaging muscle activity in motor neuron mis-wiring mutants 189
List of Figures

**Figure 1.1**: The *C. elegans* motor circuit. 9

**Figure 1.2**: Diagram of the morphology of each motor neuron class in the adult VNC 10

**Figure 1.3**: The *C. elegans* locomotion circuit 15

**Figure 1.4**: A model for stretch-regulated control of *C. elegans* locomotion 21

**Figure 2.1**: Identification of deletion sites in the putative stretch-receptor mutants 35

**Figure 2.2**: Summary of locomotion behaviour for wildtype and putative proprioceptive mutants 41

**Figure 2.3**: Body posture sequences for wildtype N2 worms and putative proprioceptive mutants 42

**Figure 2.4**: Box-plots to compare locomotion features for wildtype and putative proprioceptive mutants 44

**Figure 2.5**: Histograms for body curvature at five defined locations along the worm for wildtype N2 worms and putative stretch receptor mutants 47

**Figure 2.6**: Box-plots to compare body curvature at five defined locations along the worm for wildtype and putative proprioceptive mutants 48

**Figure 2.7**: Summary of locomotion behaviour for wildtype and fainter mutants 51

**Figure 2.8**: Body posture sequences for wildtype N2 worms and fainter mutants 52

**Figure 2.9**: Summary of locomotion behaviour for wildtype and motor neuron mis-wiring mutants 55

**Figure 2.10**: Body postures for wildtype N2 worms and motor neuron mis-wiring mutants 56

**Figure 3.1**: Image of worm line AQ2953 *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* 74

**Figure 3.2**: Image of worm line AQ2967 *ljIs133[unc-47::GCaMP3-SL2-tagRFP-T]* 75

**Figure 3.3**: Image of worm line AQ2969 *ljIs135[acr-5::GCaMP3-SL2-tag-RFP-T]* 76

**Figure 3.4**: Images of experimental and control transgenic worm lines 77

**Figure 3.5**: Picture of the calcium imaging worm tracker 84

**Figure 3.6**: Example images from the Worm Tracker 2.0 software and the Andor calcium imaging software 86

**Figure 3.7**: Calcium imaging from VD motor neurons during crawling in line AQ2967 *ljIs133[unc-47::GCaMP3-SL2-tagRFP-T]* 96
Figure 3.8: Calcium imaging from VD and DD motor neurons during crawling in line AQ2968 ljIs134[unc-47::GFP-SL2-tagRFP-T]

Figure 3.9: Calcium imaging from the AVA interneuron in line QW625 zfIs[prig-3::GCaMP3-SL2-mCherry; lin-15+] Figure 4.1: Diagram of theoretical prediction of relationship between maximal muscle activity and maximal body curvature

Figure 4.2: Images of ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms

Figure 4.3: Example crawling curvature and fluorescence ratio matrices for ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] worms

Figure 4.4: Example dorsal and ventral fluorescence ratios plotted with body curvature for ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms

Figure 4.5: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms

Figure 4.6: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms

Figure 4.7: Variance for green and red channels in freely-crawling ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] worms

Figure 4.8: Example ventral and dorsal fluorescence signals plotted with body curvature for ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] crawling worms

Figure 4.9: Example ventral and dorsal fluorescence signals plotted with body curvature over time for ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms

Figure 4.10: Correlation between green and red fluorescence and body curvature for ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] worms

Figure 4.11: Images of ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms constrained in z-axis

Figure 4.12: Example crawling curvature and fluorescence ratio matrices for
\textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} worms constrained in z-axis

\textbf{Figure 4.13}: Example dorsal and ventral fluorescence ratios plotted with body curvature for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling worms constrained in z-axis

\textbf{Figure 4.14}: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling worms constrained in z-axis

\textbf{Figure 4.15}: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling worms constrained in z-axis

\textbf{Figure 4.16}: Example ventral and dorsal fluorescence signals plotted with body curvature for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} crawling worms

\textbf{Figure 4.17}: Example ventral and dorsal fluorescence signals plotted with body curvature for \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling worms

\textbf{Figure 4.18}: Variance for green and red channels in freely-crawling \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} worms

\textbf{Figure 4.19}: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} worms

\textbf{Figure 4.20}: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} worms

\textbf{Figure 4.21}: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} backward crawling worms constrained in z-axis

\textbf{Figure 4.22}: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} backward crawling worms constrained in z-axis

\textbf{Figure 4.23}: Scatter plot of phase-shift between maximal muscle activity and maximal body curvature for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} crawling worms

\textbf{Figure 4.24}: Phase shift and bending period measurements along the body for crawling worms
Figure 4.25: Images of \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) swimming worms

Figure 4.26: Example curvature and fluorescence ratio matrices for \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) swimming worms

Figure 4.27: Example dorsal and ventral fluorescence ratios plotted with body curvature for \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) swimming worms

Figure 4.28: Variance for green and red channels in freely-swimming \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) worms

Figure 4.29: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) swimming worms constrained in z-axis

Figure 4.30: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) swimming worms constrained in z-axis

Figure 4.31: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) worms

Figure 4.32: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming \(l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) worms

Figure 4.33: Scatter plot of phase-shift between maximal muscle activity and maximal body curvature for \(l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) swimming worms

Figure 4.34: Phase shift and bending period measurements along the body for swimming worms

Figure 5.1: Images of \(nca-1(gk9);nca-2(gk5); l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(nca-1(gk9);nca-2(gk5); l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) crawling mutants

Figure 5.2: Example crawling curvature and fluorescence ratio matrices for \(nca-1(gk9);nca-2(gk5); l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(nca-1(gk9);nca-2(gk5); l{j}i{s}_{132}[pmyo-3::GFP-SL2-tagRFP-T]\) mutants

Figure 5.3: Example dorsal and ventral fluorescence ratios plotted with body curvature for \(nca-1(gk9);nca-2(gk5); l{j}i{s}_{131}[pmyo-3::GCaMP3-SL2-tagRFP-T]\)
and nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants

**Figure 5.4:** Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants

**Figure 5.5:** Example plots of dorsal and ventral muscle activity during fainting episodes for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] crawling mutants

**Figure 5.6:** Example plots of dorsal and ventral muscle activity during fainting episodes for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants

**Figure 5.7:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutations

**Figure 5.8:** Example crawling curvature and fluorescence ratio matrices for unc-79(e1068); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-79(e1068); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis

**Figure 5.9:** Example crawling curvature and fluorescence ratio matrices for unc-80(e1069); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-80(e1069); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis

**Figure 5.10:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for unc-79(e1068); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants

**Figure 5.11:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for unc-80(e1069); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants

**Figure 5.12:** Images of nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutants swimming

**Figure 5.13:** Example swimming curvature and fluorescence ratio matrices for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.14:** Example dorsal and ventral fluorescence ratios plotted with body curvature for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] swimming mutants

**Figure 5.15:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming nca-1(gk9);nca-2(gk5);
ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants

**Figure 5.16:** Example swimming curvature and fluorescence ratio matrices for unc-79(e1068); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-79(e1068); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.17:** Example swimming curvature and fluorescence ratio matrices for unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-80(e1069); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.18:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming unc-79(e1068); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]

**Figure 5.19:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]

**Figure 5.20:** Images from the EMCCD camera for unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.21:** Example crawling curvature and fluorescence ratio matrices for unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.22:** Example dorsal and ventral fluorescence ratios plotted with body curvature for unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants

**Figure 5.23:** Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants

**Figure 5.24:** Example plots of dorsal and ventral muscle activity during fixed postures for unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.25:** Example plots of dorsal and ventral muscle activity during fixed postures for unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

**Figure 5.26:** Summary of measured parameters for dorsal and ventral fluorescence
Figure 5.27: Images of unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants crawling

Figure 5.28: Example crawling curvature and fluorescence ratio matrices for unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

Figure 5.29: Example dorsal and ventral fluorescence ratios plotted with body curvature for unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants

Figure 5.30: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants

Figure 5.31: Images of vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants crawling

Figure 5.32: Example crawling curvature and fluorescence ratio matrices for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

Figure 5.33: Example dorsal and ventral fluorescence ratios plotted with body curvature for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

Figure 5.34: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants

Figure 5.35: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants
Chapter I

Introduction
Chapter I: Introduction

1.1 *Caenorhabditis elegans* as a model organism

How genes, molecules and neurons function within the nervous system to generate coordinated and complex behaviours remains one of the fundamental questions in biology. In order to answer this question it is an advantage to study an organism that is amenable to genetic analysis and where the connectivity of the nervous system can be described completely. In 1974, these requirements led Sydney Brenner to propose the use of *Caenorhabditis elegans* as a model organism in which to investigate the development and function of a simple nervous system (Brenner 1974; Brenner 1988).

Since this time, *C. elegans* has provided researchers with significant insights into a diverse range of biological processes. Three separate Nobel Prizes have recognized the worm’s importance to basic science and its role in understanding the biology of higher organisms: in 2002 for work on the genetic regulation of organ development and programmed cell death, in 2006 for the discovery of RNA interference and in 2008 for its role in the development of green fluorescent protein (GFP). Over the last ten years, advancements in molecular biology and *in vivo* imaging techniques have helped to exploit the full potential of *C. elegans* as a tool for neurobiology research. In my work, I have used a combination of genetics and newly developed methods for *in vivo* imaging of nervous system activity, to investigate how *C. elegans* coordinates its locomotion behaviour. I have investigated the modulation of this behaviour by the environment and how genetic mutations that alter movement can change the activity of the locomotion circuit.

*C. elegans* is a small, free-living soil nematode that feeds on bacteria and reproduces with a life cycle of about three days. Its advantages for study as a model system include its short life cycle, small size, ease of cultivation in the lab, compact genome, stereotypical development and transparency for imaging cells and following cell lineages. *C. elegans* is anatomically and genetically simple. Like other nematodes, *C. elegans* has an unsegmented, cylindrical body shape tapering at both ends, which is maintained by internal hydrostatic pressure. The pseudocoelomic space separates an inner and outer tube. The outer body wall tube is made up of cuticle, hypodermis, excretory system, neurons and muscles. The inner
Chapter I: Introduction

tube is made up of the pharynx, intestine and gonad (Brenner 1988). There are two sexes; hermaphrodites and males, which are both approximately one millimetre in length. After hatching, the worm develops through four larval stages (L1, L2, L3 and L4) before reaching adulthood and laying eggs. Hermaphrodites can produce both oocytes and sperm and reproduce by self-fertilization. Males arise spontaneously at low frequency (0.1%) and can mate with hermaphrodites to generate cross-progeny of up to 50% males and 50% hermaphrodites (Brenner 1974; Brenner 1988; Riddle 1997).

1.2 C. elegans neurobiology

The adult hermaphrodite has 959 somatic cells of invariant lineage, of which 302 are neurons and 95 are body wall muscle cells (Sulston and Horvitz 1977; Brenner 1988; Riddle 1997). The complete synaptic connectivity of the hermaphrodite nervous system has been characterised through serial electron micrographs (White, Southgate et al. 1986). The wiring diagram contains 6,400 chemical synapses, 900 gap junctions and 1,500 neuromuscular junctions. Between individual worms, the location of chemical synapses is about 75% reproducible (Durbin 1987). The C. elegans nervous system is composed of the nerve ring in the head, the dorsal and ventral nerve cords, and a collection of neurons in the tail. Most of the C. elegans neurons are located in the nerve ring around the pharynx, in the head. The nerve ring contains sensory neurons, interneurons and their axonal projections, whereas the neurons found in the nerve cords are mainly motor neurons (White, Southgate et al. 1976).

The 302 neurons have been assigned to 118 distinct classes, according to their morphology and synaptic connection patterns (White, Southgate et al. 1986) and each neuron is nearly structurally identical between animals (Sulston 1983). C. elegans neurons are small, around 2μm in diameter. Almost all have a simple monopolar or bipolar morphology, with unbranched processes. C elegans neurons are generated at three main developmental time points; during the proliferative phase of embryogenesis, the late L1 stage, and the late L2 stage (Riddle 1997). C. elegans neurons can be classified into four classes based on their circuitry; 1) motor neurons that synapse onto muscle, 2) sensory neurons with
specializations, 3) interneurons, which receive synapses from and send synapses to other neurons, and 4) polymodal neurons that perform more than one of these functions.

Just as in higher organisms, *C. elegans* neurons can form both chemical and electrical synapses. Chemical synapses are made *en passant* between adjacent processes. Rapid neurotransmission uses the classical neurotransmitters; acetylcholine, GABA, glutamate and monoamines (serotonin, dopamine, tyramine, and octopamine). Neurotransmitter receptors, synthesis and release are highly conserved from worms to mammals (Rankin 2002). Ion channels are similar to those in mammals but no voltage-gated sodium channels have been identified in the worm genome (Bargmann 1998). Consistent with this is the demonstration that *C. elegans* neurons fail to generate sodium action potentials (Goodman, Hall et al. 1998). The *C. elegans* genome also encodes more than 150 putative neuropeptides that are thought to modulate synaptic function (Richmond and Broadie 2002). These molecules superimpose a complex wireless network onto the existing wiring diagram (Bargmann 1998).

Within nervous systems electrical gap junctions between neurons are mostly formed at axon-to-axon and axon-to-soma contacts. Gap junctions in nematodes are made by membrane proteins called “innexins”. Twenty-five innexins have been identified in the *C. elegans* genome and these are different in their amino acid sequences to the “connexins” that form vertebrate gap junctions (Starich, Lee et al. 1996; Phelan and Starich 2001). Such connections between neurons can relay signals among connected cells, synchronize their neuronal activity and allows for cross-inhibition.

Despite its simple nervous system, *C. elegans* can perform a variety of complex and interesting behaviours, some of which were once only thought to be displayed by higher organisms. Such behaviours include chemotaxis to food sources and chemicals, thermotaxis to preferred temperatures, male mating of hermaphrodites, regulation of egg-laying by the evaluation of several environmental cues, sleeping-like behaviour on moulting between larval stages, simple forms of habituation learning to repeated mechanical and chemical stimuli and even associative learning to pair ions with the presence or absence of food
Chapter I: Introduction

(Wicks and Rankin 1996; de Bono and Maricq 2005; Raizen, Zimmerman et al. 2008; Garrity, Goodman et al. 2010).

1.3 C. elegans genetics

C. elegans is especially amenable to the genetic analysis of neuron development and function, which is essential for a comprehensive understanding of behaviour. C. elegans was the first multi-cellular organism to have its genome completely sequenced, and as a consequence it is extensively annotated. The worm has a haploid genome size of $8 \times 10^7$ nucleotide pairs, which encode around 20,000 genes. Sequence comparison with numerous other eukaryotic genomes has revealed a high degree of conservation between the genes of C. elegans and that of higher organisms. For example, there is a strong evolutionary conservation between transcription factors and the mechanisms they employ to specify motor neuron identity (Von Stetina, Treinin et al. 2005). The mammalian homolog of the transcription factor UNC-30 that specifies GABAergic neuron fate in C. elegans is Pitx2 in mammals, which also regulates GABAergic interneuron differentiation in the mammalian brain (Westmoreland, McEwen et al. 2001).

The haploid genome consists of six linkage groups, which correspond to the five autosomes (I, II, III, IV, and V) and one sex chromosome (X). Hermaphrodites are XX, whereas males are XO (Riddle 1997). Self-fertilization by the hermaphrodite means that homozygous worms generate genetically identical progeny. This allows for easy propagation of recessive mutant lines without staged crosses, and male mating allows for the movement of mutations between strains.

Mutant animals can be generated through chemical mutagenesis and exposure to ionizing radiation. Such forward genetic screens have been used in C. elegans to impartially identify novel genes in important biological processes. For example, the original screen carried out by Brenner identified around 300 mutations affecting behaviour and morphology (Brenner 1974). Of these, 77 mutations displayed altered locomotion ranging from paralysis to small defects in movement. Some of these worms were identified as having missing dorsal nerve
cords, lesions in their ventral nerve cord or defective body wall muscles. Recently, screens for more subtle locomotion defects have been carried out, for example, on the transition between crawling and swimming locomotion (Pierce-Shimomura, Chen et al. 2008). This has helped to identify genes such as \textit{unc-80} and \textit{nca-1} that are important in generating rhythmic locomotion patterns. These genes are thought to regulate motor neuron excitability by modulating the propagation of depolarization signals from the cell body to the synapse (Yeh, Ng et al. 2008).

Numerous genetic markers have been identified in the worm that can be easily used to determine linkage and map mutations of interest. Most of these markers are morphological or behavioural, and many initially identified by Brenner are still used (Brenner 1974). Many balancer chromosomes are available for each of the six chromosomes, which allow for the propagation of recessive sterile and lethal mutations.

The ability to carry out germline transformations in \textit{C. elegans} by microinjection of DNA into the gonad allows for the generation of transgenic animals that can transmit stable extrachromosomal arrays to their progeny (Mello, Kramer et al. 1991). In this way genes can be expressed in a specific subset of neurons, for example, to express a calcium indicator protein, to knockdown the expression of a gene through RNA interference (RNAi) or to express a copy of a wildtype gene to rescue a mutant phenotype.

\textbf{1.4 In vivo calcium imaging in \textit{C. elegans}}

The current challenge for \textit{C. elegans} research is mapping of the function onto the structure of the nervous system. Traditionally, electrophysiology has been used to measure the activity of neurons. This approach is technically challenging in \textit{C. elegans} due to the hydrostatic skeleton, as well as the small size and inaccessibility of the neurons. As such, it has been difficult to monitor neural activity in living animals. A solution to this problem came with the development of genetically-encoded calcium indicators (GECIs), which can be targeted to small numbers of neurons using cell-specific promoters, to monitor \textit{in vivo} neural activity.
The GECI cameleon has been extensively used in *C. elegans* to monitor the activity of neurons (Kerr, Lev-Ram et al. 2000; Suzuki, Kerr et al. 2003; Hilliard, Apicella et al. 2005). Cameleon uses fluorescence resonance energy transfer (FRET) to signal changes in cellular calcium concentration (Miyawaki, Llopis et al. 1997; Miyawaki, Griesbeck et al. 1999). Cameleon consists of four domains; cyan fluorescent protein (CFP), calmodulin (that binds calcium ions), M13 (a calmodulin binding domain) and yellow fluorescent protein (YFP). On depolarization of a neuron, the cytoplasmic concentration of calcium is high; calmodulin binds calcium ions and attracts M13, bringing the two fluorophores CFP and YFP together to allow for FRET. In this process, the donor fluorophore CFP is excited, and the emission from CFP excites the acceptor fluorophore YFP. The ratio of YFP to CFP fluorescence gives a ratiometric measure of calcium concentration.

Recently much effort has been directed into improving the properties of GECIs to facilitate the imaging of neural activity. For single fluorescent protein GECIs, such as the GCaMPs, the fluorescence of the circularly-permuted fluorescent protein is modulated by changes in the environment of the chromophore on calcium binding. The newly-developed single fluorescent protein calcium indicator GCaMP3 has a higher baseline fluorescence (x3), increased dynamic range (x3), higher affinity for calcium, faster kinetics and improved photostability compared to the previous version GCaMP2 (Tian, Hires et al. 2009). It has been successfully used to monitor sensory-evoked calcium transients in the *C. elegans* AWC
\textsuperscript{ON} neuron. In order to correct for movement artefacts on imaging, such single fluorescent protein indicators must be coupled, either physically to or by co-expression with, a reference fluorescence protein whose fluorescence does not change with cellular calcium concentration. Ratiometric measurements of calcium transients with GCaMP3 can be achieved by co-expression with, or fusion to, a reference fluorescence protein with an excitation and emission spectrum distinct from that of GCaMP3, such as red fluorescent protein (RFP) or DsRed (Piggott, Liu et al. 2011).
1.5 The C. elegans locomotion circuit

Movement can be viewed as the main output of the C. elegans nervous system, where behaviour results from the efficient integration of many different sensory inputs (Hobert 2003). The lineage, morphology and connectivity of the C. elegans locomotion circuit have been described at high resolution. The locomotory behaviour of C. elegans consists of “crawling” on a solid surface and “swimming” in liquid. These sinusoidal movement patterns result from alternating ventral and dorsal contractions of the longitudinal body wall muscles (Brenner, 1974), which are organised into 2 dorsal and 2 ventral rows, each consisting of 23 or 24 muscle cells (Sulston and Horvitz 1977; Riddle 1997; Altun, Chen et al. 2009) (see Figure 1.1A). A small subset of neurons control worm locomotion (White, Southgate et al. 1986). A total of 113 of the 302 C. elegans neurons are motor neurons that fall into 8 distinct classes (AS, VA, VB, VC, VD, DA, DB, DD), which are defined based on morphological and synaptic connection similarities.

The VA, VB, VC and VD classes of motor neuron innervate the ventral body wall muscles, whereas the DA, DB, DD and AS classes send commissures across the body to innervate the dorsal body wall muscles (White, Southgate et al. 1976; White, Southgate et al. 1986). The A and B motor neurons are acetylcholinergic and excitatory (Riddle 1997; Duerr, Han et al. 2008). The D motor neurons are GABAergic and inhibitory (Mcintire, Jorgensen et al. 1993; Mcintire, Jorgensen et al. 1993). VC4 and 5 motor neurons release several neurotransmitters (acetylcholine, neuropeptides and possibly serotonin) and synapse with the vulval muscles (White, Southgate et al. 1986; Weinshenker, Garriga et al. 1995). Within each motor neuron class there is very little overlap in the output regions of adjacent cells onto the body wall muscle (White, Southgate et al. 1976).

The majority of these motor neurons have their cell bodies distributed along the length of the ventral nerve cord (VNC) (White, Southgate et al. 1976) and their activity depends on input from the command interneurons; AVA, AVB, AVD, AVE in the head, and PVC in the tail (White, Southgate et al. 1986; Zheng, Brockie et al. 1999) (see Figure 1.1B). Synaptic innervation of motor neurons by command interneurons occurs en passant along the length
Chapter I: Introduction

of the VNC. A-class motor neurons extend their axonal processes anteriorly and synapse with the command interneurons AVA (by gap junctions and chemical synapses), AVD (by chemical synapses) and AVE (by chemical synapses). B-class motor neurons extend their axonal processes posteriorly and synapse with the command interneurons AVB (by gap junctions) and PVC (by chemical synapses) (White, Albertson et al. 1978) (See Figure 1.2).

Figure 1.1: The C. elegans motor circuit. A: Organization of body-wall muscle into 2 dorsal and 2 ventral rows (taken from (Von Stetina, Treinin et al. 2005), adapted from (Francis, Mellem et al. 2003)). B: Organization of command interneurons in the head and tail, which synapse with motor neurons in the ventral nerve cord. Motor neurons extend process in the ventral nerve cord and in the dorsal nerve cord via commissures (taken from (Von Stetina, Treinin et al. 2005), adapted from (Dixon and Roy 2005)).
Figure 1.2: Diagram of the morphology of each motor neuron class in the adult VNC. 5 classes extend commissures to the DNC (DD, VD, DA, DB and AS) and 3 classes are restricted to the VNC (VA, VB, VC). Green arrows represent input from other neurons; red triangles represent NMJs with body wall muscle cells (taken from (Von Stetina, Treinin et al. 2005)).
D-class motor neurons are postsynaptic only to other motor neurons and extend their axonal projections both anteriorly and posteriorly along the nerve cords. VD motor neurons innervate ventral muscles and receive input from excitatory motor neurons on the dorsal side; the DA and DB neurons. DD motor neurons innervate dorsal muscle and receive input from excitatory motor neurons on the ventral side; the VA and VB neurons (White, Southgate et al. 1976; White, Southgate et al. 1986).

As well as linear information flow from command interneurons, to motor neurons, to muscle, there is also much lateral signalling within the locomotion network. There are extensive reciprocal connections between the command interneurons through chemical and electrical synapses (White, Southgate et al. 1986). Gap junctions electrically connect adjacent motor neurons of the same class, as well as adjacent muscle cells in each dorsal and ventral row (White, Southgate et al. 1986). Motor neurons may also form gap junctions to motor neurons of a different class in the same region along the worm body, but rarely form gap junctions with motor neurons of a different region, even though their processes can be very closely located (White, Southgate et al. 1986).

*C. elegans* motor neurons are generated during two distinct developmental periods; firstly around mid-embryogenesis and secondly towards the end of the first larval stage (Sulston 1976; Sulston and Horvitz 1977; Sulston 1983). The DA, DB and DD motor neurons are present in the VNC at hatching and all three classes extend commissures to the DNC (Sulston et al., 1983). As in the adult worm, the DA and DB neurons innervate dorsal muscles, whereas the DD neurons innervate ventral muscles during the L1 stage only (White, Albertson et al. 1978).

The VA, VB, VD, AS and VC motor neurons are added to the VNC post-embryonically towards the end of the L1 stage (Sulston 1976; Sulston and Horvitz 1977; Riddle 1997). The processes from these neurons insert themselves into the VNC where they form contacts with the appropriate command interneurons and muscle cells. After these neurons are born, the DD neurons reverse their synaptic polarity, receiving input from the new VA and VB neurons, whilst providing output to dorsal muscles. This occurs without any structural change in their
Chapter I: Introduction

process placement (White, Albertson et al. 1978) and is an example of the plasticity exhibited by the *C. elegans* nervous system as it develops.

*C. elegans* has 95 body wall muscle cells, of which 14 are generated post-embryonically. These muscle cells are striated and classified as multiple sarcomere muscles because they have attachment points to the hypodermis and cuticle along their length. In contrast to vertebrates and insects, *C. elegans* muscles do not undergo membrane fusion, and they receive inputs from motor neurons by extending long, thin muscle arms to synapse onto the neuron processes that run along the nerve cords or in the nerve ring (Stretton 1976; Sulston and Horvitz 1977; Dixon and Roy 2005). Of the 95 body wall muscle cells, there are 16 head muscles that synapse with motor neurons exclusively in the nerve ring, and 16 neck muscles that synapse with motor neurons both in the nerve ring and to the nearest nerve cord. The remaining 63 body wall muscles synapse exclusively to the nearest motor neurons in the nerve cord (Altun, Chen et al. 2009). Muscle arm development is highly stereotypical. At hatching, muscle cells have around 2 arms, but this increases to 3 or more by the adult stage. Muscles in rows closest to the ventral and dorsal nerve cord have more arms than the row further out. Post-embryonic muscle arm outgrowth times with and depends on the birth of the 53 extra motor neurons at the end of the L1 stage (Dixon and Roy 2005).

1.6 Genetic specification of the motor circuit

The highly reproducible development, morphology and connectivity of the *C. elegans* locomotion circuit indicate a strong role for genetic control. In support of this, many mutants with abnormal patterns of locomotion and inappropriate motor neuron traits have been identified (Brenner 1974).

Genes encoding transcription factors are of particular interest because these control neuron identity through the regulation of multiple downstream target genes. In *C. elegans*, as in both invertebrate and vertebrate systems, specific transcription factors have been assigned to the stages of motor neuron differentiation, from the generation of neuronal progenitors to the terminal divisions that generate individual motor neuron classes (Brody and
Odenwald 2002; Shirasaki and Pfaff 2002; Von Stetina, Treinin et al. 2005). Mutations within such genes could be used to investigate how specific mis-wiring of neurons in the motor circuit affects motor neuron activity, muscle activity and subsequent locomotion.

For example, the *unc-55* gene encodes a COUP domain steroid hormone nuclear receptor transcription factor that is required for VD neurons to make their normal synapses with ventral muscle. In *unc-55* mutants VD neurons adopt DD neuron output onto dorsal muscle, resulting in uncoordinated locomotion (Walthall 1990). The UNC-55 protein is selectively expressed in VD neurons (Zhou and Walthall 1998). The transcription factor may function in the VDs to prevent expression of genes that result in the development of DD-like dorsal outputs because ectopic expression of UNC-55 in DD motor neurons prevents the polarity reversal of these neurons at the end of L1 stage and keeps the DDs with their ventral output connection (Shan, Kim et al. 2005).

Similarly, the *unc-4* gene encodes a paired-class homeodomain transcription factor that regulates synaptic connections of the VA neurons. In *unc-4* mutants, the VA neurons VA2 to VA10 receive synaptic input from the forward command interneurons that usually synapse onto B motor neurons (White, Southgate et al. 1992). The normal inputs to VA neurons from AVA, AVD and AVE are replaced by connections to AVB and PVC (both the chemical synapses and gap junctions). These worms cannot move backwards, coiling dorsally when they try. Forward movement is also impaired but to a lesser extent. The UNC-4 protein is expressed in the VA neurons and is hypothesized to turn off VB genes by a transcriptional repressor mechanism (Miller and Niemeyer 1995). This is supported by the finding that UNC-4 function depends on physical interaction with UNC-37, a transcriptional co-repressor protein, and that VB-specific reporter genes (*acr-5, del-1, glr-4*) are ectopically expressed in VA neurons in *unc-4* and *unc-37* mutants (Miller, Niemeyer et al. 1993; Pflugrad, Meir et al. 1997; Winnier, Meir et al. 1999).

The *vab-7* gene encodes the *C. elegans* homolog of the Even-skipped (Eve) homeodomain protein, which specifies DB motor neuron identity (Ahringer 1996; Esmaeili, Ross et al. 2002). In *vab-7* mutants DB neuron axons change polarity and project anteriorly along the
Chapter I: Introduction

DNC rather than posteriorly, so becoming morphologically identical to DA neurons. Worms carrying this mutation display uncoordinated forward locomotion. This result suggests that the VAB-7 protein functions to prevent DB motor neurons from adopting a DA fate. Eve homologs in flies and mice also function to control neuron identity and axon polarity in the motor circuit (Landgraf, Roy et al. 1999; Moran-Rivard, Kagawa et al. 2001). Interestingly, in each organism, Eve represses a transcription factor that specifies an alternative neuron class. Such conservation of transcription factor function from nematodes to mammals suggests that there is an early shared mechanism for motor circuit neuron fate determination.

1.7 Current models for how the motor circuit coordinates locomotion

The wiring diagram provides a starting point for all of the proposed models for C. elegans locomotion. The decision to move forwards or backwards is thought to be regulated by input from the command interneurons, with oscillatory activity between ventral and dorsal neurons in each class coordinating body undulations.

VA and VB neurons are believed to provide excitatory input to make ventral muscles contract, whilst DA and DB neurons provide excitatory input to make dorsal muscles contract. As the D class motor neurons are postsynaptic to excitatory A and B class motor neurons and their outputs are onto the diametrically opposite muscles, they are proposed to function in a negative feedback loop in which motor neuron activity causing muscle contraction on one side of the body results in inhibition and relaxation on the other side (White, Albertson et al. 1978; Mcintire, Jorgensen et al. 1993).

Forward movement is believed to be promoted by input from AVB and PVC interneurons onto DB and VB neurons, whereas backward movement is promoted by input from AVA, AVD and AVE interneurons onto DA and VA neurons (Riddle 1997). As such, the interneurons and motor neurons generating forward and backward movement are often considered as distinct neural circuits, in what has become termed the “dedicated circuit model” (Chalfie, Sulston et al. 1985; Faumont, Rondeau et al. 2011) (See Figure 1.3).
Chapter I: Introduction

Figure 1.3: The *C. elegans* locomotion circuit. Hexagons represent the command interneurons that synapse with excitatory motor neurons; AVA, AVD and AVE with DA and VA motor neurons for backward movement, AVB and PVC with DB and VB motor neurons for forward movement. Arrows represent chemical synapses, and blunt-ended lines indicate gap junctions. Input to D motor neurons is only from other motor neurons; DD motor neurons receive input from ventral motor neurons (VA and VB) and innervate dorsal muscle, VD motor neurons receive input from dorsal motor neurons (DA and DB) and innervate ventral muscle. This cross-inhibitory circuit coordinates muscle excitation on one side of the body with relaxation on the other side of the body. (Taken from (Von Stetina, Treinin et al. 2005), adapted from (Chalfie 1988)).

Laser ablation studies have been used to assign specific interneurons and motor neurons to either the forward or reverse circuits. On laser ablation of each class of motor neuron in L1 worms, it was found that; without DA neurons worms could not move backward but could move forward normally, without DB neurons worms could not move forward but could move backward normally, and without DD motor neurons the worms were severely...
uncoordinated in both directions (Chalfie, Sulston et al. 1985). These data suggest that, in
the L1 locomotion circuit, DA neurons are required for backward movement, DB neurons
are required for forward movement and DD neurons are required for both forward and
backward movement. Laser ablation experiments have not been performed on VA, VB and
VD motor neurons in the adult locomotion circuit, but the shared morphology and
connectivity with DA, DB and DD motor neurons suggests comparable roles for these
neurons in the forward and reverse circuits.

A role for A class motor neurons in backward movement is also supported by an experiment
in which a toxic MEC-4 (DEG/ENaC) cation channel subunit is expressed under the control of
the unc-4 promoter (Harbinder, Tavernarakis et al. 1997). This channel causes elevated ion
influx, swelling and degeneration of the VA, DA and VC motor neurons. It results in
uncoordinated animals that are unable to move backwards when touched on the head,
which is similar to the phenotype of unc-4 mutants that lack differentiated VA motor
neurons.

Laser ablation results have also assigned the command interneurons into separate forward
and reverse circuits. Ablation of both AVA and AVD impairs backward movement, whereas
ablation of AVB and PVC impairs forward movement (Chalfie, Sulston et al. 1985; Wicks and
Rankin 1995). The PVC and AVD interneurons are considered to have a modulatory role
because ablation of either interneuron alone has little effect on locomotion, but phenotypes
are exacerbated if they are co-ablated with the corresponding command interneuron (AVD
with AVA and PVC with AVB) (Chalfie, Sulston et al. 1985). These results are consistent with
the wiring diagram, where the A class “backward” neurons synapse with AVA and AVD, and
the B class “forward” neurons synapse with AVB and PVC (White, Southgate et al. 1986).

Neurophysiological evidence for distinct forward and reverse locomotion circuits is limited.
Over the last two years, several calcium imaging studies have been carried out on the C.
elegans locomotion circuit to try to test this hypothesis.

One study has found that A and B class motor neuron activity correlates with the direction
of movement, but not with the faster bending cycle (Haspel, O'Donovan et al. 2010). This
study found that VB and DB neurons were co-active during forward locomotion, whereas VA neurons were only active during backward locomotion. VA and VB neurons were not observed to be co-active during locomotion. Instead they displayed antagonistic changes in activity; a decrease in calcium level in VB and DB neurons coincides with an increase in calcium levels in VA neurons, and occurs before the worm switches from forward to backward locomotion. A corresponding increase in calcium level in VB and DB neurons coincides with a decrease in calcium levels in VA neurons, and occurs before the worm switches from backward to forward locomotion. These results are consistent with the earlier laser ablation studies by (Chalfie, Sulston et al. 1985) that assigned A motor neurons to backward locomotion and B motor neurons to forward locomotion.

DB and VB neurons are hypothesized to display alternating activity correlating with dorsal and ventral body undulations, but this was not observed in the study. However, this study was carried out on semi-restrained worms, using the calcium indicator cameleon YC2.60 that has a slow off time of 5.2 seconds, and with an imaging speed of 10Hz. It is possible that restraining the worm in an agarose pad between the head and vulva could affect the activity of motor neurons at the recording sites. Furthermore, the decay time constant of YC2.60 may be too slow to resolve faster oscillations in VB and DB activity. It is also possible that calcium signals in the cell body are decoupled from calcium signals in the neuronal processes and at the neuromuscular junction. Therefore, these limitations in imaging cannot exclude models in which VB and DB neuron activity alternates during forward locomotion, but their results are only partially consistent with the distinct circuit model.

A more recent study has found that the calcium activity of the ventral motor neurons VA and VB do oscillate in phase with ventral bends, and the calcium activity of the dorsal motor neurons DA and DB also oscillate in phase with dorsal bends (Faumont, Rondeau et al. 2011). These results are consistent with the dedicated circuit model, in which a motor neuron has a role in producing contraction on the side of the worm that it innervates.

Surprisingly, this study also finds that A motor neuron calcium activity oscillates during forward movement, and B motor neuron calcium activity oscillates during backward
movement. The dedicated circuit model predicts that these neurons should be inactive during these movements; B forward neurons should be inactive during backward locomotion, and A backward neurons should be inactive during forward locomotion. The cause of the observed activity is unclear, but it suggests that such a model may be an oversimplification and that the control of locomotion may be less modular than previously thought (Faumont, Rondeau et al. 2011). Indeed, there are many lateral connections within the locomotion circuit that could provide extensive communication between the forward and reverse circuits: between the interneurons themselves, between adjacent motor neurons of the same and different classes and between adjacent muscle cells.

One study has suggested that it could be an imbalance between the activities of the A and B motor neurons that results in directional movement (Kawano, Po et al. 2011). This study found that forward movement occurs when B neuron activity is greater than A neuron activity, and backward movement occurs when A neuron activity is greater than B neuron activity. A reciprocal change in the mean activity levels between the two neuron classes results in a change in the direction of movement. This study also found that \textit{unc-7(e5)} and \textit{unc-9(fc16)} gap junction mutants could not establish a pattern where B neuron activity was greater than A neuron activity and this correlated with uncoordinated kinking locomotion on attempts to crawl forward. Calcium imaging analysis from these mutants indicates that AVA interneurons are hyper-activated, whilst AVB interneurons display reduced activity. Restoring AVA-to-A motor neuron gap junctions reduces AVA activity and also reduces the chemical excitation of A motor neurons by AVA, suggesting that it is the reciprocal activation of interneurons which leads to reciprocal changes in the activities of A and B classes of motor neuron.

The dedicated circuit model predicts that forward command interneurons should be active during forward locomotion and reverse command interneurons should be active during backward locomotion. Four studies have reported increased calcium activity in the reverse command interneuron AVA during backwards movement (Chronis, Zimmer et al. 2007; Ben Arous, Tanizawa et al. 2010; Faumont, Rondeau et al. 2011; Kawano, Po et al. 2011) and decreased calcium activity in AVA has been observed during forward movement (Faumont,
Chapter I: Introduction

Rondeau et al. 2011; Kawano, Po et al. 2011). Conversely, increased calcium activity has been observed in the forward command interneuron AVB during forward movement and decreased calcium activity during backward movement (Faumont, Rondeau et al. 2011; Kawano, Po et al. 2011). No study has observed oscillations in the calcium activity of command interneurons with bending of the worm body, suggesting that these interneurons themselves are not directly part of the pattern generator for worm locomotion.

The dedicated circuit model is also supported by mutant phenotypes in which the motor neurons are mis-wired. The \textit{unc-4} mutant phenotype with an inability to move backward correlates with VA backward motor neurons receiving incorrect synaptic inputs from AVB and PVC forward command interneurons (White, Southgate et al. 1992). Similarly, the uncoordinated forward movement of \textit{vab-7} mutant worms correlates with the reversal in polarity of the DB motor neuron processes so that they become morphologically like DA neurons (Ahringer 1996; Esmaeili, Ross et al. 2002). Essentially, \textit{unc-4} worms have lost functional backward motor neurons innervating ventral body wall muscle and \textit{vab-7} worms have lost functional forward motor neurons innervating the dorsal body wall muscle.

1.8 The generation and propagation of sinusoidal bending waves

Two remaining major questions regarding the \textit{C. elegans} locomotion circuit are which neurons are responsible for the observed oscillatory activity of ventral cord motor neurons and whether there is a role for proprioceptive feedback in generating or modulating locomotion. Due to their observed oscillatory activity, the ventral cord motor neurons are candidates for the generation and propagation of the sinusoidal wave along the body. It is also possible that worms sense changes in stretch and tension within their bodies during locomotion. Such sensory feedback information could be used to coordinate the degree and timing of alternating contraction and relaxation of muscle (White, Southgate et al. 1986; Tavernarakis, Shreffler et al. 1997).

The \textit{C. elegans} D class GABAergic motor neurons could provide cross-inhibitory input to coordinate the dorsal and ventral contraction of muscles. However, direct measurement of
Chapter I: Introduction

C. elegans D motor neurons to detect rhythmic activity in locomotion has not been performed. Furthermore, these neurons seem to be dispensable for movement as unc-30 mutants that lack GABA signalling are capable of forward movement. Only when poked do these worms display a “shrinker” phenotype, in which dorsal and ventral muscles contract simultaneously and the worm shortens in length (Mcintire, Jorgensen et al. 1993; Mcintire, Jorgensen et al. 1993; Jin, Hoskins et al. 1994). These results suggest that GABA signalling is not required for wave propagation in a moving animal, but it does reduce the amplitude of the body waves (Mcintire, Jorgensen et al. 1993). D neurons appear most important for resetting an animal’s posture, when the phase of excitation and bending is being set up along the body, for example on changing direction or starting rapid movement (Jorgensen 1995).

The A and B class excitatory neurons could also potentially generate sinusoidal movement. Neuron processes that are morphologically unspecialized and synapse-free have been proposed to transduce proprioceptive stimuli such as stretch. Both the A and B class of motor neurons have elongated processes extending well past the active zone of the neuromuscular junction that could act as stretch receptors (White, Southgate et al. 1986; Hall and Russell 1991). Their processes orient in opposite directions and so the direction of electrical excitation travelling through them during a body bend could be determined by their anatomy.

In such a proprioceptive model, stretch of the body is proposed to activate mechanically gated channels in the motor neuron processes, leading to an influx of cations and potentiation of the motor neuron signal to the muscle that is next to be contracted (Tavernarakis, Shreffler et al. 1997) (See Figure 1.4). The activation of stretch receptors in adjacent motor neurons by local bending could propagate the wave of contraction down the body. The gap junctions present between motor neurons of the same class could also aid propagation of the electrical activity.
A similar proprioceptive property has been demonstrated for the interneuron DVA, where stretch is transduced by the \textit{trp-4} membrane channel (Li, Feng et al. 2006). The DVA neuron is located in the tail and extends a process to anterior regions of the body (White, Southgate et al. 1986). Calcium imaging experiments have shown that bending of the body generates calcium transients in DVA (Li, Feng et al. 2006). Mutation of the TRP-4 protein or laser ablation of DVA produces animals that bend their bodies deeper and more frequently than wildtype (Wicks, Roehrig et al. 1996; Riddle 1997; Li, Feng et al. 2006). This locomotion defect can be rescued by expression of TRP-4 in DVA (Li, Feng et al. 2006). A small number of A and B motor neurons are postsynaptic to DVA. Proprioceptive input during movement could maintain the activity of motor neurons during movement in both forward and backward directions and provide an explanation for observed A neuron activity during forward movement and B neuron activity during backward movement (Faumont, Rondeau et al. 2011).
Chapter I: Introduction

In this model of *C. elegans* locomotion, it is unclear how the command interneurons could trigger oscillations in motor neuron activity because the transmission of electrical excitation down a command interneuron axon is predicted to be fast enough to excite all postsynaptic motor neurons simultaneous (Niebur and Erdos 1993). It is possible that interneuron stimulation of motor neurons is sub-threshold, and so local oscillations in motor neuron activity generated by stretch are required for neurotransmitter release at the neuromuscular junction.

Body wall muscles may also sense the amount of stretch within them and modulate their contraction (Liu, Schrank et al. 1996). Mathematical modelling with early electrophysiological data suggested that body wall muscle cells function as simple actuators and generate only graded membrane potentials (Jospin, Jacquemond et al. 2002; Boyle and Cohen 2008). However, more recent experiments have shown that these cells can fire all-or-none action potentials (Jospin, Jacquemond et al. 2002; Gao and Zhen 2011; Liu, Chen et al. 2011). Calcium transients can occur in the absence of neural inputs (Liu, Chen et al. 2011) and worms in which acetylcholine and GABA receptors have been genetically ablated can still perform some locomotion (Francis, Evans et al. 2005; Touroutine, Fox et al. 2005). These results suggest that body wall muscle by itself can produce some locomotion without neural inputs.

The synchronous activity of neighbouring body wall muscle cells could result from either neural inputs or from muscle electrical coupling. Electrophysiological recordings in wildtype worms have shown that calcium transients are synchronous among neighbouring muscle cells, and that this synchronization is mediated by gap junctions formed by the innexin UNC-9, as the mean gap junction conductance and the proportion of calcium transients is 60% lower in *unc-9(fc16)* mutants (Liu, Chen et al. 2011). These defects are rescued by expressing UNC-9 specifically in muscle. Calcium imaging from adjacent body wall muscle cells using the calcium indicator GCaMP2, shows that calcium transients are synchronous among neighbouring muscles cells in wildtype worms but asynchronous in *unc-9* mutants. Synchrony of calcium transients in muscle cells does not depend on synaptic transmission as pharmacological blockade of acetylcholine receptors (by TBC) and GABA receptors (by
gabazine) did not change the synchrony of calcium transients. Muscle gap junctions are therefore suggested to play an important role in synchronizing muscle activity and coordinating body bending.

**1.9 Putative proprioceptive molecules in *C. elegans***

The sinusoidal waveform of *C. elegans* locomotion can be modified by a number of mutations in genes that are thought to be involved in proprioception. In addition to the *trp-4* gene expressed in the DVA interneuron, there are several candidate genes that have been suggested to encode the stretch receptor molecules in the motor neurons.

The genes *unc-8* and *del-1* encode ion channel subunits belonging to the degenerin/vertebrate epithelial sodium ion channel (DEG/ENaC) super-family. Degenerins are membrane proteins that are hypothesized to form mechanically-gated ion channels (Driscoll and Chalfie 1991). Members of this family are found in both vertebrates and invertebrates (de la Rosa, Canessa et al. 2000; Kellenberger and Schild 2002). They all have cytoplasmic amino and carboxy termini and a large extracellular domain that contains three cysteine-rich regions (Garcia-Anoveros, Ma et al. 1995). The degenerins have been suggested to function in *C. elegans* mechanosensation (Suzuki, Kerr et al. 2003; O'Hagan, Chalfie et al. 2005) and proprioception (Tavernarakis, Shreffler et al. 1997).

The *unc-8* and *del-1* genes are co-expressed in motor neurons. The gene *unc-8* is expressed in the A, B and D classes of motor neurons (as well as ASH, FLP sensory neuron, PVM touch neuron, AVA, AVB, AVD and PVC command interneurons), and the gene *del-1* is expressed exclusively in VA and VB motor neurons (and FLP sensory neuron) (Tavernarakis, Shreffler et al. 1997). However, analysis of the *unc-8* loss-of-function mutant phenotype has revealed only a modest defect in locomotion, with reduced wavelength and amplitude of body bends (Tavernarakis, Shreffler et al. 1997), and *del-1* loss-of-function animals move superficially like wildtype, although their locomotion has not be analysed in detail.
Other mutations have been identified that result in worms displaying phenotypes that are more consistent with a defect in proprioception. Worms that carry mutations in the \textit{unc-79} or \textit{unc-80} genes or mutations in both the \textit{nca-1} and \textit{nca-2} genes display a “fainting” locomotion phenotype. These worms are capable of moving normally, but they fail to sustain locomotion and display long periods of halting (Sedensky and Meneely 1987; Humphrey, Hamming et al. 2007). Fainting is robustly elicited during crawling escape responses to mechanical stimulation such as tapping. These worms also exhibit an abnormal swimming phenotype, becoming paralyzed upon immersion in liquid (Pierce-Shimomura, Chen et al. 2008). This is an interesting observation, considering that any external forces exerted on the body of the worm will be very small in an aqueous environment, providing little proprioceptive feedback. For both the crawl and swim “fainter” phenotypes, mutant worms freeze their body posture after exactly one dorsal-ventral bend propagates along the body during an attempt to switch from a slower to a faster form of locomotion. From these behavioural results it has been hypothesized that these four genes function together to regulate the transition from slow to fast forms of locomotion.

The four genes encode voltage-insensitive cation leak channel subunits and their expression is localized to motor neuron axon regions but not synapses (Yeh, Ng et al. 2008). The \textit{unc-79} and \textit{unc-80} genes encode large novel proteins, with single orthologs in flies, mice and humans (Humphrey, Hamming et al. 2007). \textit{unc-79} and \textit{unc-80} mutant worms display normal levels of \textit{nca-1} and \textit{nca-2} gene mRNA transcripts but greater than ten-fold decrease in protein expression. UNC-79 and UNC-80 proteins share a central armadillo motif that may be important in protein-protein binding interactions (Pierce-Shimomura, Chen et al. 2008). The \textit{unc-79} and \textit{unc-80} genes display overlapping expression in VA and VB motor neurons, as well as head and tail neurons.

The \textit{nca-1} and \textit{nca-2} genes encode \textit{C. elegans} homologs of the $\alpha$1 subunit of voltage-gated calcium and sodium channels but they have a reduced number of charged amino acids in the voltage-sensing fourth transmembrane domain. The expression patterns of the \textit{nca-1} and \textit{nca-2} genes overlap with those of \textit{unc-79} and \textit{unc-80} in the cholinergic motor neurons of the VNC, suggesting that they may function together in these neurons.
Immunofluorescent staining shows that all four reciprocally stabilize each other’s expression (Yeh, Ng et al. 2008). The proteins are thought to form a novel ion channel complex called “NCA” of unknown composition, but with \textit{nca-1} and \textit{nca-2} hypothesized to be the pore-forming subunits and \textit{unc-79} and \textit{unc-80} hypothesized to be auxiliary components. In this way, the channel is thought to regulate the activation of motor neuron synapses and so regulate locomotion.

Electrophysiological studies on the fainter worms have recorded spontaneous and evoked postsynaptic currents in body wall muscles as an indirect measure for the activities of presynaptic cholinergic and GABAergic motor neurons (Yeh, Ng et al. 2008). These investigations demonstrate that \textit{nca-1}; \textit{nca-2} and \textit{unc-80} loss-of-function mutations result in reduced spontaneous and evoked currents in body wall muscle. Calcium imaging using cameleon in the hermaphrodite–specific egg-laying neuron (HSNs) cell body and processes shows that although neuron cell body spike frequency is normal, these mutant worms have decreased synaptic spike frequency and the initiation of calcium transients is disrupted. A gain-of-function mutation in the \textit{nca-1} has also been identified. These worms display loopy, exaggerated body bends. The same experiments show that this mutation significantly increases the amplitude of calcium transients, suggesting increased membrane excitability and enhanced activation of calcium channels at the HSN synapse.

Such results suggest that the \textit{C. elegans} “NCA” channel complex regulates pre-synaptic activation of motor neurons by modulating the transmission of depolarizing signals from the cell body, along the axon, to the synapse. Indeed, the mammalian “NCA” channel ortholog has been identified as a sodium leak channel that acts to raise the cell resting potential to greater than the equilibrium potential for potassium ions (Lu, Su et al. 2007). The \textit{C. elegans} “NCA” channel may drive the motor neuron membrane potential close to its excitation threshold, facilitating the activation of other channels along the motor neuron axon and synapse.
Chapter I: Introduction

1.10 Thesis aims

The simple and well-defined structure of the *C. elegans* locomotion circuit allows the investigation of the genetic and neural rules that control the creation and function of this network. Despite the robust wiring diagram, it is still not fully understood how the motor circuit actually coordinates locomotion. The aim of this thesis is to investigate the molecular and neuromuscular mechanisms underlying locomotion in *C. elegans*. My research has the following objectives:

- To quantitatively analyse the locomotion behaviour of wildtype worms and compare this behaviour to that displayed by the putative proprioceptive mutants *unc-8* and *del-1*, the fainter mutants *nca-1, nca-2, unc-79* and *unc-80* and the motor neuron mis-wiring mutants *vab-7, unc-3, unc-4, unc-30, unc-55, unc-104* with the aim of correlating the hypothesized function of these genes with any observed locomotion defects.

- To develop a whole-worm tracking microscope for simultaneous behavioural analysis and neuromuscular calcium imaging.

- To construct transgenic lines expressing GCaMP3 and a reference fluorescent protein in body wall muscle and the A, B and D classes of motor neuron to allow ratiometric calcium imaging from these cells.

- To image calcium activity in the body wall muscle of wildtype worms during crawling and swimming locomotion, measure the phase relationship between maximal calcium activity and maximal body curvature and compare these experimental measurements to theoretical predictions.
Chapter I: Introduction

- To image calcium activity in the three classes of motor neurons in wildtype worms during crawling and swimming locomotion, measure the phase relationship between maximal calcium activity and maximal body curvature and compare these experimental measurements to theoretical predictions.

- To image the neuromuscular activity in the fainter mutants nca-1, nca-2, unc-79 and unc-80 during crawling and swimming locomotion and compare this activity to that observed in wildtype worms, with the aim of identifying how activity in the locomotion circuit changes during fainting episodes.

- To image neuromuscular activity during crawling in the motor neuron mis-wiring mutants vab-7, unc-4 and unc-55 and compare this activity to that observed in wildtype worms, with the aim of correlating specific motor neuron mis-wiring defects with changes in the pattern of neuromuscular activity that could underlie their observed locomotion phenotypes.
Chapter II

Quantitative Analysis of Locomotion Behaviour
2.1 Abstract

The use of machine vision analysis to quantitatively describe worm behaviour allows for the precise description of phenotypes that result from genetic mutations. In this Chapter, I quantitatively analyse the locomotion behaviour of wildtype worms and compare this behaviour to that displayed by putative proprioceptive mutants, fainter mutants and motor neuron mis-wiring mutants. I show here that the putative proprioceptive mutant unc-8 displays reduced locomotion compared to wildtype whereas del-1 displays increased locomotion compared to wildtype, although both retain a wildtype-like waveform and body curvature. The double mutant unc-8;del-1 displays wildtype locomotion but exhibits reduced body curvature at its head and tail.

Behavioural analysis of the fainter mutants shows that the double mutant nca-1;nca-2 exhibits the most severe fainting phenotype, the fainting phenotype of unc-79 is more severe than that of unc-80 and all three mutants display significantly reduced body curvature along their lengths. Worms carrying either nca-1 or nca-2 mutations display wildtype locomotion, although nca-1 exhibits reduced body curvature at the head and tail.

I observe that vab-7 mutants show uncoordinated backward as well as forward movement, suggesting that functional B class motor neurons are also important in the execution of smooth backward movement, and that unc-4 mutants show impaired spontaneous forward movement, suggesting that functional A class motor neurons are also important in the execution of smooth forward movement. Disruption of D class motor neuron function in unc-30 worms reduces locomotion and reduces body bending, whereas imbalanced D class motor neuron function in unc-55 worms reduces locomotion and increases body bending, showing that appropriate GABAergic signalling from D class motor neurons is essential for efficient forward and backward locomotion and for the proper coordination of body bending in C. elegans.
Chapter II: Quantitative Analysis of Locomotion Behaviour

2.2 Introduction

2.2.1 Machine Vision to Quantitatively Analyse Locomotion Behaviour

The use of machine vision analysis to quantitatively describe worm behaviour allows for the precise description of phenotypes that result from genetic mutations (Baek, Cosman et al. 2002; Geng, Cosman et al. 2003). In previous work, Eviatar Yemini and Tadas Jucikas built a tracking and imaging system that could follow an individual worm automatically and record the worm’s changing posture over time (unpublished). In this Chapter, I use the system they developed to assess the locomotion of wildtype worms and compared this behaviour to the putative stretch receptor mutants unc-8 and del-1, the fainter mutants nca-1, nca-2, unc-79 and unc-80, and the motor neuron mis-wiring mutants vab-7, unc-3, unc-4, unc-30, unc-55 and unc-104, with the aim of correlating the hypothesized function of these genes with any observed locomotion defects.

Worm Tracker 2.0 allows for the high-resolution phenotypic analysis of single worms. The tracker consists of a high-magnification camera and illumination system that is moved automatically by a motorized stage to keep the worm within the centre of the field-of-view of the video recording. The worm plate is kept stationary to avoid accelerations and any vibrations that would affect worm behaviour. In the video analysis process the worm shape is segmented from its background in each frame of the video recording, the worm outline and midline is determined, and the head and tail is automatically identified. The sequence of segmented worm postures is then used to calculate a set of features that describe the worm’s locomotion.

2.2.2 Genes hypothesized to function in proprioception during locomotion

It has been hypothesized that the unc-8 and del-1 genes could be stretch-sensitive DEG/ENaC ion channels that could contribute to C. elegans proprioception and modulate locomotion in response to body bending (Tavernarakis, Shreffler et al. 1997). As these genes are only co-expressed in the ventral A and B motor neurons, this hypothesis would require that these subunits form homotrimeric channels. Given this, I decided to quantitatively
assess the locomotion of worms carrying deletions in either of these genes to see whether I could identify any defect in the degree or timing of body bending that would indicate such a role in proprioception.

Furthermore, given their co-expression, it is possible that the UNC-8 and DEL-1 protein subunits co-assemble to form a mechanically-gated ion channel that modulates worm sinusoidal locomotion (Tavernarakis, Shreffler et al. 1997). If this is true, an unc-8(e15lb145);del-1(ok150) double mutant, lacking functional copies of both protein subunits, could be predicted to display a more severe locomotion defect than either single mutant. To investigate this question, I made an unc-8(e15lb145);del-1(ok150) double mutant line and assessed its locomotion along with wildtype N2 worms and the single mutants unc-8(e15lb145) and del-1(ok150).

2.2.3 Genes regulating the activation of motor neuron synapses

Several other mutations have been identified that result in worms displaying phenotypes that could be consistent with a defect in proprioception. Worms that carry mutations in the unc-79 or unc-80 genes or mutations in both the nca-1 and nca-2 genes display a “fainting” locomotion phenotype. (Sedensky and Meneely 1987; Humphrey, Hamming et al. 2007). I decided to assess the locomotion of these mutants to determine the severity of fainting in each strain and identify whether mutations in these genes affect the degree of body bending during periods of normal movement that could indicate a role for these genes in generating proper bend waveform.

2.2.4 Genes regulating the wiring of the locomotion circuit

C. elegans mutants could be used to investigate how specific mis-wiring of neurons in the motor circuit affects motor neuron activity, muscle activity and subsequent locomotion. I chose to analyse the behavioural phenotypes of six locomotion mutants; vab-7, unc-3, unc-4, unc-30, unc-55 and unc-104, with the aim of correlating observed movement defects to identified changes in their motor neuron wiring. Such a study should indicate functional roles for each class of motor neuron in the locomotion circuit.
The \textit{vab-7}, \textit{unc-3} and \textit{unc-4} genes specify wiring of the cholinergic motor neurons in \textit{C. elegans}. In \textit{vab-7(e1562)} mutants DB neuron axons change polarity and project anteriorly along the DNC rather than posteriorly. I chose to analyze this mutant to determine what effects the loss of dorsally innervating B motor neurons has on movement and bend waveform. The \textit{unc-3} gene encodes a protein homologous to immunoglobulin domain-containing transcription factors that is required for VNC pioneering, axon path-finding, and for the expression of CHA-1, the choline acetyltransferase enzyme, and UNC-17, the acetylcholine vesicular transporter. In these mutants the VNC shows defasciculation, with neuromuscular junctions at ectopic sites and inappropriate synapses with interneurons (Herman 1987; Wightman, Baran et al. 1997; Prasad, Ye et al. 1998). I chose to analyze this mutant to determine what effect the disruption of acetylcholinergic motor neuron wiring has on locomotion and body bending. In \textit{unc-4} mutants, the VA neurons VA2 to VA10 receive synaptic input from the forward command interneurons that usually synapse onto B motor neurons (White, Southgate et al. 1992). I decided to analyze this mutant to determine what effect the inappropriate input to VA motor neurons by forward command interneurons has on forward and backward movement and bend waveform.

The \textit{unc-30} and \textit{unc-55} genes have roles in the specification of GABAergic motor neurons in \textit{C. elegans}. The \textit{unc-30} gene controls the terminal differentiation of all nineteen GABAergic motor neurons in \textit{C. elegans} through regulating the expression of the \textit{unc-25} gene that encodes the GABA biosynthetic enzyme glutamic acid decarboxylase (GAD), and the \textit{unc-47} gene that encodes the transmembrane vesicular GABA transporter (Eastman, Horvitz et al. 1999; Cinar, Keles et al. 2005). These mutants display changes in GABA neuron morphology and the pattern of synapse formation (Mcintire, Jorgensen et al. 1993; Mcintire, Jorgensen et al. 1993). I chose to analyze the \textit{unc-30} mutant to determine what effect the disruption of GABAergic motor neuron wiring has on locomotion and body bending. In \textit{unc-55} mutants VD neurons adopt DD neuron output onto dorsal muscle (Walthall 1990). I chose to analyze the \textit{unc-55} mutant to investigate the effect of increased dorsal inhibition and reduced ventral inhibition on worm locomotion and waveform.

The \textit{unc-104} gene encodes a kinesin-like motor protein homologous to the human axonal transporter of synaptic vesicles (ATSV) (Hall and Hedgecock 1991). The UNC-104 protein is
Chapter II: Quantitative Analysis of Locomotion Behaviour

required for the anterograde axonal transport of synaptic vesicles and the differentiation of pre- and post-synaptic specializations at inhibitory neuromuscular junctions. In *unc-104(e1265)* mutants, axons form less synapses, with smaller active zones and fewer vesicles than in wildtype worms. The mutation is predicted to disrupt the function of many or all classes of neuron in *C. elegans*. In addition, UNC-104 is required for synapse remodelling of DD motor neurons at the end of the L1 stage; in *unc-104(e1265)* mutants the DD neurons continue to innervate ventral muscle (Park, Watanabe et al. 2011). I decided to analyze the locomotion of this mutant to determine the effect of disrupted signalling by all motor neuron classes on locomotion and how increased ventral inhibition and reduced dorsal inhibition affects the bend waveform.
2.3 Methods

2.3.1 Strains

Strains were maintained at 22°C, using standard methods (Brenner 1974). Strains used in this study include N2 (Bristol, England); CB151 unc-3(e151) X; CB120 unc-4(e120) II; MP145 unc-8(e15lb145) IV; CB845 unc-30(e191) IV; CB402 unc-55(e402) I; CB1068 unc-79(e1068) III; CB1069 unc-80(e1069) V; CB1265 unc-104(e1265) II; MP145 unc-8(e15lb145) IV; NC279 del-1(ok150) X; VC12 nca-1(gk9) IV; VC9 nca-2(gk5) III; VC12 nca-1(gk9) IV; VC9 nca-2(gk5) III; NC279 del-1(ok150) X; CB1562 vab-7(e1562) III. All strains were out-crossed with N2 males at least 4 times.

2.3.2 Genotyping of strains

Genotyping was carried out as described in Methods Chapter III 3.4 Molecular Biology.

2.3.2.1 Identification of deletion sites in the putative stretch-receptor mutants unc-8(e15lb145) and del-1(ok150)

PCR amplification experiments carried out by Tavernarakis et al. 1997, showed that the unc-8(e15lb145) null mutation harbours a deletion within cosmid R13A1_4 boundaries that removes cysteine-rich domain (CRD) CRDII and much of CRDIII. In order to be able to accurately follow the deletion during genotyping, I identified the deletion breakpoint sites. Primer sets outside of cysteine-rich domains CRDII and CRDIII were designed (see Chapter III 3.4 Primers) and a 915bp deletion was identified by sequencing the PCR product generated from genomic DNA. This 915bp deletion incorporates most of the coding region between CRDII and CRDIII and almost all of CRDIII (see Figure 2.1).
A. *unc-8(e15lb145)*

![Diagram A](image1)

B. *del-1(ok150)*

![Diagram B](image2)

**Figure 2.1: Identification of deletion sites in the putative stretch-receptor mutants.** A: *unc-8(e15lb145)*, B: *del-1(ok150)*. Structure of the *unc-8* and *del-1* genes; boxes indicate exons and thin lines indicate introns. Deletion sites are marked in red. Degenerin domains indicated are the predicted membrane-spanning domains MSDI and MSDII and cysteine-rich domains CRDI, CRDII and CRDIII, marked in green. The extracellular region implicated in channel closing is marked in orange as EXTR (Garciaanoveros, Ma et al. 1995; Tavernarakis, Shreffler et al. 1997).

A presumptive null allele *del-1(ok150)* is available from the CGC with a predicted 2kb deletion but the site and extent of the deletion is unknown. In order to be able to accurately follow the deletion during genotyping, I identified the deletion breakpoint sites. A 2047bp deletion was identified by sequencing the PCR product generated from genomic DNA using primers listed by the CGC as flanking the deleted region (see Chapter III 3.4 Primers). This deletion removes all of the coding regions corresponding to exons 5, 6, 7, 8, 9 and 10 (see Figure 2.1).
2.3.2.2 Identification of mutation sites in the fainter mutants nca-1(gk9), unc-79(e1068) and unc-80(e1069)

The lines nca-1(gk9) and nca-2(gk5) are two deletion alleles generated by the C. elegans Gene Knockout Consortium. The mutations are predicted to remove part of the pore-forming domain and result in null alleles. The nca-2(gk5) mutation is a 2970bp deletion in the genomic sequence. The nca-1(gk9) mutation is reported to be a 2287bp deletion and a 128bp insertion by the C. elegans Gene Knockout Consortium. When carrying out duplex PCR for genotyping with primers outside of the predicted deletion site I noticed both wildtype and mutant length bands present in the PCR products. Sequencing of the mutant PCR product instead revealed a 2138bp deletion and a 139bp insertion in the genomic sequence. Generation of the nca-1(gk9);nca-2(gk5) double mutant still produced a line displaying a robust fainting phenotype, indicating that the nca-1(gk9) mutation remains a functional null allele.

The exact molecular nature of the unc-79(e1068) and unc-80(e1069) mutations have not been published. Primer sets were designed for both of these genes to amplify overlapping 1kb fragments from genomic DNA. The unc-79(e1068) allele was found to contain a CGA to TGA substitution in the 7th codon in the first exon of the gene model E03A3.6b. The position of this substitution site agrees with the location of a genomic fragment previously shown to give successful transgenic rescue of the unc-79 mutant phenotype (Humphrey, Hamming et al. 2007). This mutation results in the introduction of a stop codon where there should be a codon for the amino acid arginine and which would be predicted to result in the production of a truncated protein product.

The unc-80(e1069) allele was found to contain a G to A substitution at the first base of intron 29 of the gene model F25C8.3a. This mutation changes the conserved 5’-GT splice site boundary to 5’-AT. Mutation of G to A at the 5’ splice site is known to be especially deleterious, blocking the second step in the splicing process (3’ cleavage and exon ligation) and causing accumulation of the lariat intermediate (Parker and Siliciano 1993). This is expected to prevent production of mature mRNA and seriously impair gene expression.
2.3.3 Genetic Manipulations

2.3.3.1 Generation of nca-1(gk9); nca-2(gk5) double mutant

The VC12 nca-1(gk9) IV;VC9 nca-2(gk5) III double mutant was made by crossing N2 males into nca-2(gk5) hermaphrodites. Males heterozygous for the nca-2(gk5) mutation were crossed with nca-1(gk9) hermaphrodites to generate F1 progeny. F1 progeny were singled, and the resulting F2s were screened for the “fainting” phenotype. These F2s were singled and one candidate plate was identified as being homozygous for the “fainting” phenotype. The presence of both mutations was confirmed by PCR.

2.3.3.2 Generation of unc-8(e15lb145); del-1(ok150) double mutant

The MP145 unc-8(e15lb145) IV;NC279 del-1(ok150) X double mutant was made by generating del-1(ok150) males by heat-shocking del-1(ok150) hermaphrodites at L4 stage for 6 hours at 30°C. The resulting del-1(ok150) males were crossed to unc-8(e15lb145) hermaphrodites. The resulting F1 progeny were singled and 25 F2 worms were singled from these plates. One double mutant line was identified through PCR.

2.3.4 Locomotion Assays

Single worm locomotion assays were carried out using Worm Tracker 2.0 v2.0.4 (developed by E. Yemini at MRC LMB, UK, unpublished) and the data analysed using Worm Analysis Toolbox v1.1.4 (developed by T. Jucikas at MRC LMB, UK, unpublished). Worms were staged as late L4s the evening before tracking. Adult worms were tracked on 3.5cm plates with low peptone nematode growth medium (NGM). Plates were stored at 4°C until needed and then left out to reach room temperature overnight and dry with the lids on. A 20μl spot of fresh OP50 bacterial suspension was placed in the middle of each plate and allowed to dry for 30 minutes with the lids on. One worm was picked to each plate. After 30 minutes, worms were tracked and their behaviour recorded for 15 minutes. For each genotype, a total of at least 18 individual worms were tracked over 3 days.
2.3.5 Measurement of Locomotion Features

Video analysis of worm locomotion recordings involved segmentation of the worm from its background in each video frame, followed by identification of the worm outline, midline, head and tail. The sequence of segmented worm postures was used to calculate a set of features that describe the worm’s locomotion. These features include:

**Mean Forward and Backward Velocity (μm/second)**

Distance travelled in a defined time period (15 minutes for locomotion assays). A 3/4 second derivative (i.e. the worm’s position subtracted from its position 3/4 seconds later) is taken. Velocity is positive when the worm moves forward and negative when the worm moves backward. Forward velocity is defined as greater than +10μm/sec, backward velocity is defined as less than -10μm/sec. Pausing is defined as velocities smaller than ±10μm/sec.

**Bend Wavelength (μm)**

Average length of one full period of a sinusoidal wave created by the worm (calculated as the feature TrackWavelength as described in (Feng, Cronin et al. 2004)).

**Distance Moved Forward and Backward (mm)**

Total distance moved in a defined time period (15 minutes for locomotion assays). The distance is calculated by taking the worm centroid for each frame and computing the Euclidian distance between these points.

**Time Spent Moving Forward, Backward and Pausing (second)**

Time spent moving forward is determined from the number of frames in which velocity is positive and time spent moving backward is determined from the number of frames in which velocity is negative. Forward velocity is greater than +10μm/sec, backward velocity is
less than -10μm/sec. Time spent pausing is determined from the number of frames in which velocities are smaller than ±10μm/sec.

**Body Curvature (degrees)**

The angle formed on the worm skeleton between points 1/12\(^{th}\) of the skeleton length in either direction of the point to which the curvature is assigned. Curvature is measured at five points along the body; head (1/6\(^{th}\) distance along body from tip of nose), neck (2/6\(^{th}\) distance along body from tip of nose), mid-body (3/6\(^{th}\) distance along body from tip of nose), hips (4/6\(^{th}\) distance along body from tip of nose) and tail (5/6\(^{th}\) distance along body from tip of nose).

**2.3.6 Statistical Analysis**

Standard statistical tests were performed using Matlab functions. A one-way analysis of variance (ANOVA) compared the distribution of mean values (one per individual worm) from each population of worms against that of each other population. Each ANOVA tested the null hypothesis that the mean of the mean values from each population were the same. Multiple comparison tests were then performed to indentify which pairs of means were significantly different. A significance level of α = 0.05 was used and significant results are indicated with a black star. Box and whisker plots have lines at the lower quartile, median and upper quartile values. Whiskers extend up to 1.5 times the inter-quartile range. Outliers are data with values beyond this range and are displayed with a red + sign.
2.4 Results

2.4.1 Locomotion behaviour of Wildtype N2 worms and the Putative Stretch Receptor Mutants \textit{unc-8(e15lb145)} and \textit{del-1(ok150)}

Whereas a detailed analysis of the DEL-1 mutant phenotype has not yet been reported, previous analysis of the \textit{unc-8(e15lb145)} null mutant phenotype reported that these worms display a significant reduction in wavelength and amplitude of body bends as determined from measuring tracks inscribed on a bacterial lawn (Tavernarakis, Shreffler et al. 1997). Bend wavelength was reported to be reduced approximately 4-fold compared to wildtype, and bend amplitude was reported to be reduced approximately 3.5-fold compared to wildtype.

The locomotion of wildtype N2 worms, and \textit{unc-8(e15lb145)}, \textit{del-1(ok150)} and \textit{unc-8(e15lb145);del-1(ok150)} mutants is summarised in Figures 2.2 and 2.3. Analysis of bend wavelength with Worm Tracker 2.0 shows that wildtype N2 worms have an average bend wavelength of 761.9μm. The mutants \textit{unc-8(e15lb145)} and \textit{unc-8(e15lb145);del-1(ok150)} have a smaller average bend wavelength compared to wildtype, at 750.6μm and 739.8μm respectively, whereas the mutant \textit{del-1(ok150)} has a larger average bend wavelength than wildtype, at 798.9μm (see Figure 2.4A). However, unlike the previous report by Tavernarakis et al., none of these differences are statistically significant. This suggests that, at least under the described imaging conditions, these putative proprioceptive mutants do not display a waveform that is significantly different in shape to wildtype N2 worms.
## Chapter II: Quantitative Analysis of Locomotion Behaviour

**Figure 2.2: Summary of locomotion behaviour for wildtype and putative proprioceptive mutants.** Reported values are the mean ± standard deviation for each locomotion feature measured. At least 18 animals were tested for each genotype. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.

<table>
<thead>
<tr>
<th>Locomotion Feature</th>
<th>N2</th>
<th>unc-8</th>
<th>del-1</th>
<th>unc-8;del-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Forward Velocity (μm/sec)</td>
<td>56.0 ± 34.9</td>
<td>36.0 ± 30.6</td>
<td>*87.2 ± 33.7</td>
<td>57.4 ± 45.9</td>
</tr>
<tr>
<td>Mean Backward Velocity (μm/sec)</td>
<td>-57.0 ± 25.6</td>
<td>*-37.5 ± 28.1</td>
<td>-72.9 ± 26.4</td>
<td>-50.1 ± 33.8</td>
</tr>
<tr>
<td>Mean Wavelength (μm)</td>
<td>761.9 ± 81.7</td>
<td>750.6 ± 79.0</td>
<td>798.9 ± 75.6</td>
<td>739.8 ± 65.3</td>
</tr>
<tr>
<td>Distance Moved Forward (mm)</td>
<td>41.8 ± 26.2</td>
<td>27.2 ± 23.8</td>
<td>*61.0 ± 27.9</td>
<td>46.4 ± 35.1</td>
</tr>
<tr>
<td>Distance Moved Backward (mm)</td>
<td>19.0 ± 5.0</td>
<td>*13.8 ± 4.0</td>
<td>20.6 ± 4.5</td>
<td>17.0 ± 4.6</td>
</tr>
<tr>
<td>Time Spent Moving Forward (s)</td>
<td>465.4 ± 124.2</td>
<td>*365.2 ± 155.9</td>
<td>550.4 ± 117.8</td>
<td>458.1 ± 159.9</td>
</tr>
<tr>
<td>Time Spent Moving Backward (s)</td>
<td>195.9 ± 42.4</td>
<td>178.4 ± 33.6</td>
<td>195.1 ± 49.2</td>
<td>182.8 ± 46.7</td>
</tr>
<tr>
<td>Time Spent Pausing (s)</td>
<td>224.3 ± 95.8</td>
<td>*355.4 ± 158.0</td>
<td>153.4 ± 82.3</td>
<td>258.0 ± 149.2</td>
</tr>
<tr>
<td>Curvature at head (degrees)</td>
<td>20.0 ± 1.9</td>
<td>19.5 ± 3.4</td>
<td>20.8 ± 1.8</td>
<td>*17.8 ± 1.6</td>
</tr>
<tr>
<td>Curvature at neck (degrees)</td>
<td>19.0 ± 1.8</td>
<td>19.8 ± 3.0</td>
<td>19.4 ± 1.2</td>
<td>18.8 ± 1.4</td>
</tr>
<tr>
<td>Curvature at mid-body (degrees)</td>
<td>11.0 ± 1.8</td>
<td>11.5 ± 3.6</td>
<td>10.4 ± 1.6</td>
<td>10.0 ± 1.8</td>
</tr>
<tr>
<td>Curvature at hips (degrees)</td>
<td>17.4 ± 2.4</td>
<td>18.7 ± 3.5</td>
<td>17.0 ± 1.6</td>
<td>*14.7 ± 1.9</td>
</tr>
<tr>
<td>Curvature at tail (degrees)</td>
<td>18.0 ± 2.4</td>
<td>19.3 ± 3.4</td>
<td>17.9 ± 2.0</td>
<td>*15.1 ± 1.6</td>
</tr>
</tbody>
</table>
Figure 2.3: Body posture sequences for wildtype N2 worms and putative proprioceptive mutants. Example image sequences from automatic tracking analysis, shown at 1 second intervals over a total time of 5 seconds. Worms are imaged crawling on OP50 food, at 30fps for a total time of 15 minutes. The segmented worm is shown with outline and midline overlaid and the head (green) and tail (red) marked. The vulva is indicated with a red dot close to the mid-body. A: Wildtype N2, B: unc-8(e15lb145), C: del-1(ok150), D: unc-8(e15lb145);del-1(ok150).
The mean velocity of movement can be used to assess the efficiency of locomotion (distance moved during a defined time period). Wildtype N2 worms move with a mean forward velocity of 56.0μm/sec and a mean backward velocity of -57.0μm/sec over the 15 minute assay period (see Figure 2.4B and 2.4C). The mutant \textit{unc-8(e15lb145)} spends significantly less time moving forward than wildtype (see Figure 2.4D) and significantly more time pausing (see Figure 2.4F). They do move forward slower than wildtype, but this difference in mean forward velocity does not reach statistical significance. However, \textit{unc-8(e15lb145)} worms do move backward significantly slower than wildtype and in accordance with this result, these worms move a significantly smaller distance backward than wildtype over the 15 minute assay period (see Figures 2.4B and 2.4G). Taken together, these results suggest that \textit{unc-8(e15lb145)} worms are somewhat less active than wildtype worms, but that they retain a normal waveform during movement.

Interestingly, although the mutant \textit{del-1(ok150)} spends a comparable amount of time moving forward, backward and pausing to wildtype (see Figures 2.4D, E and F) and moves at a similar backward velocity to wildtype (see Figure 2.4C), it displays a significantly faster forward mean velocity of 87.2μm/sec and moves a significantly larger total distance forward in the 15 minute assay period (see Figures 2.4B and 2.4G). Such a result suggests that \textit{del-1(ok150)} worms are more active than wildtype worms. This result is unexpected for a putative proprioceptive mutant in which impaired locomotion would be predicted.

The double mutant \textit{unc-8(e15lb145);del-1(ok150)} moves at a comparable forward and backward velocity to wildtype worms, and covers a similar total distance (see Figures 2.4B, C, G and H). This result suggests that the modest impairment in locomotion displayed by \textit{unc-8(e15lb145)} worms can be rescued by the hyperactive locomotion displayed by \textit{del-1(ok150)} worms to produce wildtype locomotion behaviour in the \textit{unc-8(e15lb145);del-1(ok150)} double mutant.
Chapter II: Quantitative Analysis of Locomotion Behaviour

A: Mean Bend Wavelength

B: Mean Forward Velocity

C: Mean Backward Velocity

D: Mean Time Spent Moving Forward

E: Mean Time Spent Moving Backward

F: Mean Time Spent Pausing

G: Mean Distance Moved Forward

H: Mean Distance Moved Backward
**Figure 2.4:** Box-plots to compare locomotion features for wildtype and putative proprioceptive mutants. Each box has lines at the lower quartile (blue), median (red) and upper quartile values (blue). Whiskers extend from each end of the box to adjacent data values. Outliers are red crosses with values outside 1.5 times the interquartile range.

A: Mean Bend Wavelength, B: Mean Forward Velocity, C: Mean Backward Velocity, D: Mean Time Spent Moving Forward, E: Mean Time Spent Moving Backward, F: Mean Time Spent Pausing, G: Mean Distance Moved Forward, H: Mean Distance Moved Backward.
In suggested models for *C. elegans* proprioception, stretch of the body is proposed to activate mechanically-gated channels in the motor neuron processes, leading to an influx of cations and potentiation of the motor neuron signal to the muscle that is next to be contracted. The activation of these stretch receptors in adjacent motor neurons by local bending could be used to coordinate the degree and timing of alternating contraction and relaxation of muscle (White, Southgate et al. 1976; Tavernarakis, Shreffler et al. 1997). Such models would predict that any proprioceptive mutant should display body bends that are altered in magnitude or timing. To investigate this, I assessed the magnitude of curvature displayed by wildtype N2 worms and the putative stretch receptor mutants at five defined points along their body; at the head, neck, mid-body, hips and tail.

Wildtype N2 worms display a mean curvature of 20.0° at the head, 19.0° at the neck, 11.0° at the mid-body, 17.4° at the hips and 18.0° at the tail (see Figures 2.2, 2.5 and 2.6). Curvature likely decreases towards the middle of the worm’s body because the curvature here is constrained to some degree by the adjacent segments of the worm body. Curvature at the head and neck is likely higher than at the tail due to the greater extent of innervation from muscles that allow a greater range of movement, for example during foraging.

Despite exhibiting comparable bend waveform and forward and backward mean velocities to wildtype worms, the double mutant *unc-8(e15lb145);del-1(ok150)* exhibits reduced curvature compared to wildtype all the way along its body (see Figure 2.6). This reduction reaches statistical significance at both ends of the body; head (17.8°), hips (14.7°) and tail (15.1°) (see Figure 2.6A, D and E). This result is in agreement with the hypothesis that the *unc-8* and *del-1* genes may function together to modulate the extent of body bending in *C. elegans*, as single mutants for these genes do not display any defect in the degree of body curvature along their length (see Figure 2.6).
Figure 2.5: Histograms for body curvature at five defined locations along the worm for wildtype N2 worms and putative stretch receptor mutants. Curvature values are plotted in degrees against their frequency of occurrence for head (blue), neck (green), mid-body (red), hips (cyan) and tail (purple). **A:** Wildtype N2, **B:** unc-8(e15lb145), **C:** del-1(ok150), **D:** unc-8(e15lb145);del-1(ok150).
Figure 2.6: Box-plots to compare body curvature at five defined locations along the worm for wildtype and putative proprioceptive mutants. Each box has lines at the lower quartile (blue), median (red) and upper quartile values (blue). Whiskers extend from each end of the box to adjacent data values. Outliers are red crosses with values outside 1.5 times the interquartile range. A: Body Curvature at Head, B: Body Curvature at Neck, C: Body Curvature at Mid-body, D: Body Curvature at Hips, E: Body Curvature at Tail.
2.4.2 Locomotion behaviour of the fainter mutants \textit{nca-1}(gk9), \textit{nca-2}(gk5), \textit{unc-79(e1068)} and \textit{unc-80(e1069)}

Previous phenotypic analysis of the mutants \textit{unc-79(e1068)}, \textit{unc-80(e1069)} and the double mutant \textit{nca-1(gk9);nca-2(gk5)} has reported that these mutants each display robust fainting behaviours (Pierce-Shimomura, Chen et al. 2008; Yeh, Ng et al. 2008). Worms carrying the \textit{unc-79(e1068)} and \textit{unc-80(e1069)} mutations were described as having identical phenotypes, moving forward with a slower frequency, amplitude and propagation of body bends compared to wildtype but with a well-coordinated waveform. Additionally, both \textit{nca-1(gk9)} and \textit{nca-2 (gk5)} worms have been reported to display short fainting-like episodes during crawling (Pierce-Shimomura, Chen et al. 2008).

In agreement with previous reports, data collected with Worm Tracker 2.0 shows that the fainter mutants \textit{unc-79(e1068)}, \textit{unc-80(e1069)} and \textit{nca-1(gk9);nca-2(gk5)} display less efficient locomotion than wildtype worms (see Figures 2.7 and 2.8). The behavioural results suggests that the mutant \textit{unc-79(e1068)} and the double mutant \textit{nca-1(gk9);nca-2(gk5)} display the strongest fainting behaviours, with the double mutant displaying the most severe phenotype. Both of these worm strains display significantly reduced mean forward and backward velocities compared to wildtype N2 worms. Although they spend a comparable amount of time moving backward compared to wildtype, they spend significantly less time moving forward and significantly more time pausing. As such, they cover significantly less distance moving forward and backward compared to wildtype worms.

The fainter mutant \textit{unc-80(e1069)} does exhibit a slower forward and backward velocity compared to wildtype but this does not reach statistical significance. In contrast to previously published results, this result suggests that the \textit{unc-80(e1069)} fainter phenotype is not as severe as that displayed by the mutant \textit{unc-79(e1068)}.

Worms carrying the \textit{unc-79(e1068)} and \textit{unc-80(e1069)} mutations show significantly reduced body curvature all the way along their bodies compared to wildtype worms. In addition, worms carrying the \textit{unc-79(e1068)} mutation show a significantly greater bend wavelength.
compared to wildtype worms. The double mutant \textit{nca-1(gk9);nca-2(gk5)} also displays significantly reduced body curvature at the head, neck, hips and tail, although not at the mid-body. These results suggest that even during periods of “normal” movement, there are defects in the extent of body bending in these worms.

The \textit{nca-1(gk9)} and \textit{nca-2(gk5)} single mutants spend a comparable amount of time moving forward, backward and pausing to wildtype worms, as well as displaying similar mean forward and reverse velocities and bend wavelengths. Fainting behaviour in these worms was not observed as previously reported (Pierce-Shimomura, Chen et al. 2008). In support of this, worms carrying the \textit{nca-2(gk5)} allele show comparable body curvature to wildtype at all five measured points along their body. However, despite displaying phenotypically wildtype locomotion, the mutant \textit{nca-1(gk9)} does display significantly reduced body curvature at its extremities; at the head, hips and tail.
**Chapter II: Quantitative Analysis of Locomotion Behaviour**

<table>
<thead>
<tr>
<th></th>
<th>N2</th>
<th>nca-1</th>
<th>nca-2</th>
<th>nca-1,nca-2</th>
<th>unc-79</th>
<th>unc-80</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Forward Velocity (μm/sec)</strong></td>
<td>54.8 ± 31.1</td>
<td>59.0 ± 44.4</td>
<td>50.7 ± 29.8</td>
<td>*24.0 ± 14.9</td>
<td>*31.0 ± 13.8</td>
<td>45.1 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>-57.5 ± 29.7</td>
<td>-53.7 ± 33.9</td>
<td>-51.6 ± 24.8</td>
<td>*-30.7 ± 26.5</td>
<td>*-30.3 ± 11.5</td>
<td>-44.6 ± 17.8</td>
</tr>
<tr>
<td><strong>Mean Backward Velocity (μm/sec)</strong></td>
<td>779.4 ± 86.4</td>
<td>778.0 ± 81.9</td>
<td>768.5 ± 92.7</td>
<td>794.9 ± 97.6</td>
<td>*837.4 ± 74.5</td>
<td>807.9 ± 85.4</td>
</tr>
<tr>
<td></td>
<td>-57.5 ± 29.7</td>
<td>-53.7 ± 33.9</td>
<td>-51.6 ± 24.8</td>
<td>*-30.7 ± 26.5</td>
<td>*-30.3 ± 11.5</td>
<td>-44.6 ± 17.8</td>
</tr>
<tr>
<td><strong>Mean Wavelength (μm)</strong></td>
<td>779.4 ± 86.4</td>
<td>778.0 ± 81.9</td>
<td>768.5 ± 92.7</td>
<td>794.9 ± 97.6</td>
<td>*837.4 ± 74.5</td>
<td>807.9 ± 85.4</td>
</tr>
<tr>
<td><strong>Distance Moved Forward (mm)</strong></td>
<td>44.4 ± 27.5</td>
<td>46.2 ± 35.2</td>
<td>40.9 ± 26.5</td>
<td>*18.3 ± 7.8</td>
<td>*25.5 ± 11.5</td>
<td>32.6 ± 13.9</td>
</tr>
<tr>
<td><strong>Distance Moved Backward (mm)</strong></td>
<td>18.5 ± 5.9</td>
<td>16.1 ± 4.5</td>
<td>13.8 ± 7.0</td>
<td>*12.1 ± 4.0</td>
<td>*13.5 ± 3.9</td>
<td>15.7 ± 5.4</td>
</tr>
<tr>
<td><strong>Time Spent Moving Forward (s)</strong></td>
<td>491.2 ± 157.5</td>
<td>454.2 ± 168.9</td>
<td>525.6 ± 155.7</td>
<td>*287.0 ± 91.6</td>
<td>*360.3 ± 93.7</td>
<td>424.8 ± 116.5</td>
</tr>
<tr>
<td><strong>Time Spent Moving Backward (s)</strong></td>
<td>191.1 ± 73.9</td>
<td>159.0 ± 37.4</td>
<td>151.8 ± 60.9</td>
<td>158.3 ± 42.4</td>
<td>164.7 ± 35.6</td>
<td>174.7 ± 63.0</td>
</tr>
<tr>
<td><strong>Time Spent Pausing (s)</strong></td>
<td>207.0 ± 118.9</td>
<td>252.4 ± 132.8</td>
<td>221.6 ± 149.2</td>
<td>*421.7 ± 141.4</td>
<td>*373.9 ± 90.0</td>
<td>*299.4 ± 100.2</td>
</tr>
<tr>
<td><strong>Curvature at head (degrees)</strong></td>
<td>20.6 ± 2.3</td>
<td>*18.9 ± 2.1</td>
<td>21.7 ± 2.4</td>
<td>*15.6 ± 1.5</td>
<td>*15.6 ± 1.5</td>
<td>*16.3 ± 1.6</td>
</tr>
<tr>
<td><strong>Curvature at neck (degrees)</strong></td>
<td>19.4 ± 1.8</td>
<td>18.0 ± 3.9</td>
<td>19.6 ± 2.0</td>
<td>*17.2 ± 3.0</td>
<td>*15.2 ± 1.9</td>
<td>*15.5 ± 1.7</td>
</tr>
<tr>
<td><strong>Curvature at mid-body (degrees)</strong></td>
<td>11.0 ± 1.8</td>
<td>9.8 ± 1.3</td>
<td>10.0 ± 2.2</td>
<td>9.9 ± 2.4</td>
<td>*8.3 ± 2.6</td>
<td>*8.4 ± 1.7</td>
</tr>
<tr>
<td><strong>Curvature at hips (degrees)</strong></td>
<td>17.1 ± 1.7</td>
<td>*14.1 ± 1.6</td>
<td>15.5 ± 2.5</td>
<td>*14.6 ± 4.3</td>
<td>*12.6 ± 2.2</td>
<td>*14.9 ± 2.5</td>
</tr>
<tr>
<td><strong>Curvature at tail (degrees)</strong></td>
<td>17.8 ± 1.9</td>
<td>*15.0 ± 1.8</td>
<td>16.4 ± 3.0</td>
<td>*15.5 ± 4.1</td>
<td>*13.1 ± 2.5</td>
<td>*15.7 ± 2.8</td>
</tr>
</tbody>
</table>

**Figure 2.7: Summary of locomotion behaviour for wildtype and fainter mutants.** Reported values are the mean ± standard deviation for each locomotion feature measured. At least 18 animals were tested for each genotype. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
Chapter II: Quantitative Analysis of Locomotion Behaviour

Wildtype N2

\[\begin{array}{cccccc}
0\text{s} & 1\text{s} & 2\text{s} & 3\text{s} & 4\text{s} & 5\text{s} \\
\end{array}\]

nca-1(gk9)

\[\begin{array}{cccccc}
0\text{s} & 1\text{s} & 2\text{s} & 3\text{s} & 4\text{s} & 5\text{s} \\
\end{array}\]

nca-2(gk5)

\[\begin{array}{cccccc}
0\text{s} & 1\text{s} & 2\text{s} & 3\text{s} & 4\text{s} & 5\text{s} \\
\end{array}\]

nca-1(gk9); nca-2(gk5)

\[\begin{array}{cccccc}
0\text{s} & 1\text{s} & 2\text{s} & 3\text{s} & 4\text{s} & 5\text{s} \\
\end{array}\]

unc-79(e1068)

\[\begin{array}{cccccc}
0\text{s} & 1\text{s} & 2\text{s} & 3\text{s} & 4\text{s} & 5\text{s} \\
\end{array}\]

unc-80(e1069)

\[\begin{array}{cccccc}
0\text{s} & 1\text{s} & 2\text{s} & 3\text{s} & 4\text{s} & 5\text{s} \\
\end{array}\]
Figure 2.8: Body posture sequences for wildtype N2 worms and fainter mutants. Example image sequences from automatic tracking analysis, shown at 1 second intervals over a total time of 5 seconds. Worms are imaged crawling on OP50 food, at 30fps for a total time of 15 minutes. The segmented worm is shown with outline and midline overlaid and the head (green) and tail (red) marked. The vulva is indicated with a red dot close to the mid-body.

A: Wildtype N2, B: nca-1(gk9), C: nca-2(gk5), D: nca-1(gk9);nca-2(gk5), E: unc-79(e1068), F: unc-80(e1069).
2.4.3 Locomotion behaviour of the mis-wiring mutants *vab-7(e1562)*, *unc-3(e151)*, *unc-4(e120)*, *unc-30(e191)*, *unc-55(e402)* and *unc-104(e1625)*

The locomotion behaviour of the six lines with mis-wired motor neurons (*vab-7*, *unc-3*, *unc-4*, *unc-30*, *unc-55* and *unc-104*) is summarised in Figures 2.9 and 2.10, along with that of wildtype.

Previous analysis of *vab-7(e1562)* locomotion reported that these mutants are severely uncoordinated, exhibiting defects in forward locomotion as expected from loss of dorsally- innervating B class motor neurons that are thought to coordinate forward movement. In response to head touch, these worms are observed to display normal backward movement but in response to tail touch, they curl ventrally and are unable to move forward normally (Ahringer 1996; Esmaeili, Ross et al. 2002).

Analysis of *vab-7(e1562)* locomotion with Worm Tracker 2.0 confirms that these worms display uncoordinated forward locomotion. They have a significantly reduced forward velocity compared to wildtype worms and spend significantly less time moving forward and significantly more time pausing, indicating a dramatically reduced efficiency in forward locomotion. However, despite spending a comparable amount of time moving backward to wildtype, *vab-7(e1562)* worms also display a significant reduction in their mean backward velocity. This result suggests that functional DB motor neurons may also be required in the coordination of smooth backward movement. These worms display a comparable bend wavelength to wildtype and only display significantly increased curvature at the mid-body.
### Chapter II: Quantitative Analysis of Locomotion Behaviour

<table>
<thead>
<tr>
<th></th>
<th>N2</th>
<th>vab-7</th>
<th>unc-3</th>
<th>unc-4</th>
<th>unc-30</th>
<th>unc-55</th>
<th>unc-104</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Forward Velocity</strong> (μm/sec)</td>
<td>63.8 ± 42.7</td>
<td>*31.7 ± 19.9</td>
<td>*12.6 ± 6.5</td>
<td>*19.1 ± 10.2</td>
<td>*24.2 ± 19.7</td>
<td>*30.6 ± 24.0</td>
<td>*3.4 ± 1.3</td>
</tr>
<tr>
<td><strong>Mean Backward Velocity</strong> (μm/sec)</td>
<td>-63.6 ± 43.2</td>
<td>*-26.2 ± 14.1</td>
<td>*-6.7 ± 2.8</td>
<td>*-9.5 ± 5.7</td>
<td>*-18.1 ± 7.9</td>
<td>*-25.3 ± 10.8</td>
<td>*-3.6 ± 1.7</td>
</tr>
<tr>
<td><strong>Mean Wavelength</strong> (μm)</td>
<td>767.5 ± 79.6</td>
<td>805.7 ± 89.6</td>
<td>739.6 ± 95.4</td>
<td>779.5 ± 93.9</td>
<td>724.7 ± 88.3</td>
<td>792.9 ± 105.4</td>
<td>*638.6 ± 134.0</td>
</tr>
<tr>
<td><strong>Distance Moved Forward (mm)</strong></td>
<td>50.2 ± 33.6</td>
<td>*24.8 ± 19.1</td>
<td>*7.7 ± 12.8</td>
<td>*17.0 ± 15.4</td>
<td>*18.3 ± 20.1</td>
<td>*24.7 ± 16.2</td>
<td>*0.6 ± 0.3</td>
</tr>
<tr>
<td><strong>Distance Moved Backward (mm)</strong></td>
<td>19.6 ± 5.3</td>
<td>*12.4 ± 3.2</td>
<td>*2.3 ± 3.3</td>
<td>*10.4 ± 2.7</td>
<td>*9.8 ± 3.4</td>
<td>*0.9 ± 0.4</td>
<td>*0.4 ± 0.4</td>
</tr>
<tr>
<td><strong>Time Spent Moving Forward (s)</strong></td>
<td>492.8 ± 146.8</td>
<td>*361.3 ± 174.7</td>
<td>*228.9 ± 112.6</td>
<td>*346.3 ± 173.5</td>
<td>*285.7 ± 136.4</td>
<td>*364.8 ± 169.5</td>
<td>*202.3 ± 179.7</td>
</tr>
<tr>
<td><strong>Time Spent Moving Backward (s)</strong></td>
<td>185.9 ± 49.3</td>
<td>178.4 ± 63.5</td>
<td>*54.9 ± 26.1</td>
<td>*77.4 ± 42.6</td>
<td>177.7 ± 44.4</td>
<td>160.5 ± 38.1</td>
<td>*74.2 ± 116.2</td>
</tr>
<tr>
<td><strong>Time Spent Pausing (s)</strong></td>
<td>210.2 ± 123.0</td>
<td>*359.2 ± 177.5</td>
<td>*615.1 ± 117.2</td>
<td>*475.1 ± 176.9</td>
<td>*435.4 ± 141.6</td>
<td>*373.5 ± 159.1</td>
<td>*622.4 ± 207.7</td>
</tr>
<tr>
<td><strong>Curvature at head (degrees)</strong></td>
<td>20.3 ± 2.3</td>
<td>19.4 ± 3.2</td>
<td>*17.3 ± 2.8</td>
<td>*23.7 ± 4.1</td>
<td>*15.8 ± 2.0</td>
<td>18.7 ± 2.6</td>
<td>*16.5 ± 4.0</td>
</tr>
<tr>
<td><strong>Curvature at neck (degrees)</strong></td>
<td>19.1 ± 1.8</td>
<td>19.8 ± 2.8</td>
<td>*16.1 ± 3.4</td>
<td>19.8 ± 2.8</td>
<td>*15.6 ± 2.2</td>
<td>18.1 ± 1.9</td>
<td>*24.3 ± 7.1</td>
</tr>
<tr>
<td><strong>Curvature at mid-body (degrees)</strong></td>
<td>10.8 ± 1.9</td>
<td>*15.3 ± 3.1</td>
<td>*14.3 ± 3.3</td>
<td>*14.8 ± 3.7</td>
<td>11.2 ± 2.5</td>
<td>*13.3 ± 1.9</td>
<td>*17.8 ± 5.4</td>
</tr>
<tr>
<td><strong>Curvature at hips (degrees)</strong></td>
<td>16.6 ± 2.5</td>
<td>20.0 ± 5.3</td>
<td>*28.3 ± 7.5</td>
<td>*22.4 ± 7.0</td>
<td>17.1 ± 3.2</td>
<td>18.7 ± 2.9</td>
<td>*28.2 ± 10.5</td>
</tr>
<tr>
<td><strong>Curvature at tail (degrees)</strong></td>
<td>17.4 ± 2.5</td>
<td>19.4 ± 5.6</td>
<td>*28.5 ± 7.6</td>
<td>*23.7 ± 9.5</td>
<td>17.1 ± 3.4</td>
<td>21.1 ± 3.2</td>
<td>*26.3 ± 11.2</td>
</tr>
</tbody>
</table>

Figure 2.9: Summary of locomotion behaviour for wildtype and motor neuron mis-wiring mutants. Reported values are the mean ± standard deviation for each locomotion feature measured. At least 18 animals were tested for each genotype. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
Chapter II: Quantitative Analysis of Locomotion Behaviour

Wildtype N2

\[\text{0s} \quad 1s \quad 2s \quad 3s \quad 4s \quad 5s\]

\textit{nca-1}(gk9)

\[\text{0s} \quad 1s \quad 2s \quad 3s \quad 4s \quad 5s\]

\textit{nca-2}(gk5)

\[\text{0s} \quad 1s \quad 2s \quad 3s \quad 4s \quad 5s\]

\textit{nca-1}(gk9);\textit{nca-2}(gk5)

\[\text{0s} \quad 1s \quad 2s \quad 3s \quad 4s \quad 5s\]
Chapter II: Quantitative Analysis of Locomotion Behaviour

Wildtype N2

vab-7(e1562)

unc-3(e151)

unc-4(e120)

unc-30(e191)

unc-55(e402)

unc-104(e1265)
Chapter II: Quantitative Analysis of Locomotion Behaviour

Figure 2.10: Body postures for wildtype N2 worms and fainter mutants. Example image sequences from automatic tracking analysis, shown at 1 second intervals over a total time of 5 seconds. Worms are imaged crawling on OP50 food, at 30fps for a total time of 15 minutes. The segmented worm is shown with outline and midline overlaid and the head (green) and tail (red) marked. The vulva is indicated with a red dot close to the mid-body.

A: Wildtype N2, B: vab-7(e1562), C: unc-3(e151), D: unc-4(e120), E: unc-30(e191), F: unc-55(e402), G: unc-104(e1265).

Worms carrying the unc-3(e151) mutation have been reported to display normal head movement but are unable to propagate smooth bends along their body (Herman 1987). Previous phenotypic analysis of unc-4 worms has shown that these worms cannot move backwards and instead coil dorsally when they try, though the head and tail are able to bend both dorsally and ventrally. Forward movement is also impaired but to a less severe extent (White, Southgate et al. 1992).

Locomotion analysis with Worm Tracker 2.0 shows that worms carrying the unc-3(e151) and unc-4(e120) mutations display severe defects in locomotion compared to wildtype worms. They show a significant reduction in mean forward and backward velocity compared to wildtype. They both also spend significantly less time moving forward, less time moving backward and more time pausing. Even though both unc-3(e151) and unc-4(e120) worms exhibit mean wavelengths of body bends that are comparable to wildtype worms, worms carrying the unc-3(e151) mutation display a significantly reduced body curvature at the head and neck and significantly greater body curvature at the mid-body, hips and tail, compared to wildtype worms. Worms carrying the unc-4(e120) mutation display a significantly greater curvature at the head, mid-body, hips and tail than wildtype worms.

These results agree with the observation that unc-3(e151) worms cannot propagate smooth, coordinated bends along their bodies (Herman 1987). However, in contrast to reports that these worms display normal head movements, data from Worm Tracker 2.0 suggests that unc-3(e151) worms cannot move their heads and necks to the same extent as wildtype worms. Furthermore, it appears that spontaneous forward movement of unc-4(e120) worms is severely impaired, indicating that functional VA motor neurons may have a role in
the coordination of forward movement as well as backward movement. Taken together, these results indicate that differentiated cholinergic neurons and proper organization of the VNC is essential for efficient forward and backward locomotion, maintaining wildtype body posture and the smooth propagation of bends along the worm body.

The locomotion of unc-30(e191) and unc-55(e402) worms has not been analysed in detail. When initiating reversals, unc-30(e191) worms exhibit the shrinker phenotype, in which both the ventral and dorsal sides of the worm contract at the same time (Jin, Hoskins et al. 1994). During shrinking, the magnitude of contraction for the dorsal and ventral muscle was equal and no locomotory imbalance was observed (Walthall and Plunkett 1995). In contrast, unc-55(e402) worms cannot sustain backward movement and instead coil ventrally in response to head touch. This behavioural response is hypothesized to be caused by less inhibition of ventral muscle and more inhibition of dorsal muscle on VD neuron mis-wiring (Walthall 1990).

Results from Worm Tracker 2.0 show that both unc-30(e191) and unc-55(e402) GABA neuron mis-wiring mutants are significantly less active than wildtype worms, displaying highly reduced mean forward and backward velocities. They spend a comparable amount of time moving backward to wildtype but significantly less time moving forward and significantly more time pausing. Both strains display a mean wavelength that is comparable to wildtype.

However, worms carrying the unc-30(e191) mutation display significantly reduced body curvature at the head and neck compared to wildtype worms, whereas unc-55(e402) mutants display a significant increase in body curvature at the mid-body. These results suggest that unc-30(e191) worms exhibit a similar behavioural phenotype to unc-25(e156) worms, in which disruption of GABA motor neuron signalling reduces the extent of body bending (McIntire et al., 1993), although this defect seems to be specifically localized to the anterior portion of unc-30(e191) worms. In contrast, the greater extent of body bending in unc-55(e402) worms agrees with the hypothesis that VD motor neuron mis-wiring causes less inhibition of ventral muscle and more inhibition of dorsal muscle, resulting in unbalanced body wall muscle excitation and coiling (Walthall 1990; Walthall and Plunkett...
1995). Taken together, these results show that appropriate GABAergic signalling from D class motor neurons is essential for efficient forward and backward locomotion and for the proper coordination of body bending in \textit{C. elegans}.

Worms carrying mutations within the \textit{unc-104} gene are reported to display severely uncoordinated locomotion, although robust feeding, growth and reproduction (Hall and Hedgecock 1991). These worms fail to alternate dorsal and ventral bending and they are observed to assume tightly coiled postures with all dorsal or ventral muscles contracted simultaneously for several minutes at a time (Hall and Hedgecock 1991). Such a locomotion phenotype closely resembles that displayed by \textit{cha-1} mutants lacking the enzyme acetylcholine transferase required for acetylcholine synthesis, and could reflect their shared disruption of functional cholinergic synapses (Hall and Hedgecock 1991).

The \textit{unc-104(e1265)} mutant displays the most severe disruption in locomotion of all the mis-wiring mutants assayed, moving less than a total of 1mm forward or backwards during the 15 minute assay period. Furthermore, \textit{unc-104(e1265)} worms are the only strain to show a significant reduction in bend wavelength compared to wildtype worms. In accordance with this result, \textit{unc-104(e1265)} displays significant differences in body curvature all the way along its body length compared to wildtype. Although curvature at the head is less than for wildtype, curvature at the neck, mid-body, hips and tail is significantly higher and likely accounts for the observed decrease in bend wavelength for this strain. Such an increase in body curvature from the neck posteriorly, agrees with the imbalance in body postures observed in these worms, where all dorsal or ventral muscles contract simultaneously. A reduction in curvature at head could result from the disrupted functioning of motor neurons innervating the head muscle, thereby reducing the extent of movement.

All six motor neuron mis-wiring mutants display a significant reduction in both forward and backward mean velocity compared to wildtype N2 worms. All these worm strains move forwards and backwards a total distance that is significantly less than wildtype worms during the assay period. They also all spend significantly more time pausing, confirming that the mis-wiring of the locomotion circuit exhibited by these strains dramatically reduces their efficiency of movement.
2.5 Discussion

In this study, I have quantitatively characterized the locomotion behaviour of fourteen mutant strains and compared this to wild type N2 worms.

2.5.1. The role of proprioception in locomotion behaviour

I investigated the effect on locomotion of mutations in the DEG/ENaC proteins UNC-8 and DEL-1, predicted to function together to form a mechanosensory channel for proprioception in the VA and VB motor neurons. I showed that the putative proprioceptive mutant \textit{unc-8} displays reduced locomotion compared to wildtype whereas, unexpectedly, \textit{del-1} displays increased locomotion compared to wildtype, although both retain a wildtype-like waveform and body curvature. Despite exhibiting reduced body curvature at its head and tail, the double mutant \textit{unc-8;del-1} displays otherwise wildtype locomotion.

If UNC-8/DEL-1 channels are expressed on the predicted stretch-sensitive portion of motor neuron processes and do contribute to proprioception, they are hypothesized to respond to stretch by depolarizing the neuron and potentiating neurotransmitter release at the neuromuscular junction. This would result in stronger muscle contraction and deeper body bends. No strains displayed a dramatic alteration in either the degree or timing of body bending, as would be predicted from a proprioceptive defect. These results suggest either the UNC-8 and DEL-1 proteins are not involved in proprioceptive modulation of worm posture, or if they are, then proprioception may only have a subtle role in modulating the extent of body bending during worm locomotion. It would be possible to test these hypotheses further by calcium imaging of VA and VB motor neurons and body wall muscle. Furthermore, it has been shown that mutations in the paraoxonase-like protein MEC-6 can suppress semi-dominant \textit{unc-8} phenotypes (neuronal swelling and severe uncoordination) suggesting that MEC-6 is required for UNC-8 channel function (Shreffler, Magardino et al. 1995). If this is true, it would interesting to assess the locomotion of a \textit{unc-8;del-1;mec-6} triple mutant.
2.5.2 The role of cation leak channels in locomotion behaviour

Behavioural analysis of the fainter mutants showed that the double mutant \textit{nca-1(gk9);nca-2(gk5)} exhibits the most severe fainting phenotype, that the fainting phenotype of \textit{unc-79(e1069)} is more severe than that of \textit{unc-80(e1068)} and that all three mutations significantly reduce body curvature along the length of the worm. Worms carrying either \textit{nca-1(gk9)} or \textit{nca-2(gk5)} mutations display wildtype locomotion, although \textit{nca-1(gk9)} exhibits reduced body curvature at the head and tail. These results show that mutations within these genes not only result in fainting episodes, but also affect the curvature of the body during episodes of normal locomotion.

All four genes encode voltage-insensitive cation leak channel subunits and their expression overlaps in the cholinergic motor neurons of the VNC and is localized to the predicted stretch-sensitive portion of motor neuron processes (Yeh, Ng et al. 2008). The \textit{nca-1} and \textit{nca-2} genes encode \textit{C. elegans} homologs of the \(\alpha 1\) subunit of voltage-gated calcium and sodium channels, whereas the \textit{unc-79} and \textit{unc-80} genes encode large novel proteins with a central armadillo motif that may be important in protein-protein binding interactions (Humphrey, Hamming et al. 2007; Pierce-Shimomura, Chen et al. 2008). The proteins are therefore thought to form novel ion channel complexes called “NCA”, with \textit{nca-1} and \textit{nca-2} hypothesized to be pore-forming subunits and \textit{unc-79} and \textit{unc-80} hypothesized to be auxiliary components. In this way, the channels are believed to regulate the activation of motor neuron synapses and so regulate locomotion.

The \textit{nca-1(gk9);nca-2(gk5)} mutant displayed the most severe fainting phenotype. Each of the NCA-1 and NCA-2 proteins contain the four domains found in voltage-gated sodium and calcium channels, which suggests that separate NCA-1 and NCA-2 channels can exist independently, rather than being part of one complex. This hypothesis is consistent with the additive fainting phenotype observed in the double mutant. The tracking results also suggest that NCA-1 and NCA-2 channels could functionally compensate for each other, as no strong locomotion phenotype was observed in either of the single mutants.
In comparison, both \textit{unc-79(e1068)} and \textit{unc-80(e1069)} worms display less severe fainting phenotypes, consistent with their hypothesized roles as auxiliary components that regulate the NCA channel through localization of the pore-forming subunit. Also in agreement with this hypothesis is the observation that expression of UNC-80 enhances current through NCA-1 channels in transfected HEK293T cells (Yeh, Ng et al. 2008).

Previous studies report a significant decrease in synaptic activity in the neurons of \textit{nca-1(gk9);nca-2(gk5)} and \textit{unc-80(e1069)} worms (Yeh, Ng et al. 2008), which correlates with the observed fainting behaviour of these strains. The synaptic activity in \textit{unc-79(e1068)} worms has not been determined, but it might be predicted to show a similar reduction. Based on these results, the NCA channel is hypothesized to drive motor neuron membrane potential close to its excitation threshold, facilitating the activation of other channels at the motor neuron axon or synapse. It should be noted that motor neuron activity was not directly recorded in this study. Instead, electrophysiological recordings of currents in body wall muscle were taken as a read-out for acetylcholinergic and GABAergic neurons and calcium imaging was carried out on the cell bodies and axons of the serotonergic egg-laying HSN neurons in glued worms. To further test the hypothesized role of the NCA channel in motor neurons it will be essential to directly monitor motor neuron and muscle activity in these mutants during unrestrained locomotion encompassing episodes of fainting as well as normal movement.

It is interesting to observe that mutations predicted to reduce motor neuron excitability (\textit{unc-8;del-1, nca-1;nca-2, unc-79} and \textit{unc-80}), do result in reduced body curvature. This result suggests that motor neuron excitability does have a role to play in determining the extent of body bending in \textit{C. elegans}. For mutations in which a subtle locomotion phenotype is observed (\textit{unc-8;del-1} and \textit{nca-1}), it is mainly the extremities of the worm, the head and tail, which are affected. These points exhibit the largest range of curvatures in wildtype worms, and coupled with the greater extent of muscle innervation in the head and neck, it might be expected that a reduction in motor neuron activity affects these regions more severely.
2.5.3 The roles of the A, B and D motor neuron classes in locomotion

I also investigated the effect on locomotion of mutations that affect motor neuron specification and wiring. I observed that \textit{vab-7(e1562)} mutants show uncoordinated backward and well as forward movement, suggesting that functional B class motor neurons are also important in the execution of smooth backward movement, and that \textit{unc-4(e120)} mutants show impaired spontaneous forward movement, suggesting that functional A class motor neurons are also important in the execution of smooth forward movement. Such results agree with very recent calcium imaging studies suggesting that B motor neurons continue to display oscillatory activity during backward movement and that A motor neurons continue to display oscillatory activity during forward movement (Faumont, Rondeau et al. 2011), and highlights that the forward and backward motor circuits may not be as distinct as previously thought.

I observe that disruption of D class motor neuron function in \textit{unc-30(e191)} worms reduces locomotion and reduces body bending, resulting in a phenotype similar to that described for \textit{unc-25(e156)} worms lacking GABA neurotransmission. In contrast, imbalanced D class motor neuron function in \textit{unc-55(e402)} worms reduces locomotion and increases body bending. This is hypothesized to be caused by less inhibition of ventral muscle and more inhibition of dorsal muscle on VD neuron mis-wiring (Walthall 1990) though this is yet to be confirmed experimentally. These results confirm that appropriate GABAergic signalling from D class motor neurons is essential for efficient forward and backward locomotion and for the proper coordination of body bending in \textit{C. elegans}.

I found that \textit{unc-104(e1265)} worms display the most severe defects in locomotion of all strains assayed, hardly moving a distance of one body length during recordings. They have increased body bending from the neck to the tail resulting in a bend waveform of reduced wavelength. These worms have a locomotion phenotype that closely resembles that of \textit{cha-1} worms, notably the coiling and tight S-shaped body postures. This could point to the importance of the A and B cholinergic neurons in coordinating the wildtype bend waveform required for efficient locomotion. However, it is possible that, similar to the \textit{unc-55} phenotype, the tight coiling is due to unbalanced D class motor neuron activity. For these
worms it is hard to disentangle those phenotypes caused by D neuron mis-wiring and those caused by decreased neurotransmission brought about by the synaptic vesicle trafficking defect.

### 2.5.4 Utility of Machine Vision for quantifying behavioural phenotypes

The use of machine vision analysis to quantitatively describe worm behaviour allows for the precise description of phenotypes that result from genetic mutations. Using Worm Tracker 2.0 it has been possible to discern and measure subtle locomotion phenotypes for several strains I have assayed, for example, the reduced body curvature at the head and tail for \textit{nca-1(gk9)} and the double mutant \textit{unc-8(e15lb145);del-1(ok150)}. Such subtle phenotypes are unlikely to be detected through the observation of worm behaviour by eye. Increasing the ability of Worm Analysis Toolbox v1.1.4 to identify other behavioural features, such as reversals, coiled shapes and head foraging movements, would give better phenotypic characterization of mutants and could suggest further roles for the genes I have assessed, as well as others, in worm locomotion.

Coupling the quantitative analysis of behaviour with calcium imaging would provide a powerful method with which to investigate the relationship between motor neuron activity, muscle activity and locomotion features. As of yet, the activity of D class cross-inhibitory motor neurons during worm locomotion has not been reported. Furthermore, experimental confirmation of observed oscillations in the activity of A and B motor neurons with body bending will be essential for confirming the current model for \textit{C. elegans} locomotion. In addition, it is unclear which neurons are responsible for such oscillations in activity and whether there is a role for proprioceptive feedback in the modulation of locomotion. Ultimately, to answer these questions it will be necessary to image motor neuron and body wall muscle activity in freely behaving wildtype worms as well as strains with mutations in genes predicted to affect motor neuron activity and motor neuron wiring.
Chapter III

Construction of GCaMP3 transgenic lines and calcium imaging worm tracker
3.1 Abstract

In this Chapter I describe methods to construct the GCaMP3 expression vectors and transgenic *C. elegans* lines to allow imaging from the body wall muscle and A, B and D motor neuron classes in the worm locomotion circuit. Transgenic lines were made to express GCaMP3 in body wall muscle and the B and D classes of motor neuron, but attempts at expression of GCaMP3 in A class motor neurons were unsuccessful.

I describe the hardware and software involved in developing the calcium imaging worm tracker, which allows simultaneous whole-worm tracking and calcium imaging. This design is based on the high-resolution, single-worm tracker, Worm Tracker 2.0, developed by E. Yemini and T. Jucikas, MRC LMB, UK (http://www.mrc-lmb.cam.ac.uk/wormtracker/, unpublished).

I describe the assays for calcium imaging in freely-crawling and freely-swimming worms and the post-acquisition processing of the calcium imaging image sequences, as well as the software for the quantitative analysis of body wall muscle and motor neuron fluorescence. I describe the methods for checking laser stability, applying a flat-field correction to the images to correct for uneven laser illumination in the field-of-view, correcting fluorescence bleed-through of the red filter and checking for saturation of the fluorescence signal.

As of yet, preliminary imaging results from D and B classes of motor neurons have been inconclusive and I briefly present these results at the end of this Chapter.
3.2 Strains

Strains were maintained at 22°C, using standard methods (Brenner 1974). Strains used in this study include:

N2 (Bristol, England)
MP145, unc-8(e151b145) IV
CB151 unc-3(e151) X
CB120 unc-4(e120) II
CB845 unc-30(e191) IV
CB402 unc-55(e402) I
DR1089 unc-77(e625) IV
CB1068 unc-79(e1068) III
CB1069 unc-80(e1069) V
CB1265 unc-104(e1265) II
VC12 nca-1(gk9) IV
VC9 nca-2(gk5) III
VC12 nca-1(gk9) IV; VC9 nca-2(gk5) III
NC279 del-1(ok150) X
MP145 unc-8(e151b145) IV; NC279 del-1(ok150) X
CB1562 vab-7(e1562) III
DA438 bli-1(e937) I; rol-6(e187) II; daf-2(e1368) vab-7(e1562) III; unc-31(e928) IV;
dpy-11(e224) V; lon-2(e678) X
XJ45 kfEx[che-3::GCaMP3]
QW625 zfIs42[prig-3::GCaMP3-SL2-mCherry;lin-15+]
AQ2953 ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2954 ljIs132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2955 nca-1(gk9);nca-2(gk5) ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2956 nca-1(gk9);nca-2(gk5) ljIs132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2957 unc-79(e1068) ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2958 unc-79(e1068) ljIs132[pmyo-3::GFP-SL2-tagRFP-T]
3.3 Molecular Biology

3.3.1 Genotyping

Template DNA for Polymerase Chain Reaction (PCR) was prepared by worm lysis. Lysis solution was prepared by mixing 900μl nuclease-free water (Mediatech, Inc., US), 100ul 10 x PCR Buffer (Invitrogen, Life Technologies Ltd., US) and 10μl Proteinase K (Sigma US, P2308) at a concentration of 20mg/ml in a glycerol-based buffer. 10 – 20 adult worms were placed in 10μl of the lysis solution and frozen at -80°C for 20 minutes and thawed at room temperature. The worm solution was then placed in a BioRad Thermal Cycler and heated at 65°C for 1 hour (lysis step), followed by 95°C for 15 minutes (denaturing step for Proteinase K). The resulting worm DNA preparation was stored at -20°C until required.

PCR to confirm genotype was performed using the worm DNA preparation and Taq Polymerase (New England Biolabs Inc., US). For a 30μl PCR reaction, reagents were mixed in the following volume and order:
15.8μl Nuclease-free water (Mediatech, Inc., US)
2μl 10 x CoralLoad PCR buffer with 15mM MgCl₂ (Qiagen, US)
0.6μl 25mM MgCl₂ (Qiagen, US)
1μl Primers (Integrated DNA Technologies, Inc. US)
0.5μl 10mM dNTPs
0.1μl Taq Polymerase (5,000 Units/ml, NEB)
10μl Worm DNA preparation

PCR programmes varied according to the length of the DNA fragment to be amplified, with the following general scheme:

1 94°C 3min
2 94°C 30 sec
3 55-65°C 45 sec
4 72°C 1 min/kb
5 go to 2 35x
6 72°C 10min
7 4°C forever

PCR reactions for cloning and sequencing were performed in the same manner, but using Expand High Fidelity enzyme (Roche Ltd., US).

DNA sequencing (Molecular Biology Shared Resources, Janelia Farm, US) was used to confirm genotype and sequences of cloned DNA fragments. Chromatograms from sequencing runs were visualized in A plasmid Editor v1.13 (ApE v1.13 for Windows, M. Wayne Davis, free software).

Restriction endonuclease digests of miniprepped DNA were used to identify correct clones. 10μl digest reactions were run for 3 hours at 37°C:
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

3μl Nuclease-free water (Mediatech, Inc., US)
1μl 10 x NEB Enzyme buffer (New England Biolabs Inc., US)
5μl DNA
1μl Restriction endonuclease (New England Biolabs Inc., US)

PCR amplifications and restriction endonuclease digests were analysed by agarose gel electrophoresis. Agarose gels were made with 1% UltraPure Agarose (Invitrogen, Life Technologies Ltd., UK), 1 x Tris-Acetate EDTA (TAE) electrophoresis buffer (Mediatech, Inc., US), stained with ethidium bromide (5μl/100ml, Thermo Fisher Scientific, Inc., US) and run at 110V for 60 minutes. A photograph of each gel was taken under UV lighting.

3.4 Primers

The following primers were generated during my thesis, listed along with their name and use:

- **del-1 int for**: AAACCAACTGACCCAAGGTG (sequencing del-1(ok150))
- **del-1 int rev**: TATCTAGGGTGCCGCAAAACC (sequencing del-1(ok150))
- **del-1 for**: CGAGTTAAAGCGGTAGGGAAG (genotyping del-1(ok150))
- **del-1 rev wt**: CGAGCAATCCGAGTAAACAGGT (genotyping del-1(ok150))
- **del-1 rev mut**: GCAACTTACTTCTGGCTCATAC (genotyping del-1(ok150))
- **unc-8 lf for CDRII**: ACAGTATGAAGCCAGGAGGTG (sequencing unc-8(e15lb145))
- **unc-8 lf rev CDRIII**: GACTGGGCCATGCAATGCA (sequencing unc-8(e15lb145))
- **unc-8 lf for wt**: ACAAGAAGGAACCTGTCATACACC (sequencing unc-8(e15lb145))
- **unc-8 lf rev mut**: CTTCATCTCTCTGTGTTGGCTTT (sequencing unc-8(e15lb145))
- **unc-8 lf for wt**: ACAAGAAGGAACCTGTCATACACC (genotyping unc-8(e15lb145))
- **unc-8 lf rev wt**: CTGCTGCTTCTGTTGTTGGCATG (genotyping unc-8(e15lb145))
- **unc-8 lf rev mut**: CTTCATCTCTCTGTGTTGGCTTT (genotyping unc-8(e15lb145))
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

\[
\begin{align*}
\text{nca-1 seq for} & \quad \text{ATGCCTCGGTAGTCTTTTATGACG} & \text{sequencing nca-1(gk9)} \\
\text{nca-1 seq rev} & \quad \text{CCACACGTCTCTGTCAGTTT} & \text{sequencing nca-1(gk9)} \\
\text{nca-1 for wt} & \quad \text{GTGATTGGAGAAAAACACACAATCTGGATTTAC} & \text{genotyping nca-1(gk9)} \\
\text{nca-1 rev wt} & \quad \text{CGATGACATTTTTGAAACATTAACGATC} & \text{genotyping nca-1(gk9)} \\
\text{nca-1 for mut} & \quad \text{GATGAGAAAAACACGTGTAATGTTGG} & \text{genotyping nca-1(gk9)} \\
\text{nca-1 rev mut} & \quad \text{GAAACATTAATGACGTTTGAGACTGG} & \text{genotyping nca-1(gk9)} \\
\text{nca-2 for wt} & \quad \text{GGACCCCGAAAGAAATGGGA} & \text{genotyping nca-2(gk5)} \\
\text{nca-2 rev wt} & \quad \text{CATCTGTCCAGCCTTTTGTGTTA} & \text{genotyping nca-2(gk5)} \\
\text{nca-2 rev mut} & \quad \text{CAGATCATAGGTAACAACGCAG} & \text{genotyping nca-2(gk5)} \\
\text{unc-79 for 1} & \quad \text{CCCTTCCGTCTGTAGTGTCC} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 rev 1} & \quad \text{CTCCAGATTCTCTTGGCTAA} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 for 2} & \quad \text{GTGTAATGGCAACACATGGTG} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 rev 2} & \quad \text{GCTTCAACAAGTGACCAG} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 for 3} & \quad \text{GACAATGGAATCACACCAACG} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 rev 3} & \quad \text{CTATCGGCAATGCACTCGGA} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 for 4} & \quad \text{CTCGGTGGATGCTGAAGTGAAG} & \text{sequencing unc-79(e1068)} \\
\text{unc-79 rev 4} & \quad \text{GGATGTTTTGTTGTTGTGAACC} & \text{sequencing unc-79(e1068)} \\
\text{unc-80 for} & \quad \text{TGCTACAGACCTACGGACTAGAC} & \text{sequencing unc-80(e1069)} \\
\text{unc-80 rev} & \quad \text{GAGTTTCCTGATTTGGGAGC} & \text{sequencing unc-80(e1069)} \\
\text{pmyo-3 for NotI} & \quad \text{CACGCCGCCGCCGCCTATATAAGTTCTTGGAATAAA} & \text{cloning myo-3 promoter} \\
\text{pmyo-3 rev BamHI} & \quad \text{CACGGATCCCTCTAGATGGATCTAGTGCTGGG} & \text{cloning myo-3 promoter} \\
\text{punc-47 for NotI} & \quad \text{CACGCCGCCGCCAAAAATGGACAAAAACACCTTTCTTGG} & \text{cloning unc-47 promoter} \\
\text{punc-47 rev BamHI} & \quad \text{CACGGATCCCTCTGTAATGAAATAATGGCACTGG} & \text{cloning unc-47 promoter} \\
\text{pacr-5 for NotI} & \quad \text{CACGCCGCCGCCCAATGGCAATGCTGGTTC} & \text{cloning acr-5 promoter} \\
\text{pacr-5 rev BamHI} & \quad \text{CACGGATCCCTCAGTACGCTTAATGGAATTAATGACGCTG} & \text{cloning acr-5 promoter} \\
\text{punc-4 for NotI} & \quad \text{CACGCCGCCGCCGAACTGGGATATAATTTCTAAC} & \text{cloning unc-4 promoter} \\
\text{punc-4 rev BamHI} & \quad \text{CACGGATCCACCGATCTTTTTTCACTTTTTTGGAG} & \text{cloning unc-4 promoter}
\end{align*}
\]
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Cloning Site</th>
<th>Cloning Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>TAGAGGATCCCGGGATG</td>
<td>CCATGTGCACGGAATTGG</td>
<td>XmaI</td>
<td>cloning GFP into pSM</td>
</tr>
<tr>
<td>GFP</td>
<td>CCATGTCGACGGAATTGG</td>
<td>CTACATCCATTTAAGCTCATCTCTAAG</td>
<td>Sall</td>
<td>cloning GFP into pSM</td>
</tr>
<tr>
<td>GC3</td>
<td>CACACCGGTATGGTTGATTCAAGTGAAGAATG</td>
<td>CCATGTCGACGGAATTGG</td>
<td>AgeI</td>
<td>cloning GC3 into pGP-13.1</td>
</tr>
<tr>
<td>GC3</td>
<td>CACGAATTCCTTTTTTTATTTAGGGTCATCTTTTGAACG</td>
<td>CACGGAATTCTTATTTAGCCGTCATCTTTTGAACG</td>
<td>EcoRI</td>
<td>cloning GC3 into pGP-13.1</td>
</tr>
<tr>
<td>RFP</td>
<td>CACACCGGTATGGTTGATTCAAGTGAAGAATG</td>
<td>CACGGAATTCCTTTTTATTTAGGGTCATCTTTTGAACG</td>
<td>AgeI</td>
<td>cloning RFP into pGP-13.1</td>
</tr>
<tr>
<td>RFP</td>
<td>CACGAATTCCTTTTTATTTAGGGTCATCTTTTGAACG</td>
<td>CACGGAATTCCTTTTTATTTAGGGTCATCTTTTGAACG</td>
<td>EcoRI</td>
<td>cloning RFP into pGP-13.1</td>
</tr>
<tr>
<td>YC3.60</td>
<td>CACGGAATTCGATAGCCATATTCAAACGGGAACG</td>
<td>CACGGAATTCGATAGCCATATTCAAACGGGAACG</td>
<td>BsmHI</td>
<td>cloning YC3.60</td>
</tr>
<tr>
<td>YC3.60</td>
<td>CACGGAATTCGATAGCCATATTCAAACGGGAACG</td>
<td>CACGGAATTCGATAGCCATATTCAAACGGGAACG</td>
<td>PmeI</td>
<td>cloning YC3.60</td>
</tr>
</tbody>
</table>

**Primers for unc-4::mCherry-GCaMP3 fusion construct:**

- **punc-4 rev AgeI**
  - CACACCGGTACCGATCATTTTCACTTTTTGGAAG
  - cloning unc-4 promoter

- **Age.mCherry_U**
  - GGGACCCTGCTATGGAAGCAAGGCCGGAGGAGGAATCAAAACTTGCCCATCA

- **GC3.Bam.mCherry_L**
  - CTACTTGAATCAACCATGCGGATCCCTTTTGTACAGCTCCATGCCTGACGGCTGCTGGCGGCCCTCGGCGCTCGTA

- **mCherry.Bam.GC3_U**
  - TCCACCGGCAGCTGGACGCTGTATAAGGAGATCGCCGATGGTTATGATTCTAGAAGAAATGGGATCAAAAACA

- **RI.Stp.GC3_L**
  - GGCGAATTCCTTTTATGCGGTCACTCATGAAAGCAACTCCTCGTA

- **mCh-GC3 fused 1**
  - GGGTGGGAAGTGGTGGATGATT

- **mCh-GC3 fused 2**
  - GGACCGAGCCCAAGATTATTC

- **mCh-GC3 fused 3**
  - GAAGGCGAGATCAGCGAG
3.5 Construction of GCaMP3 and GFP expression vectors

3.5.1 Generation of body-wall muscle lines \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}

The \textit{myo-3} promoter region was obtained from plasmid pDEST-myo-3p (Kuroyanagi, Kobayashi et al. 2006), a gift from H. Kuroyanagi. A 2.3kb NotI/BamHI fragment was ligated to worm codon-optimized GCaMP3 and tagRFP-T (DNA2.0 Inc., USA), gifts from L. Looger, within the vector pSM (C. Bargmann, Rockefeller University), a derivative of vector pPD49.26 (A. Fire, Stanford University). A 870bp Xmal/EcoRI GFP fragment was ligated in place of the GCaMP3 within the vector pSM, to generate a control construct. Transgenic lines were obtained by germline injection into N2 wildtype worms with the plasmid \textit{pmyo-3::GCaMP3-SL2-tagRFP-T} at a concentration of 50ng/μl, along with 100ng/μl 1kb DNA Ladder (Invitrogen, Life Technologies Ltd., UK) to increase the DNA concentration and enhance the efficiency of transgene formation and expression (R. Branicky, personal communication). The construct expresses in the body-wall muscle and vulval muscle of hermaphrodites (see Figure 3.1).

Figure 3.1: Image of worm line AQ2953 \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]}. This line expresses GCaMP3 and tagRFP-T in body wall and vulval muscle. The image shows tag-RFP-T fluorescence detected by Andor calcium imaging camera.
3.5.2 Generation of D class GABAergic motor neuron lines *ljIs133*[unc-47::GCaMP3-SL2-tagRFP-T] and *ljIs134*[unc-47::GFP-SL2-tagRFP-T]

The *unc-47* promoter region was obtained from plasmid pTNZ025A, a gift from Y. Tanizawa, and should express in the D class neurons (McIntire et al. 1997). A 1.2kb NotI/BamHI fragment was ligated to worm codon-optimized GCaMP3 and tagRFP-T within the vector pSM. A 870bp XmaI/EcoRI GFP fragment was ligated in place of the GCaMP3 within the vector pSM, to generate a control construct. Transgenic lines were obtained by germline injection into N2 wildtype worms with the plasmid *P unc-47::GCaMP3-SL2-tagRFP-T* at a concentration of 50ng/μl, along with 100ng/μl 1kb DNA Ladder (15615-016, Invitrogen, Life Technologies Ltd., UK). The construct expresses in the inhibitory GABAergic VD and DD neurons (see Figure 3.2).

![Figure 3.2: Image of worm line AQ2967 *ljIs133*[unc-47::GCaMP3-SL2-tagRFP-T]. This line expresses GCaMP3 and tagRFP-T in D class motor neurons. The image shows tag-RFP-T fluorescence detected by Andor calcium imaging camera with VD and DD neurons labelled.](image)
3.5.3 Generation of B class acetylcholinergic motor neuron lines \textit{ljis135[acr-5::GCaMP3-SL2-tag-RFP-T]} and \textit{ljis136[acr-5::GFP-SL2-tagRFP-T]}

The \textit{acr-5} promoter region was obtained from plasmid pJR7, a gift from D. Miller and should express in the B class neurons (Winnier, Meir et al. 1999). A 4.2kb NotI/BamHI fragment was ligated to worm codon-optimized GCaMP3 and tagRFP-T, within the vector pSM. A 870bp Xmal/Sall GFP fragment was ligated in place of the GCaMP3 within the vector pSM, to generate a control construct. Transgenic lines were obtained by germline injection into N2 wildtype worms with the plasmid \textit{Pacr-5::GCaMP3-SL2-tagRFP-T} at a concentration of 50ng/\mu l, along with 100ng/\mu l 1kb DNA Ladder (Invitrogen, Life Technologies Ltd., UK). The construct expresses in the VB and DB excitatory acetylcholinergic neurons (see Figure 3.3).

\textbf{Figure 3.3: Image of worm line AQ2969 \textit{ljis135[acr-5::GCaMP3-SL2-tag-RFP-T]}.} This line expresses GCaMP3 and tagRFP-T in B class motor neurons. The image shows tag-RFP-T fluorescence detected by Andor calcium imaging camera with VB and DB neurons labelled.
## Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

<table>
<thead>
<tr>
<th>Strain</th>
<th>Green Channel</th>
<th>Red Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]}</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>\textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]}</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>\textit{ljls133[punc-47::GCaMP3-SL2-tagRFP-T]}</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>\textit{ljls134[punc-47::GFP-SL2-tagRFP-T]}</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>\textit{ljls135[pacr-5::GCaMP3-SL2-tag-RFP-T]}</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>\textit{ljls136[pacr-5::GFP-SL2-tagRFP-T]}</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3.4: Images of experimental and control transgenic worm lines.** Left image shows green fluorescence (GCaMP3 in experimental worms and GFP in control worms). Right image shows red fluorescence (RFP in experimental and control worms).
3.5.4 Strategies for making A class motor neuron lines

All strategies for expressing calcium indicators in the A class motor neurons were unsuccessful. These included the generation and microinjection of the following constructs:

1. $\text{Punc-4}::\text{GCaMP3-SL2-tagRFP-T}$
2. $\text{Punc-4}::\text{GCaMP3}$ and $\text{unc-4}::\text{tagRFP-T}$
3. $\text{Punc-4}::\text{GCaMP3}$ and $\text{unc-4}::\text{mCherry}$
4. $\text{Punc-4}::\text{GCaMP5.003}$ and $\text{unc-4}::\text{mCherry}$
5. $\text{Punc-4}::\text{YC3.60}$
6. $\text{unc-4}::\text{mCherry-GCaMP3}$ fusion

The $\text{unc-4}$ promoter region was obtained from plasmid pNE1 (Pflugrad, Meir et al. 1997), a gift from D. Miller, and should express in A class neurons (Miller, Shen et al. 1992). A 2.7kb NotI/BamHI fragment was ligated to worm codon-optimized GCaMP3 and tagRFP-T, within the vector pSM to generate a $\text{Punc-4}::\text{GCaMP3-SL2-tagRFP-T}$ expression vector. Transgenic lines were obtained by germline injection into N2 wildtype worms with the plasmid $\text{Punc-4}::\text{GCaMP3-SL2-tagRFP-T}$ at a concentration of 20ng/μl, along with 100ng/μl 1kb DNA Ladder (Invitrogen, Life Technologies Ltd., UK). It was noted that tagRFP-T expression was dim and often non-specific, in places where the promoter does not usually drive expression. GCaMP3 expression was extremely dim and not observable in the majority of worms. It was further noticed that injecting at DNA concentrations greater than 20ng/ul produced transgenic worms that displayed the $\text{unc-4}$ mutant phenotype. These worms could move forwards normally, but could not move backwards and instead coiled dorsally.

The separate $\text{Punc-4}::\text{GCaMP3}$ and $\text{Punc-4}::\text{tagRFP-T}$ constructs were made by ligating the $\text{unc-4}$ promoter region, GCaMP3 coding region and tagRFP-T coding region into plasmid pGP-13.1, a gift from D. Kim (GECI project, Janelia Farm Research Campus, US.). A 2.7kb BamHI/NotI fragment of the $\text{unc-4}$ promoter was ligated to either a 1.2kb AgeI/EcoRI fragment of GCaMP3, or a 700bp AgeI/EcoRI fragment of tagRFP-T, within the pGP-13.1 vector. Transgenic lines were obtained by germline injection into N2 wildtype worms with a
mixture of both plasmids at a concentration of 50ng/μl. The same dim and non-specific tagRFP-T and GCaMP3 expression patterns were observed as for the Punc-4::GCaMP3-SL2-tagRFP-T construct.

The Punc-4::GCaMP5.003 construct was made by ligating a 2.7kb BamHI/NotI fragment of the unc-4 promoter region into plasmid pGP-13.2, containing the GCaMP5.003 coding region. Transgenic lines were obtained by germline injection into N2 wildtype worms with a mixture of this plasmid and an Punc-4::mCherry plasmid, a gift from R. Skelton (Miller lab, Vanderbilt University, US), both at a concentration of 50ng/μl. The same dim and non-specific expression pattern was observed as for the two constructs above.

Worm codon-optimized YC3.60 coding sequence was obtained from plasmid pJ207, a gift from D. Kim (GECI project, Janelia Farm Research Campus, US.). A 800bp BamHI/Pmel fragment was ligated to the unc-4 promoter in the Punc-4::mCherry plasmid. Injection into wildtype N2 worms of the plasmid unc-4::YC3.60 failed to produce any transgenic F1 worms.

A Punc-4::mCherry-GCaMP3 fusion construct was made by overlapping PCR to fuse the coding region of mCherry to the coding region of GCaMP3. A 2.7kb BamHI/AgeI fragment of the unc-4 promoter region was TOPO cloned into the pCR8/GW/TOPO vector (Invitrogen, Life Technologies Ltd., US). A 700bp AgeI/BamHI fragment of the mCherry coding region and a 1.2kb BamHI/EcoRI fragment of the GCaMP3 coding region were amplified in a first round of PCR. These fragments were then joined by overlap PCR in a second round of amplification using just the two outer primers (BamHI-mCherry and EcoRI-GCaMP3). A BamHI cloning site was introduced between the mCherry and GCaMP3 coding regions and also provides a short flexible linker to join the two proteins (JM Knapp, personal communication). The 1.9kb AgeI/EcoRI fused mCherry-GCaMP3 fragment was TOPO cloned into the pCR8/GW/TOPO vector (Invitrogen, Life Technologies Ltd., US). The 2.7kb BamHI/AgeI unc-4 TOPO fragment was ligated with the 1.9kb AgeI/EcoRI fused mCherry-GCaMP3 TOPO fragment and with the 3.2kb BamHI/EcoRI backbone fragment of the Punc-4::tagRFP-T plasmid, in a 2-way ligation. Injection into wildtype N2 worms of the plasmid Punc-4::mCherry-GCaMP3 failed to produce any transgenic F1 worms.
3.6 Integration of extrachromosomonal arrays

Integration of body wall muscle, D class motor neuron and B class motor neuron GCaMP3 and GFP expression vectors was achieved by UV irradiation. L4 stage worms were incubated with 0.03mg/ml trioxsalen (3902-71-4, Sigma Aldrich Inc., US) in DMSO (D8418-100ML, Sigma Aldrich Inc., US) for 15 minutes. Worms were subjected to 1 second UV irradiation (~1000 µJ/cm²) using the DAPI filter on a fluorescence microscope. Worms were then allowed to recover for 5 hours and 40 transgenic young adults were singled. These worms were removed from plates after 1 day, and after an additional day the plates were scored for dead eggs. After a further 2 days, 200 F1 worms were singled from plates containing some, but not all, dead eggs. After 4 days, F1 plates with greater than 75% transmission of fluorescence and non-mosaic expression pattern were selected and 3 worms were singled from each of these plates, giving approximately 600 F2 plates. These F2 plates were allowed to almost starve and then were screened for 100% transmission of fluorescence and non-mosaic expression. 3 F3 worms were singled from each candidate integration plate. Integrants underwent 10 generations of singling and backcrossing before use in experiments. Integrant lines used in experiments were:

lijIs131[Pmyo-3::GCaMP3-SL2-tagRFP-T] line 13 out of 15 integrants
lijIs132[Pmyo-3::GFP-SL2-tagRFP-T] produced by a spontaneous integration event
lijIs133[Punc-47::GCaMP3-SL2-tagRFP-T] line 11 out of 11 integrants
lijIs134[Punc-47::GFP-SL2-tagRFP-T] line 2 out of 20 integrants
lijIs135[Pacr-5::GCaMP3-SL2-tag-RFP-T] line 1 out of 6 integrants
lijIs136[Pacr-5::GFP-SL2-tagRFP-T] line 7 out of 7 integrants
3.7 Mapping the integration site for body wall muscle GCaMP3 construct

3.7.1 Two point mapping for linkage analysis

It was noted that the fluorescent construct \textit{ljIs131[Pmyo-3::GCaMP3-SL2-tagRFP-T]} could not be crossed into strain \textit{unc-8(e15lb145)}. It was hypothesized that this fluorescent construct was integrated onto the same chromosome as the \textit{unc-8} mutation; chromosome IV. This was confirmed by linkage analysis using the strain DA438 \textit{bli-1(e937) I; rol-6(e187) II; daf-2(e1368) vab-7(e1562) III; unc-31(e928) IV; dpy-11(e224) V; lon-2(e678) X}. \textit{Pmyo-3::GCaMP3-SL2-tagRFP-T} males were generated by crossing N2 males with \textit{Pmyo-3::GCaMP3-SL2-tagRFP-T} hermaphrodites. The fluorescent males were then crossed into strain DA438. 12 F1 worms that displayed wildtype locomotion and fluorescence were singled from this cross for 2 days. From among the F2s, 50 worms that displayed the marker for the mutation on each chromosome were picked to a single plate under a light microscope. The numbers of worms displaying fluorescence were then counted. Fluorescence acts as a dominant mutation. For unlinked mutations, this number should be \(\frac{3}{4}\). For linked mutations, the number will be much less. The results were as follows:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Mutation</th>
<th>No. of fluorescent worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Bli</td>
<td>39/59</td>
</tr>
<tr>
<td>II</td>
<td>Rol</td>
<td>53/53</td>
</tr>
<tr>
<td>III</td>
<td>Vab</td>
<td>63/69</td>
</tr>
<tr>
<td>IV</td>
<td>Unc</td>
<td>2/148</td>
</tr>
<tr>
<td>V</td>
<td>Dpy</td>
<td>42/50</td>
</tr>
<tr>
<td>X</td>
<td>Lon</td>
<td>29/52</td>
</tr>
</tbody>
</table>

From the recombination frequency of fluorescent and unc worms (2/148) it was calculated that the site of integration, with a 95% confidence interval, is between 0.08 and 2.46 mu from the chromosome IV marker, \textit{unc-31}.
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

3.7.2 Three point mapping

3 point mapping of the \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} integration site was carried out using the strain DR190, \textit{dpy-13(e184); unc-24(e138)IV}. \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} males were generated by crossing N2 males with \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} hermaphrodites. The fluorescent males were then crossed into strain DR190. 45 F1 worms heterozygous for the \textit{dpy-13} and \textit{unc-24} mutations, and that displayed wildtype locomotion and fluorescence, were singled from this cross over 2 days. From among the F2s, dpy non-unc and unc non-dpy recombinant worms were picked. Of the 82 dpy non-unc worms identified, all were fluorescent. Of the 96 unc non-dpy worms, only 1 worm was fluorescent.

Pooling the results from both types of recombinants gives 177 recombination events occurring between \textit{dpy-13} and the integration site, and 1 recombination event between the integration site and \textit{unc-24}. Therefore, the integration site is \(1/178\) the distance between \textit{unc-24} (at genetic position 3.51 on chromosome IV) and \textit{dpy-13} (at genetic position 0.00 on chromosome IV). \(3.51/178 = 0.0197. 3.51 - 0.0197 = 3.49\) mu. The integration site is at genetic position 3.49 on chromosome IV. The 95% confidence interval for the map distance, based on the binomial distribution, is 3.48 mu to 3.51 mu.

3.8 Crossing of integrated arrays into mutant lines

Mutant lines carrying integrated arrays were generated by first crossing N2 males into each integrated line. Fluorescent males carrying the integrated array were then crossed into mutant lines. 3 F1 worms heterozygous for the mutation and integrated array were singled. F2s worms were singled from these plates if they were fluorescent and displayed either an uncoordinated or fainter phenotype, as appropriate for the mutation of interest. If it was not possible to follow any obvious phenotype, 12 F2 worms were singled and those plates homozygous for the mutation were identified by PCR. Worms then underwent homozygosing for fluorescence.
3.9 Calcium Imaging Setup

3.9.1 Calcium imaging hardware components and configuration

3.9.1.1 Tracking Stage

T-LSR075A Motorized Linear Slides (Zaber Technologies Inc., US) support a 0.5 inch x 18 inch x 12 inch aluminium breadboard stage (custom made by Janelia Farm Instrument Design and Fabrication) and give automated x-y movement (see Figure 3.5). The stage supports an iXon 885 EMCCD calcium imaging camera (Andor Technology Plc., US).

3.9.1.2 Fluorescence Excitation

Fluorescence excitation is provided by two optically pumped semiconductor lasers (see Figure 3.2). A Sapphire™ 488nm laser (Coherent, Inc., Germany) provides excitation for green fluorescence and a Sapphire™ 561nm laser (Coherent, Inc., Germany) provides excitation for red fluorescence. Each laser beam is free-space coupled into a high power fused silica single-mode fibre optic patchcord (QSMJ-3AF, 3A-488-3.5/125-3AS-1 Oz Optics Ltd., US) via a FibrePort Collimator (PAF-X-7A, f = 7.5mm, 350-700nm, Thorlabs, US). Each fibre output is connected to a lens tube (SM1L20, Thorlabs, US) via a fibre adaptor cap (S120-FC, Thorlabs, US). Laser excitation and tracking lighting is delivered to the sample plane using a two dichroic mirror system. The laser excitation is delivered to the sample plane by passing the beams up through the calcium imaging objective lens. A BrightLine® single-edge dichroic mirror (FF510-Di01-25x36, Semrock, Inc.) reflects the 488nm laser and transmits the 561nm laser onto a second BrightLine® single-edge dichroic mirror (FF580-FDi0125x36, Semrock, Inc.). This second dichroic mirror reflects both laser beams onto a pellicle beamsplitter (BP108, uncoated 8:92 R:T, Thorlabs, US) positioned between the tube lens and the 2x objective.
Figure 3.5: Picture of the calcium imaging worm tracker. Tracking hardware is listed to the left of the image and imaging hardware is listed to the right of the image.
3.9.1.3 Fluorescence Emission Detection

Emitted fluorescence is recorded through an MVX Plan Apochromat 2x objective (Olympus America, Inc., working distance 20mm, numerical aperture 0.5). The fluorescence image is split into a green channel and a red channel by an Optosplit II Image Splitter (Cairn Research Ltd., UK) containing a BrightLine® single-edge laser-flat dichroic mirror (Di01-R561-25x36, Semrock, Inc.) that transmits light wavelengths above 561nm. The two channels are each projected onto one half of the EMCCD camera chip. Green emission is filtered at 498nm to 553nm wavelengths by a Brightline® single-band pass filter (FF03-525/50, Semrock, Inc.) and red emission is filtered at 581nm to 619nm wavelengths by a Brightline® single-band pass filter (FF01-600/37-25, Semrock, Inc.). The presence of an additional StopLine® quad-notch filter (NF01-405/488/561/638-25x05.0, Semrock, Inc.) in the green channel light path blocks excitation light from the lasers. 100mm and 250mm focal lenses positioned between the output end of the optic fibre patch cord and dichroic mirrors projects a concentrated laser spot onto the worm at the sample plane. A tube lens (MVX-TLU from MVX10 Macroview Microscope, Olympus America, Inc.) between the Optosplit II Beamsplitter and the 2x objective brings parallel light rays from the 2x objective together at the intermediate image plane. The total magnification produced by the combination of the objective lens and tube lens was calculated as 3.95.

3.9.2 Worm Tracking

A Dino-Lite Pro AM413T USB camera (Dino-Lite Digital Microscopes, Netherlands) is used for automated worm tracking (see Figure 3.6). Tracking lighting is provided by a red LED (M627L1 627nm 500mW, Thorlabs, US) controlled by a T-Cube LED driver (LEDD1B, 0-1200mA 12V, Thorlabs, US) and filtered at 633nm to 647nm by a Brightline® single-band pass filter (FF01-640/14-25, Semrock, Inc.). The same filter is also used to regulate light wavelengths reaching the USB tracking camera. The red light passes through the BrightLine® single-edge dichroic mirror (FF580-FDi0125x36, Semrock, Inc.) that reflects both laser beams, so that all three light beams reach the pellicle membrane and are reflected together onto the sample plane.
Figure 3.6: Example images from the Worm Tracker 2.0 software and the Andor calcium imaging software. An example image from the Worm Tracker 2.0 worm tracking software is shown on the left and an example image from the Andor calcium imaging software is shown on the right. The top image displays the red channel (RFP fluorescence) and the lower image displays the green channel (GCaMP3 fluorescence in experimental worms or GFP fluorescence in control worms).
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

3.9.3 Software

3.9.3.1 Worm tracking Software

Automated tracking of single worms was performed using the Worm Tracker 2.0 Software Version 2.0.3.1 (developed by E. Yemini, MRC LMB, UK, http://www.mrc-lmb.cam.ac.uk/wormtracker/, unpublished).

The following settings were used on the Worm Tracker 2.0 tracking software for tracking:

- Stage speed = 200
- Stage Acceleration = 1
- Centroid boundary bounding box = 5 x 5
- Resolution 640 x 480
- Tracking manual threshold = 80
- Vignette correction on
- Convert to grey-scale
- LED set to 350mA

3.9.3.2 Calcium Imaging Software

Calcium imaging was performed using Andor Solis Imaging Software (Andor Technology Plc., US). Output images from this software were saved as 32-bit .dat files and imported into ImageJ for further processing.

The following settings were used on the Andor Solis Imaging Software during imaging:
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

3.10 Calcium Imaging Assays

Worms for calcium imaging assays were staged as L4s by picking 10 adult worms to a plate. Worms were allowed to lay eggs for 4 hours between 5pm and 9pm and then assayed two days later at L4 stage. True freely moving crawling assays were performed by picking single worms to a 100μl NGM pad. Each pad was allowed to cool and dry for 5 minutes and then a single worm was picked to the pad, tracked and imaged. Crawling assays with worm movement confined in the z-axis were carried out as above, with the worm imaged between the NGM pad and a coverslip. Crawling movement was unhindered with S-shaped dorsal and ventral bends propagating from the head towards the tail over time. Swimming assays were carried out in M9 buffer with 20% dextran w/w (Sigma US 95771), whilst minimizing movement in the z-axis by placing the worm between a microscope slide and a coverslip, with an additional coverslip acting as a spacer. Swimming movement was unhindered with C-shaped dorsal and ventral bends propagating from the head towards the tail over time.

The following laser settings were used for imaging each line:
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

Crawling Assays

<table>
<thead>
<tr>
<th>Strain</th>
<th>Red channel (mW/mm²)</th>
<th>Green channel (mW/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>ljIs132[pmyo-3::GFP-SL2-tagRFP-T]</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>ljIs133[unc-47::GCaMP3-SL2-tagRFP-T]</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>ljIs134[unc-47::GFP-SL2-tagRFP-T]</td>
<td>0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>ljIs135[acr-5::GCaMP3-SL2-tag-RFP-T]</td>
<td>0.07</td>
<td>0.10</td>
</tr>
<tr>
<td>ljIs136[acr-5::GFP-SL2-tagRFP-T]</td>
<td>0.14</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Swimming Assays

<table>
<thead>
<tr>
<th>Strain</th>
<th>Red channel (mW/mm²)</th>
<th>Green channel (mW/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>ljIs132[pmyo-3::GFP-SL2-tagRFP-T]</td>
<td>0.14</td>
<td>0.02</td>
</tr>
</tbody>
</table>

For muscle imaging, an EM gain of 3 was used on the calcium imaging camera. For neuron imaging, an EM gain of 5 was used on the calcium imaging camera.

3.11 Post-processing of calcium image sequences

3.11.1 Checking stability of laser illumination over time and between powers

Laser illumination was shown to be stable over time and between laser powers using a fluorescent dye sample (Alexa Fluor 561nm for the red channel, FITC for the green channel). Mean pixel values were compared from two 300 frame image sequences separated in time, for laser powers between 15mW and 150mW and were found to be comparable.

3.11.2 Fluorescence bleed-through correction

Calculation of the bleed-through of green fluorescence into the red channel with 488nm laser excitation was performed using strain XJ45 kfEx[che-3::GCaMP3] as the fluorescent sample. This strain expresses GCaMP3 in ciliated head neurons. Background-corrected images from the red and green channels were collected at 15mW and 75mW laser powers and the fraction of fluorescence in the red channel compared to the green channel was quantified using ImageJ software. The value of the bleed-through is dependent on the set of
emission filters. For the filter set listed above, the filter bleed-through was calculated as 10.0%. All red channel fluorescence measurements are corrected for this bleed-through by subtracting 10.0% of the fluorescence value from the corresponding image in the green channel.

3.11.3 Checking for saturation of the image

The following custom-written ImageJ script was used to check that the fluorescence signal in the red and green channels did not saturate. This was done by taking a maximum intensity projection of each image sequence, subtracting a value of 15,000 (corresponding to the pixel value range on the Andor camera settings) and setting the minimum pixel value to 0 (as the data is 32-bit floating point).

ImageJ Script to check for saturation of fluorescence signal:

```java
Dialog.create("Load Image Sequence for checking saturation");
Dialog.addNumber("start:", 0);
Dialog.addNumber("stop:", 1000);
Dialog.show();
Starting_Frame = Dialog.getNumber();
Number_of_Frames = Dialog.getNumber();
run("Z Project...", "start=Starting_Frame stop=Number_of_Frames projection=[Max Intensity]");
run("Subtract...", "value=15000");
run("Min...", "value=0");
```

3.11.4 Checking stability of laser illumination on stage movement

Laser illumination was found to shift with tracking movements and so a flat-field correction was applied to each image sequence to correct for this uneven laser illumination across the field of view. A 128-length image sequence of the laser illumination pattern in both channels was recorded using the fluorescent dye samples at the start and end of each imaging session. A background image without a fluorescent sample and with lasers off was also taken. An average intensity projection was performed on the image sequences and the
average illumination pattern before and after experiments was calculated. The following
custom-written ImageJ script calculates the correction images and applies them to the
image sequence. This is done by background correcting the laser illumination pattern in
each channel, measuring the maximum pixel value for each background corrected image
and dividing the background corrected image by this maximum value to give a scaling
image.

**ImageJ script to produce flat-field correction images:**

```java
home = getDirectory("home");
print("home: " + home + "Desktop\Final Laser Illumination Correction Images");
open(home + "Desktop\Final Laser Illumination Correction Images\AVG_Green channel.tif");
open(home + "Desktop\Final Laser Illumination Correction Images\AVG_Red channel.tif");
open(home + "Desktop\Final Laser Illumination Correction Images\AVG_Background.tif");
imageCalculator("Subtract create", "AVG_Green channel.tif", "AVG_Background.tif");
resultGreen = getTitle();
makeRectangle(0, 0, 1004, 501);
run("Crop");
run("Measure");
max = getResult("Max");
//close();
selectWindow("Results");
run("Close");
selectWindow(resultGreen);
print (max);
run("Divide...", "value=" + max);
saveAs("Tiff", "C:\Documents and Settings\labadmin\Desktop\Final Laser Illumination Correction Images\Green Channel Correction Image.tif");
selectWindow("AVG_Green channel.tif");
close();
selectWindow("AVG_Red channel.tif");
imageCalculator("Subtract create", "AVG_Red channel.tif", "AVG_Background.tif");
resultYellow = getTitle();
makeRectangle(0, 501, 1004, 501);
run("Crop");
```

91
The following custom-written ImageJ script applies the flat-field correction to each image of a recording by background correcting the image from each channel, and dividing this background-corrected image by the scaling image from each channel. Images were converted to 16-bit and saved as a .tiff image sequence for quantitative fluorescence analysis.

**ImageJ script to apply flat-field correction to calcium image sequence:**

```java
function zeros(param) {
    number = "" + param;
    while (lengthOf(number)<10) {
        number = "0" + number;
    }
    return number;
}
Dialog.create("Load Image Sequence for making movie");
Dialog.addNumber("Starting Frame:", 0);
Dialog.addNumber("Number of Frames:", 1000);
Dialog.show();
Starting_Frame = Dialog.getNumber();
Number_of_Frames = Dialog.getNumber();
print(Starting_Frame);
print(Number_of_Frames);
home = getDirectory("home");
```
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

print("home: " + home + "Desktop\Shadowing Correction");
setBatchMode(true);
for (i = Starting_Frame; i < Starting_Frame + Number_of_Frames; i++) {
    number = zeros(i);
    title = "acq" + number + ".dat";
    open(home + "Desktop\Shadowing Correction\" + title);
    run("Conversions...", " ");
    run("16-bit");
    open(home + "Desktop\Final Laser Illumination Correction Images\AVG_Background.tif");
    open(home + "Desktop\Final Laser Illumination Correction Images\Combined Green and Red Channel Correction Image.tif");
    imageCalculator("Subtract create 32-bit", "acq" + number + ".dat", "AVG_Background.tif");
    imageCalculator("Divide create 32-bit", "Result of acq" + number + ".dat", "Combined Green and Red Channel Correction Image.tif");
    run("Min...", "value=0");
    run("16-bit");
    saveAs("Tiff", home + "Desktop\Shadow Corrected Image Sequence\acq" + number);
    close();
    close();
    close();
}

3.12 Quantitative Analysis of Calcium Imaging Sequences

3.12.1 Muscle Calcium Imaging Analysis

Muscle calcium imaging analysis was performed using a custom Matlab script written by Rex Kerr (Janelia Farm Research Campus, US). The user inputs the head-tail and dorsal-ventral orientation of the worm. The analysis locates the edges of the worm in both the green and red channels using the “Canny” edge detection algorithm. These edges are used to create a mask over the worm, and this mask is filled to give a solid worm. The mask is used to find a first approximation of the midline of the animal and locate the two ends corresponding to the worm’s head and tail. The bright bands of muscle on each side of the midline are identified and used to fix the position of the midline. A constant-speed spline fit is used to improve the location of the midline. The background around the worm is located, measured
and used for background correction of fluorescence measurements. A bleed-through correction is also performed to correct for bleed-through of green fluorescence into the red channel. The fluorescence ratio is calculated as the background-corrected green fluorescence divided by the background- and bleed-through- corrected red fluorescence.

Pixels corresponding to the muscle of the worm are projected from the outline onto the midline of the worm to give a fluorescence and curvature measurement at a particular point along the worm body (1 = head, 100 = tail). This results in the correction for compression and stretching of the muscle on body bending being inherent in the analysis algorithm. Fluorescence measurements are normalized to the mean fluorescence. Raw curvature values are scaled by a factor of \((x \times 20 + 1)\) to allow easy visual comparison with fluorescence ratio values. Positive (upper peaks) in curvature traces correspond to dorsal body bends and negative (lower peaks) in curvature traces correspond to ventral bends.

### 3.12.2 Neuron Calcium Imaging Analysis

Neuron calcium imaging analysis was performed using a custom Matlab script written by Toufiq Parag (Janelia Farm Research Campus, US). The user inputs the initial location of each neuron to be tracked by clicking on its fluorescent centre in the first frame of the image sequence. A restricted local search for the maximum fluorescence signal around each neuron location is used for tracking each neuron in the subsequent frame. Fluorescence is measured as the sum of the pixel values within the tracked neuron location. The background around the worm is located, measured and used for background correction of fluorescence measurements. A bleed-through correction is also performed to correct for bleed-through of green fluorescence into the red channel. The fluorescence ratio is calculated as the background-corrected green fluorescence divided by the background- and bleed-through- corrected red fluorescence. For each frame, a spline fit to all neuron locations is used to calculate a value for the body curvature at each neuron location.
3.13 Preliminary imaging results from neurons

As of yet, preliminary imaging results from D and B classes of motor neurons have been inconclusive. I briefly present the current state of the work here.

A limited set of data generated from imaging the D class motor neurons in the line \( ljIs133[unc-47::GCaMP3-SL2-tagRFP-T] \) shows some oscillations in the GCaMP3 fluorescence ratio with body curvature, but this is currently not consistent throughout single traces or between multiple worms. Currently no calcium imaging studies have been conducted on the GABAergic D class motor neurons. As the D class motor neurons are postsynaptic to excitatory A and B class motor neurons and their outputs are onto the diametrically opposite muscles, they are proposed to function in a negative feedback loop in which muscle contraction on one side of the body causes inhibition and relaxation on the other side (White, Albertson et al. 1978; McIntire, Jorgensen et al. 1993). As such, D class motor neurons are hypothesized to show oscillatory activity, with VD activity occurring during a dorsal bend and DD activity occurring during a ventral bend. Imaging from VD neurons does show some increase in calcium activity towards the end of a ventral bend and when the body is starting to bend dorsally (see Figure 3.7).

A limited set of control traces from imaging VD neurons in the line \( ljIs134[unc-47::GFP-SL2-tagRFP-T] \) show no such changes in fluorescence with body bending (see Figure 3.8). There is no correlation between curvature and D neuron fluorescence for GCaMP3 experimental worms and GFP experimental worms (mean ± stdev for GCaMP3 worm 1; -0.09 ± 0.15, GCaMP3 worm 2; 0.08 ± 0.16, GFP worm 1; 0.12 ± 0.11, GFP worm 2; 0.04 ± 0.13).
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker
Figure 3.7: Calcium imaging from VD motor neurons during crawling in line AQ2967 \textit{ljls133[unc-47::GCaMP3-SL2-tagRFP-T]}. Traces plot the GCaMP3/RFP fluorescence ratio in blue and body curvature at the point of each neuron in black. Dorsal bends are positive (upper peaks in curvature traces) and ventral bends are negative (lower peaks in curvature traces). Example calcium traces from a \textit{ljls133[unc-47::GCaMP3-SL2-tagRFP-T]} worm, from tracked neurons VD3, VD4 and VD5
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker
Chapter III: Construction of GCaMP3 transgenic lines and calcium imaging worm tracker

Figure 3.8: Calcium imaging from VD and DD motor neurons during crawling in line AQ2968 ljIs134[unc-47::GFP-SL2-tagRFP-T]. Traces plot the GFP/RFP fluorescence ratio in blue and body curvature at the point of each neuron in black. Dorsal bends are positive (upper peaks in curvature traces) and ventral bends are negative (lower peaks in curvature traces). Example calcium traces from a ljIs134[unc-47::GFP-SL2-tagRFP-T] worm, from tracked neurons VD3, DD2 and VD4.

Despite constraining worm movement within the z-axis, the calcium signals so far obtained from the D class motor neurons are noisy and variable. There could be several explanations for this observation. Firstly, as a result of the need to keep the whole worm in the field of view for body wall muscle imaging, the lower magnification of the imaging setup compared to similar setups that have been used to investigate neuron activity during worm locomotion (Ben Arous, Tanizawa et al. 2010; Haspel, O'Donovan et al. 2010; Faumont, Rondeau et al. 2011; Kawano, Po et al. 2011)) could result in low signal-to-noise when trying to image from smaller motor neurons.

Secondly, GCaMP3 expression levels may not be optimal to give a high calcium signal in the motor neurons. It was noted that on construction of some GCaMP3 vectors, there was an incompatibility between the promoter and GCaMP3 expression. GCaMP3 was not successfully expressed in the A class motor neurons using the unc-4 promoter, in the AVA, AVD and AVE backward command interneurons and in the PVC forward command interneuron using the nmr-1 promoter (Brockie, Mellem et al. 2001), in only the DD motor neurons using the flp-13 promoter (Von Stetina, Watson et al. 2007), or in only the VB motor neurons using the ceh-12 promoter (Von Stetina, Fox et al. 2007). It is possible that some neurons are especially sensitive to the expression of calcium sensors, which if too high, can significantly buffer calcium transients (Hires, Tian et al. 2008). As increasing a GECI expression concentration also improves signal-to-noise, the optimal GECI concentration is therefore a balance between signal-to-noise and buffering.

As such, I decided to image AVA interneuron activity in strain QW625 zfIs[prig-3::GCaMP3-SL2-mCherry; lin-15+], a neuronal line in which GCaMP3 fluorescence is known to increase
with higher calcium concentrations during reversals (Mark Alkema and Chris Clark, personal communication). This would give verification that GCaMP3 calcium signals from neurons could be detected with the current magnification and imaging settings of the setup. On my setup I observe increases in GCaMP3 fluorescence during some but not all reversals, with increases in fluorescence also occurring during periods when the worm is moving forward (see Figure 3.9). These results further suggest that it might be possible to detect neuronal calcium signals with the current setup but that the traces collected so far have low signal and significant noise compared to those obtained from imaging body wall muscle. It may be possible to improve the signal-to-noise by modification of the hardware, the calcium imaging assays or the fluorescence analysis, or a combination of these. This is on-going work.
Figure 3.9: Calcium imaging from the AVA interneuron in line QW625 zfls[Prig-3::GCaMP3-SL2-mCherry; lin-15+]. The trace plots the GCaMP3/mCherry ratio over time with periods of backward locomotion highlighted in red.
Chapter IV

Imaging Muscle Activity in Freely Moving Wildtype Worms
4.1 Abstract

During *C. elegans* locomotion, motor neurons activate body wall muscles that in turn determine the posture of the worm. The relationship between body wall muscle activity and worm posture depends on the environment of the worm but the neuromuscular mechanisms that underlie this force-dependent adaptation of locomotion are not yet known. In this study I combine automated worm tracking with GCaMP3 calcium imaging to investigate the pattern of muscle activity in freely-moving wildtype worms during forward and backward crawling and during swimming. I measure the phase-shift of maximal muscle activity with maximal body curvature for both forms of locomotion.

I show that freely-moving worms exhibit movement within the z-axis perpendicular to the imaging plane and that this movement precludes analysis of the calcium signal in the GCaMP3 channel despite co-expression with a reference fluorescent protein.

When movement is constrained within the z-axis but worms are free to crawl forward in the x- and y- axes I show that dorsal muscle calcium signal correlates with dorsal bending, ventral muscle calcium signal correlates with ventral bending and dorsal and ventral muscle activity is out-of-phase, providing evidence for the cross-inhibition model of *C. elegans* locomotion. Maximal muscle activity and maximal body curvature show a small but significant phase-shift, with maximal muscle activity preceding maximal body curvature. Phase-shifts are comparable between dorsal and ventral muscle but the magnitude increases along the body of the worm and is significantly higher in posterior muscle compared to anterior and mid-body muscle. Such a relationship has also been observed for the longitudinal red muscle fibres of many fish species that display anguilliform locomotion similar to *C. elegans*.
The basic relationships between muscle activity and body curvature described above also apply to backward crawling and to swimming locomotion. For swimming, I detected a small but significant phase-shift where maximal curvature actually precedes maximal calcium signal. No significant difference was observed between dorsal and ventral phase-shifts but the phase-shift for anterior muscle was significantly greater than that for mid-body and posterior muscle. The muscle calcium signal is also smaller and is correlated with the shallower body bending and faster kinetics for this form of locomotion.
4.2 Introduction

4.2.1 Crawling and swimming locomotion display distinct kinematics

During *C. elegans* locomotion, motor neurons activate body wall muscles that in turn determine the posture of the worm (White, Southgate et al. 1976; Sternberg and Horvitz 1982). The relationship between body wall muscle activity and worm posture depends on the environment of the worm. When crawling on a solid medium, wildtype *C. elegans* display a sinusoidal S-shaped movement pattern. To move forward worms propagate a backward travelling wave from the head towards the tail, whereas to move backwards worms propagate a forward travelling wave from the tail towards the head. When swimming in liquid of low viscosity, wildtype *C. elegans* display a characteristic C-shaped bending posture.

Previous analysis of crawling and swimming motion has shown that these behaviours are described by unique kinematics (Pierce-Shimomura, Chen et al. 2008; Fang-Yen, Wyart et al. 2010). Swimming is characterized by shallower amplitude, faster frequency and faster speed of wave propagation along the body compared to crawling. For example, measurements on crawling worms show they exhibit a bend wavelength of $0.65 \pm 0.03$ body lengths and a bend frequency of $0.30 \pm 0.02$ Hz, whereas swimming worms exhibit a bend wavelength of $1.54 \pm 0.04$ body lengths and a bend frequency of $1.76 \pm 0.07$ Hz (Fang-Yen, Wyart et al. 2010).

It is possible that such differences in locomotion kinematics could be caused by different underlying neural activities, the forces imposed on the worm by its environment or a combination of both these factors. At the size and speed of a *C. elegans*, the forces due to surface tension that holds the crawling animal to an agar surface are approximately 10,000-fold larger than forces due to viscosity when swimming in water (Sauvage 2007). Therefore, to fully understand worm locomotion it is necessary to understand *C. elegans* locomotory
biomechanics; how muscle activity produces movement within the mechanical limits of the worm’s body and its physical environment.

4.2.2 Theoretical models relating muscle activity to body curvature

The neuromuscular mechanisms that underlie this force-dependent adaptation of locomotion are not yet known. Theoretical models attempting to relate muscle activity to body curvature take into account several different forces acting on the worm during locomotion;

1) The internal pressure of the worm from its hydrostatic skeleton that is directed outwards
2) The forces of the elastic cuticle which resists stretching and act between two points on the same side of the worm
3) The contracting forces generated by the longitudinal body wall muscles acting between two points on the same side of the worm
4) The inertial and frictional forces exerted by the environment on the worm

Inertial forces depend on the worm's mass and acceleration and are typically several orders of magnitude smaller than frictional forces and so can be neglected (Niebur and Erdos 1991). Therefore, to move the worm must exert forces on its environment which equal or exceed the frictional forces exerted by the environment on the worm.

A number of studies (Gray 1953; Gray and Lissmann 1964; Niebur and Erdos 1991) have investigated and modelled locomotion generated by sinusoidal undulations in crawling snakes and nematodes. These studies suggested that, to create a propulsive force, muscles are most active when approaching a region of increasing curvature rather than at the maximal curvature itself (see Figure 4.1). Fully stretched or fully compressed muscles (maximal and minimal body curvature) are least active and activity in the muscle rises until half of the shortening process has occurred and then begins to lower again (Gray 1953).
Hence, this gives an estimated phase-shift between maximal muscle activity and maximal body curvature on the order of $\pi/2$ (i.e. $\lambda/4$) but it could be much smaller.

Figure 4.1: Diagram showing the theoretical prediction of the relationship between maximal muscle activity and maximal body curvature for undulatory locomotion. The widths of the shaded areas indicate the strength of muscle activity. Maximal muscle activity and maximal body curvature are predicted to be phase-shifted by up to $\pi/2$. Modified from (Gray 1953).
Theoretical models based on estimates of the elasticity of the worm cuticle and the resistance of different environments have proposed how muscle activity should relate to body curvature. During crawling, the external forces exerted by the environment on the worm are comparable to those needed to bend the worm body (Fang-Yen, Wyart et al. 2010), and it is predicted that the wave of curvature is phase-shifted with respect to muscle activity, as described above (muscles should be most active before curvature is maximal). However, during swimming the external forces exerted by the environment on the worm are negligible compared to those needed to bend the worm body (Fang-Yen, Wyart et al. 2010), and it is predicted that muscle activity and curvature are proportional (muscles should be most active when curvature is maximal).

4.2.3 Preliminary evidence supporting theoretical models

In preliminary work by Beth Chen, using YC2 (yellow cameleon 2) to image muscle activity during crawling, it was found that as worms displayed one cycle of undulatory locomotion, the observed calcium transients in muscle cells also oscillated through one cycle of high and low concentration (Chen 2007). Waves of muscle activity were consistent with the direction of locomotion; backward travelling waves of muscle activity during forward locomotion and forward travelling waves of muscle activity during backward locomotion. Ventral and dorsal muscle activity was observed to be out-of-phase, with maximal calcium activity occurring just before maximal body curvature (Chen 2007).

To investigate how worm locomotion is affected by changes in the environment it would be necessary to image body wall muscle activity during crawling and swimming and measure any observed phase shift between calcium signal and body curvature. The main challenge with this experiment is that the fluorescence decay time for calcium indicators is not negligible with respect to the period of locomotion of the worm, particularly for swimming. Using the newly developed calcium indicator GCaMP3 may help reduce this problem because its fluorescence decay time (650 ± 230 ms, (Tian, Hires et al. 2009)) is significantly shorter than the other available FRET-based sensors (TNXXL, 1550 ± 640 ms for 10 APs;
D3cpV, 9500 ± 3400 ms for 10APs, (Tian, Hires et al. 2009), YC2.0, 580 ± 470 ms for 40 APs, (Miyawaki, Llopis et al. 1997; Reiff, Ihring et al. 2005)).

Although the fluorescence decay time for YC2.0 is slightly faster than that for GCaMP3, GCaMP3 additionally displays a 3-fold increase in dynamic range and a 1.3-fold increase in calcium affinity (Tian, Hires et al. 2009). Therefore, in this study I use GCaMP3 for calcium imaging with the hope that these improved properties will maximize signal-to-noise detection. I carry out calcium imaging from the body wall muscle throughout the length of wildtype worms whilst tracking the animal’s movement. I compare the relationship between muscle activity and body curvature during crawling and swimming locomotion to test the theoretical model outlined above.
4.3 Methods

4.3.1 Strains

Strains were maintained at 22°C, using standard methods (Brenner 1974). Strains used in this study include:

AQ2953  \(ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV\)
AQ2954  \(ljIs132[pmyo-3::GFP-SL2-tagRFP-T]\)

Construction of the body wall muscle GCaMP3 and GFP constructs and the worm lines were carried out as detailed in Chapter III Methods 3.5.

4.3.2 Calcium Imaging Assays and Muscle Calcium Analysis

Calcium imaging assays and muscle calcium analysis was carried out as detailed in Chapter III Methods 3.10.

4.3.3 Statistical Analysis

Standard statistical tests were performed using Matlab functions. Correlation coefficients were calculated using the Matlab function \texttt{corrcoef}. A value of +1 indicates perfect positive correlation. A value of -1 indicates perfect negative correlation. A one-sample \(t\)-test performed a \(t\)-test of the null hypothesis that data are a random sample from a normal distribution with mean 0 and unknown variance, against the alternative that the mean is not 0. Two-sample \(t\)-tests performed a \(t\)-test of the null hypothesis that two sets of data are independent random samples from normal distributions with equal means and equal but unknown variances, against the alternative that the means are not equal. A one-way analysis of variance (ANOVA) compared the distribution of mean values (one per individual worm) from each population of worms against that of each other population. Each ANOVA
tested the null hypothesis that the mean of the mean values from each population were the same. Multiple comparison tests were then performed to identify which pairs of means were significantly different. A significance level of $\alpha = 0.05$ was used and significant results are indicated with a black star.

**4.3.4 Phase-shift Measurements**

Phase shift measurements were performed in Matlab using the cross-correlation function `xcorr`. The period of the sinusoidal movement was calculated by averaging the distance between adjacent maxima in the cross-correlation sequence. The phase-shift for dorsal muscle activity was calculated as the displacement of the central maxima from a phase lag of 0. The phase-shift for ventral muscle activity was calculated as the displacement of the central minima from a phase lag of 0. A positive phase-shift indicates maximal muscle activity occurring before maximal body curvature. A negative phase-shift indicates maximal muscle activity occurring after maximal body curvature.
4.4 Results

4.4.1 Calcium imaging from the body wall muscle of freely crawling wildtype worms

Wildtype *C. elegans* crawl with an S-shaped sinusoidal movement pattern. To measure muscle activity during worm locomotion I expressed the new calcium indicator GCaMP3 (Tian, Hires et al. 2009) in body wall muscle cells under the *Pmyo-3* promoter (Kuroyanagi, Kobayashi et al. 2006). Since GCaMP3 is not a FRET-based ratiometric calcium sensor, calcium-insensitive RFP was co-expressed with GCaMP3 to act as a reference channel so that fluorescence values could be represented as a GCaMP3/RFP ratio, to correct for any movement artefacts in the z-axis. Control worms expressing calcium-insensitive GFP in place of GCaMP3 were assayed along with experimental worms, to ensure that any observed changes in fluorescence were the result of calcium changes and not due to movement artefacts. Figure 4.2 shows an example of the unprocessed time lapse images captured by the EMCCD camera for these worms crawling on an NGM pad during imaging.
Figure 4.2: Unprocessed time lapse images from the EMCCD camera for \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} worms crawling on an NGM pad. The upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and the lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.034 seconds and shown at 1 second intervals over a total time of 4 seconds.

\textbf{A:} \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} worms; \textbf{B:} \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} worms.
For each crawling worm assayed, I quantified the curvature of the animal’s midline and plotted this for 100 points along the body over the duration of the recording. Example curvature kymographs from a forward crawling experimental worm expressing calcium-sensitive GCaMP3 in body wall muscle and a forward crawling control worm expressing calcium-insensitive GFP in body wall muscle is shown in Figures 4.3A and B. Point 1 corresponds to the tip of the head and point 100 corresponds to the tip of the tail. Red (positive) curvature corresponds to dorsal bending and blue (negative) curvature corresponds to ventral bending. S-shaped dorsal and ventral bends propagate from the head toward the tail over time.

The dorsal and ventral fluorescence ratios (GCaMP3/RFP for experimental worms and GFP/RFP for control worms) are plotted for the 100 points along the body over the duration of the recording (see Figures 4.3C, D, G and H). A high fluorescence ratio, indicating high calcium levels, is plotted in black, and a low fluorescence ratio, indicating low calcium levels, is plotted in white. For GCaMP3 worms, alternation in the fluorescence ratio between lower and higher values (i.e. between black and white) is observed (see Figures 4.3C and G). This effect is less marked in GFP control worms, but some alternation does seem to be present (see Figures 4.3D and H).

The fluorescence ratio is combined with the curvature kymograph to visually assess how calcium activity changes with body curvature for dorsal muscle (see Figures 4.3E and F) and ventral muscle (see Figures 4.3I and J). Bright colour corresponds to a higher fluorescence ratio and pale colour corresponds to a lower fluorescence ratio. For ventral muscle, ventral bends approximately correspond with higher ventral fluorescence, indicated by brighter blue and paler red (see Figure 4.3I). Unexpectedly, this pattern also seems to hold for dorsal muscle (see Figure 4.3E). Dorsal bends do not obviously correspond with higher dorsal fluorescence as there is no brighter red and paler blue as would be expected.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

Body Curvature

Dorsal Fluorescence Ratio

Dorsal Fluorescence Ratio Overlaid on Curvature
Figure 4.3: Example crawling curvature and fluorescence ratio matrices for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* worms. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Curvature kymograph for *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]*;
Plotting dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body (point 25), mid-body (point 50) and posterior body (point 75) shows oscillations in fluorescence ratio with body bending for both experimental and control worms. Example experimental and control traces are shown in Figures 4.4 and 4.5. A weak positive correlation was observed between the dorsal fluorescence ratios and positive (dorsal) body curvature along the length of the worm in experimental animals (mean R value ± stdev for anterior body; 0.30 ± 0.39, mid-body; 0.12 ± 0.52, posterior body; 0.24 ± 0.36). No strong correlation was observed between the dorsal fluorescence ratio and body curvature along the length of the worm in control animals (mean R value ± stdev for anterior body; 0.00 ± 0.29, mid-body; -0.04 ± 0.27, posterior body; -0.04 ± 0.22).

In contrast, a weak negative correlation was observed between ventral fluorescence ratios and positive (dorsal) body curvature along the length of the worm in experimental animals (mean R value ± stdev for anterior body; -0.43 ± 0.30, mid-body; -0.36 ± 0.49, posterior body; -0.40 ± 0.41). No strong correlation was observed between the ventral fluorescence ratio and body curvature along the length of the worm in control animals (mean R value ± stdev for anterior body; -0.04 ± 0.19, mid-body; -0.04 ± 0.28, posterior body; 0.04 ± 0.31). Taken together, these results suggest that there might be a weak GCaMP3 calcium signal in body wall muscle in experimental worms that is not present in control worms expressing calcium-insensitive GFP in body wall muscle.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

Anterior Body

Mid-body

Posterior Body
Figure 4.4: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling worms. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. A: Anterior body for \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]}; B: Anterior Body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; C: Mid-body for \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]}; D: Mid-body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; E: Posterior Body for \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]}; F: Posterior Body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}. 
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

IjIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]  IjIs132[pmyo-3::GFP-SL2-tagRFP-T]

Anterior Body

A

Scatter plot of fluorescence ratio against curvature for anterior body

B

Scatter plot of fluorescence ratio against curvature for anterior body

Mid-body

C

Scatter plot of fluorescence ratio against curvature for mid-body

D

Scatter plot of fluorescence ratio against curvature for mid-body

Posterior Body

E

Scatter plot of fluorescence ratio against curvature for posterior body

F

Scatter plot of fluorescence ratio against curvature for posterior body
Figure 4.5: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljls132[pmyo-3::GFP-SL2-tagRFP-T]* crawling worms. For each frame in a recording, the dorsal and ventral fluorescence ratio values and body curvature are plotted as one point. Blue points indicate dorsal ratio and curvature values and red points indicate ventral ratio and curvature values. 

A: Anterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*.

Locomotion models predict that ventral and dorsal muscle activity should be out-of-phase. However, no consistent correlation was observed between dorsal and ventral muscle calcium activity in experimental worms (mean R value ± stdev for anterior body; 0.37 ± 0.32, mid-body; 0.43 ± 0.35, posterior body; 0.18 ± 0.39, see Figure 4.6). A similar result was observed for control worms (mean R value ± stdev for anterior body; 0.16 ± 0.30, mid-body; 0.21 ± 0.39, posterior body; -0.13 ± 0.31).
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

Anterior Body

A

Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

B

Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

Mid-body

C

Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

D

Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

Posterior Body

E

Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body

F

Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body
Figure 4.6: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljs132[pmyo-3::GFP-SL2-tagRFP-T]* crawling worms. For each frame in a recording, the dorsal and ventral fluorescence ratio values are plotted against each other. A: Anterior body for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljs132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljs132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljs132[pmyo-3::GFP-SL2-tagRFP-T]*.

The magnitude of the changes in the dorsal and ventral muscle fluorescence ratios in control worms was observed to be comparable to that in experimental worms (see Figure 4.4). It is possible that there is significant movement of the worm body in the z-axis perpendicular to the imaging plane during free locomotion, which could distort detection of any true GCaMP3 signal corresponding to changes in calcium concentration within body wall muscle. To investigate whether this was true, I investigated the fluorescence signals in the separate green and red channels in experimental and control worms.

Analysis of the variance of the fluorescence signal in the green and red channel shows that the ventral and dorsal GCaMP3 variances are not significantly different to the corresponding ventral and dorsal RFP variances in experimental worms (mean variance ± stdev for ventral GCaMP3; 0.041 ± 0.025, ventral RFP; 0.030 ± 0.019, dorsal GCaMP3; 0.056 ± 0.042, dorsal RFP; 0.041 ± 0.016, see Figure 4.7). As expected, the ventral and dorsal GFP variances in control worms are also not significantly different to the ventral and dorsal RFP variances (mean variance ± stdev for ventral GFP; 0.043 ± 0.026, ventral RFP; 0.047 ± 0.028, dorsal GFP; 0.034 ± 0.018, dorsal RFP; 0.037 ± 0.022). This result suggests either that GCaMP3 fluorescence is not changing significantly with changes in calcium concentration within body wall muscle cells, or that there could be significant movement artefacts in the z-axis on worm movement that are masking the calcium signal in the GCaMP3 channel. Additionally, as GCaMP3 is not an intrinsic ratiometric calcium indicator, it is also possible that movement
in the z-axis has a large impact on the fluorescence signal and that co-expression with a red reference protein does not adequately compensate for such z-axis movements.

Figure 4.7: Variance for green and red channels in freely crawling \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} worms. The variance of the fluorescence signal is plotted for green and red channels, for ventral and dorsal muscle, for GCaMP3 experimental worms (left) and GFP control worms (right). Each point represents a single recording \( n = 23 \) recordings for experimental worms and \( n = 20 \) recordings for control worms.
To further investigate the possibility that movement in the z-axis on body bending causes artefacts in the fluorescence signal, I investigated whether there was a correlation between the fluorescence in the red reference channel and body curvature during locomotion. RFP is a calcium insensitive protein and so should not display a change in fluorescence on body bending. Therefore, any changes in fluorescence observed in this channel are due to movements in the z-axis; an increase in fluorescence corresponds to a movement toward the imaging camera and a decrease in fluorescence corresponds to a movement away from the imaging camera.

In both experimental and control worms, the ventral RFP fluorescence exhibits a strong positive correlation with positive (dorsal) curvature at the mid-body of the worm (mean R value ± stdev for experimental GCaMP3 worms; 0.79 ± 0.13, control GFP worms; 0.79 ± 0.16, see Figures 4.8, 4.9 and 4.10). In contrast, the dorsal RFP fluorescence exhibits a strong negative correlation with positive (dorsal) curvature at the mid-body of the worm (mean R value ± stdev for experimental GCaMP3 worms; -0.86 ± 0.11, control GFP worms; -0.71 ± 0.23).

GFP is also a calcium insensitive protein and so should not display a change in fluorescence on body bending. However, the ventral GFP fluorescence in control worms displays the same positive relationship to body curvature as ventral RFP (mean R value ± stdev = 0.81 ± 0.13, see Figures 4.9 and 4.10), and the dorsal GFP fluorescence in control worms displays the same negative relationship to body curvature as dorsal RFP (mean R value ± stdev = -0.76 ± 0.22).
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

Anterior Body

A. Ventral GCaMP3 and RFP normalized fluorescence and curvature for anterior body

B. Dorsal GCaMP3 and RFP normalized fluorescence and curvature for anterior body

Mid-body

C. Ventral GCaMP3 and RFP normalized fluorescence and curvature for mid-body

D. Dorsal GCaMP3 and RFP normalized fluorescence and curvature for mid-body

Posterior Body

E. Ventral GCaMP3 and RFP normalized fluorescence and curvature for posterior body

F. Dorsal GCaMP3 and RFP normalized fluorescence and curvature for posterior body
Figure 4.8: Example ventral and dorsal fluorescence signals plotted with body curvature over time for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* crawling worms. Black line plots body curvature, red line plots RFP fluorescence signal and green line plots GCaMP3 fluorescence signal. 

**A:** Ventral fluorescence in anterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **B:** Dorsal fluorescence in anterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **C:** Ventral fluorescence in mid-body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **D:** Dorsal fluorescence in mid-body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **E:** Ventral fluorescence in posterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **F:** Dorsal fluorescence in posterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*.
Figure 4.9: Example ventral and dorsal fluorescence signals plotted with body curvature over time for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling worms. Black line plots body curvature, red line plots RFP fluorescence signal and green line plots GFP fluorescence signal. \textbf{A:} Ventral fluorescence in anterior body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; \textbf{B:} Dorsal fluorescence in anterior body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; \textbf{C:} Ventral fluorescence in mid-body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; \textbf{D:} Dorsal fluorescence in mid-body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; \textbf{E:} Ventral fluorescence in posterior body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}; \textbf{F:} Dorsal fluorescence in posterior body for \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}. 
Figure 4.10: Correlation between green and red fluorescence and body curvature for \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} worms.

The correlation coefficient of the fluorescence signal with body curvature is plotted for green and red channels, for ventral and dorsal muscle, for GCaMP3 experimental worms (left) and GFP control worms (right). Each point represents a single recording, \( n = 23 \) recordings for experimental worms and \( n = 20 \) recordings for control worms.
The fluorescence signals in the GCaMP3 channels of experimental worms weakly follow those of their corresponding RFP channels. Ventral GCaMP3 shows a weak positive correlation with body curvature (mean R value ± stdev = 0.23 ± 0.58, see Figure 4.10) and dorsal GCaMP3 shows a weak negative correlation with body curvature (mean R value ± stdev = -0.58 ± 0.37). These correlations are weaker than those displayed by RFP and GFP and suggest that there may be some calcium signal from the GCaMP3. It is possible that the true change in GCaMP3 fluorescence based on muscle calcium concentration could be masked by z-axis movement artefacts during worm locomotion. However, at the low x2 magnification of the imaging setup it is expected that all fluorescence signal is in the same focal plane and so should not be greatly affected by such movements. To investigate whether z-axis movement masks fluorescent signals, I decided to image worms crawling freely between an NGM pad and coverslip so that movement in the z-axis direction would be minimized.
4.4.2 Calcium imaging from the body wall muscle of freely forward crawling wildtype worms constrained in the z-axis

I re-assayed muscle activity in worms that were freely forward crawling in the x- and y- axes, whilst minimizing movement in the z-axis by placing the worm between an NGM pad and a coverslip. S-shaped crawling movement was unhindered with dorsal and ventral bends propagating from the head towards the tail over time in both experimental worms expressing GCaMP3 in body wall muscle and control worms expressing GFP in body wall muscle (see Figures 4.11 and 4.12).
Figure 4.11: Unprocessed time lapse images from the EMCCD camera for \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} worms crawling on an NGM pad and constrained in z-axis with coverslip. Upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.034 seconds and shown at 1 second intervals over a total time of 4 seconds. \textbf{A: ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]; B: ljls132[pmyo-3::GFP-SL2-tagRFP-T].}

The dorsal and ventral fluorescence ratios for experimental and control worms are shown in Figures 4.12C, D, G and H. For experimental worms, a marked alternation in the fluorescence ratio between higher and lower values (i.e. between black and white) is observed along the entire length of body. This is not observed in control worms. Combining the fluorescence ratio matrices with the curvature kymograph shows that for experimental worms, dorsal bends coincide with higher dorsal fluorescence (bright red stripes on kymograph, see Figure 4.12E) and ventral bends coincide with higher ventral fluorescence (bright blue stripes on kymograph, see Figure 4.12I). This is not observed in control worms.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

*ijs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*

**Body Curvature**

A.

“Kymograph of Body Curvature”

<table>
<thead>
<tr>
<th>Part along body (Tail—Head)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

B.

“Kymograph of Body Curvature”

<table>
<thead>
<tr>
<th>Part along body (Tail—Head)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

**Dorsal Fluorescence Ratio**

C.

“Kymograph of dorsal GCaMP3/RFP ratio”

<table>
<thead>
<tr>
<th>Part along body (Tail—Head)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

D.

“Kymograph of dorsal GFP/RFP ratio”

<table>
<thead>
<tr>
<th>Part along body (Tail—Head)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

**Dorsal Fluorescence Ratio Overlaid on Curvature**

E.

“Dorsal GCaMP3/RFP ratio overlaid on body curvature”

<table>
<thead>
<tr>
<th>Part along body (Tail—Head)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

F.

“Dorsal GFP/RFP ratio overlaid on body curvature”

<table>
<thead>
<tr>
<th>Part along body (Tail—Head)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 4.12: Example crawling curvature and fluorescence ratio matrices for \textit{ljis131[\text{pmyo-3::GCaMP3-SL2-tagRFP-T}]} \textit{and ljis132[\text{pmyo-3::GFP-SL2-tagRFP-T}]} worms constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for \textit{ljis131[\text{pmyo-3::GCaMP3-SL2-tagRFP-T}]; B: Curvature kymograph for}
Plotting dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body (point 25), mid-body (point 50) and posterior body (point 75) shows oscillations in fluorescence ratios corresponding with body bending for experimental worms along their body, but not for control worms (see Figures 4.13 and 4.14).

In experimental worms the dorsal fluorescence ratio shows a strong positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.82 ± 0.06, mid-body; 0.78 ± 0.15, posterior body; 0.79 ± 0.06). In contrast to this, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.04 ± 0.2, mid-body; -0.10 ± 0.22, posterior body; -0.01 ± 0.18).

In experimental worms the ventral fluorescence ratio shows a strong negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.81 ± 0.10, mid-body; -0.77 ± 0.11, posterior body; -0.70 ± 0.20). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.04 ± 0.24, mid-body; -0.16 ± 0.37, posterior body; 0.06 ± 0.16).
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

A

Normalized fluorescence ratios and curvature for anterior body

B

Normalized fluorescence ratios and curvature for anterior body

C

Normalized fluorescence ratios and curvature for mid-body

D

Normalized fluorescence ratios and curvature for mid-body

E

Normalized fluorescence ratios and curvature for posterior body

F

Normalized fluorescence ratios and curvature for posterior body
Figure 4.13: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljis132[pmyo-3::GFP-SL2-tagRFP-T]* crawling worms constrained in z-axis by coverslip. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. A: Anterior body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

N2; ljs[pmyo-3::GCaMP3-SL2-tagRFP-T]  N2; ljs[pmyo-3::GFP-SL2-tagRFP-T]

Anterior Body

A  Scatter plot of fluorescence ratio against curvature for anterior body

Mid-body

C  Scatter plot of fluorescence ratio against curvature for mid-body

Posterior Body

E  Scatter plot of fluorescence ratio against curvature for posterior body
Figure 4.14: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljls132[pmyo-3::GFP-SL2-tagRFP-T]* crawling worms constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values and body curvature are plotted as one point. Blue points indicate dorsal ratio and curvature values and red points indicate ventral ratio and curvature values. A: Anterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*.

Dorsal and ventral muscle activity appears out-of-phase (see Figures 4.14 and 4.15). Quantifying this by calculating the correlation in calcium signal between dorsal and ventral muscles shows that fluorescence ratios display a moderate negative correlation with respect to each other along the body (mean R value ± std dev for anterior body; -0.61 ± 0.15, mid-body; -0.66 ± 0.17, posterior body; -0.52 ± 0.21. In contrast to this, dorsal and ventral fluorescence ratios in control worms display a very weak positive correlation with respect to each other (mean R value ± std dev for anterior body; 0.26 ± 0.12, mid-body; 0.20 ± 0.09, posterior body; 0.17 ± 0.16).

Taken together, these results suggest that dorsal muscle is active during dorsal bends and ventral muscle is active during ventral bends. Furthermore, dorsal muscle activity is out-of-phase with that of ventral muscle activity and these relationships apply along the length of the worm body. As expected, control worms carrying calcium-insensitive GFP in place of GCaMP3 display no change in fluorescence that corresponds with body bending.

In 7 out of 13 experimental worms, the dorsal and ventral fluorescence signals were observed to take a more hyperbolic than linear relationship (see Figure 4.15E). Such a relationship suggests that muscle activity on one side of the worm does not begin to increase until the muscle activity on the opposite side reaches some threshold low value.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

N2; lfs[pmyo-3::GCaMP3-SL2-tagRFP-T]

Anterior Body

A

Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

B

Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

Mid-body

C

Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

D

Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

Posterior Body

E

Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body

F

Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body
Figure 4.15: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* crawling worms constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values are plotted against each other.

A: Anterior body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]*.

Plotting the separate channel fluorescence signals for experimental worms shows flat red RFP signals with body bending, whereas the green GCaMP3 signals oscillate with body bending (see Figure 4.16). In contrast, for control worms both the red RFP and green GFP signals are flat with body bending (see Figure 4.17).

Analysis of the variance of the fluorescence signal in the green and red channel shows that the ventral and dorsal GCaMP3 variances are significantly greater than the corresponding ventral and dorsal RFP variances in experimental worms (mean variance ± stdev for ventral GCaMP3; 0.179 ± 0.058, ventral RFP; 0.011 ± 0.004, dorsal GCaMP3; 0.138 ± 0.068, dorsal RFP; 0.009 ± 0.003, see Figure 4.18). In contrast to this, the ventral and dorsal GFP variances are not significantly different to the ventral and dorsal RFP variances in control worms (mean variance ± stdev for ventral GFP; 0.016 ± 0.005, ventral RFP; 0.011 ± 0.007, dorsal GFP; 0.015 ± 0.008, dorsal RFP 0.012 ± 0.012, see Figure 4.18). This result suggests that GCaMP3 fluorescence is changing significantly with changes in calcium concentration within body wall muscle cells.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

$l/s131[pmyo-3::GCaMP3-SL2-tagRFP-T]$
Figure 4.16: Example ventral and dorsal fluorescence signals plotted with body curvature over time for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* crawling worms. Black line plots body curvature, red line plots RFP fluorescence signal and green line plots GCaMP3 fluorescence signal. **A:** Ventral fluorescence in anterior body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **B:** Dorsal fluorescence in anterior body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **C:** Ventral fluorescence in mid-body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **D:** Dorsal fluorescence in mid-body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **E:** Ventral fluorescence in posterior body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **F:** Dorsal fluorescence in posterior body for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

*ijls*132[pmyo-3::GFP-SL2-tagRFP-T]

**Anterior Body**

(A) Ventral GFP and RFP normalized fluorescence and curvature for anterior body

(B) Dorsal GFP and RFP normalized fluorescence and curvature for anterior body

**Mid-body**

(C) Ventral GFP and RFP normalized fluorescence and curvature for mid-body

(D) Dorsal GFP and RFP normalized fluorescence and curvature for mid-body

**Posterior Body**

(E) Ventral GFP and RFP normalized fluorescence and curvature for posterior body

(F) Dorsal GFP and RFP normalized fluorescence and curvature for posterior body
Figure 4.17: Example ventral and dorsal fluorescence signals plotted with body curvature over time for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T] crawling worms. Black line plots body curvature, red line plots RFP fluorescence signal and green line plots GFP fluorescence signal. \textbf{A: Ventral fluorescence in anterior body for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T]; B: Dorsal fluorescence in anterior body for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T]; C: Ventral fluorescence in mid-body for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T]; D: Dorsal fluorescence in mid-body for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T]; E: Ventral fluorescence in posterior body for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T]; F: Dorsal fluorescence in posterior body for \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T].}

Figure 4.18: Variance for green and red channels in freely crawling \textit{lji}s131[pmyo-3::GCaMP3-SL2-tagRFP-T] and \textit{lji}s132[pmyo-3::GFP-SL2-tagRFP-T] worms. The variance of the fluorescence signal is plotted for green and red channels, for ventral and dorsal muscle, for GCaMP3 experimental worms (left) and GFP control worms (right). Each point represents a single recording, \(n = 12\) for experimental worms and \(n = 15\) for control worms.
Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental and control worms are shown in the tables below (see Figures 4.19 and 4.20).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Min</td>
<td>0.39 ± 0.05</td>
<td>0.42 ± 0.11</td>
<td>0.40 ± 0.12</td>
<td>0.40 ± 0.04</td>
<td>0.40 ± 0.06</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>Max</td>
<td>2.24 ± 0.39</td>
<td>2.36 ± 0.72</td>
<td>2.28 ± 0.44</td>
<td>2.07 ± 0.25</td>
<td>2.23 ± 0.45</td>
<td>2.21 ± 0.41</td>
</tr>
<tr>
<td>Range</td>
<td>1.85 ± 0.41</td>
<td>1.94 ± 0.80</td>
<td>1.88 ± 0.50</td>
<td>1.67 ± 0.24</td>
<td>1.82 ± 0.42</td>
<td>1.80 ± 0.45</td>
</tr>
<tr>
<td>StDev</td>
<td>0.38 ± 0.07</td>
<td>0.38 ± 0.10</td>
<td>0.39 ± 0.10</td>
<td>0.40 ± 0.05</td>
<td>0.40 ± 0.06</td>
<td>0.36 ± 0.07</td>
</tr>
</tbody>
</table>

Figure 4.19: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* worms. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 12 worms.
**ljIs132[pmyo-3::GFP-SL2-tagRFP-T] worms**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dorsal Fluorescence Signal</th>
<th>Ventral Fluorescence Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
<td>Mid-body</td>
</tr>
<tr>
<td>Mean</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Min</td>
<td>0.65 ± 0.08</td>
<td>0.71 ± 0.03</td>
</tr>
<tr>
<td>Max</td>
<td>1.48 ± 0.14</td>
<td>1.48 ± 0.18</td>
</tr>
<tr>
<td>Range</td>
<td>0.84 ± 0.19</td>
<td>0.77 ± 0.19</td>
</tr>
<tr>
<td>StDev</td>
<td>0.12 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

**Figure 4.20: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* worms.** Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 15 worms.
For wildtype worms expressing GCaMP3 in body wall muscle, the normalized and averaged crawling fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.40 \pm 0.08$, a maximum value of $2.23 \pm 0.44$, a range of $1.83 \pm 0.47$ and a standard deviation of $0.38 \pm 0.07$. For control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.66 \pm 0.06$, a maximum value of $1.49 \pm 0.14$, a range of $0.82 \pm 0.17$ and a standard deviation of $0.13 \pm 0.02$.

Experimental worms show a significantly larger range in the fluorescence signal compared to control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. This result is expected as GFP and RFP fluorescence should not change on body curvature with changes in calcium concentration. For experimental worms, there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, or between anterior, mid-body and posterior regions, suggesting that body wall muscle activity is comparable on both sides of the worm and along the length of the worm body.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

4.4.3 Calcium imaging from the body wall muscle of backward crawling wildtype worms

Continuous backward movement for several undulations of body bending was not observed during calcium imaging. To investigate whether the relationships observed between muscle activity and body curvature for forward crawling locomotion apply to backward crawling locomotion, I combined measurements of muscle activity and body curvature for brief periods of backward movement from multiple worms for both experimental and control recordings.

In experimental worms the dorsal fluorescence ratio shows a strong positive correlation with positive (dorsal) curvature along the length of the worm (mean R value for anterior body; 0.74, mid-body; 0.72, posterior body; 0.71, see Figure 4.21). In contrast to this, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value for anterior body; -0.01, mid-body; -0.08, posterior body; -0.10, see Figure 4.21).

In experimental worms the ventral fluorescence ratio shows a strong negative correlation with positive (dorsal) curvature along the length of the worm (mean R value for anterior body; -0.77, mid-body; -0.58, posterior body; -0.65, see Figure 4.21). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value for anterior body; 0.08, mid-body; -0.12, posterior body; 0.00, see Figure 4.21).

Dorsal and ventral muscle activity appears out-of-phase with each other (see Figures 4.21 and 4.22). Quantifying this by calculating the correlation in calcium signal between dorsal and ventral muscles shows that the fluorescence ratios display a moderate negative correlation with respect to each other along the length of the worm body (mean R value for anterior body; -0.48, mid-body; -0.31, posterior body; -0.34). In contrast to this, dorsal and ventral fluorescence ratios in control worms display very weak positive correlation with
respect to each other (mean R value ± stdev for anterior body; 0.13, mid-body; 0.23, posterior body; 0.28).

Taken together, these results suggest that the relationships observed between muscle activity and body curvature for forward crawling locomotion also apply to backward crawling locomotion. Dorsal muscle calcium activity is high during dorsal body bends and ventral muscle calcium activity is high during ventral body bends. Furthermore, dorsal muscle calcium activity is out-of-phase with that of ventral muscle calcium activity and these relationships apply along the length of the worm body. As expected, control worms carrying calcium-insensitive GFP in place of GCaMP3 display no change in fluorescence that corresponds with body bending during backward locomotion.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

\(ijl131[pmyo-3::GCaMP3-SL2-tagRFP-T]\) \(ijl132[pmyo-3::GFP-SL2-tagRFP-T]\)

**Anterior Body**

\(A\) Scatter plot of fluorescence ratio against curvature for anterior body

**Mid-body**

\(C\) Scatter plot of fluorescence ratio against curvature for mid-body

**Posterior Body**

\(E\) Scatter plot of fluorescence ratio against curvature for posterior body
Figure 4.21: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljis132[pmyo-3::GFP-SL2-tagRFP-T]* backward crawling worms constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values and body curvature are plotted as one point. Blue points indicate dorsal ratio and curvature values and red points indicate ventral ratio and curvature values. A: Anterior body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

\[ \text{Image: Scatter plots of dorsal against ventral normalized fluorescence ratio for anterior body:} \]

\[ \text{Image: Scatter plots of dorsal against ventral normalized fluorescence ratio for mid-body:} \]

\[ \text{Image: Scatter plots of dorsal against ventral normalized fluorescence ratio for posterior body:} \]
Figure 4.22: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for \(ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(ljs132[pmyo-3::GFP-SL2-tagRFP-T]\) backward crawling worms constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values are plotted against each other. A: Anterior body for \(ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]\); B: Anterior body for \(ljs132[pmyo-3::GFP-SL2-tagRFP-T]\); C: Mid-body for \(ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]\); D: Mid-body for \(ljs132[pmyo-3::GFP-SL2-tagRFP-T]\); E: Posterior body for \(ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]\); F: Posterior body for \(ljs132[pmyo-3::GFP-SL2-tagRFP-T]\).
4.4.4 Measuring the phase-shift between muscle activity and body curvature during crawling

Cross-correlations were measured between muscle activity and body curvature for anterior, mid-body and posterior body during forward crawling locomotion. The phase-shift for dorsal muscle activity was calculated as the displacement of the central maxima from a phase lag of 0. The phase-shift for ventral muscle activity was calculated as the displacement of the central minima from a phase lag of 0. A positive phase-shift indicates maximal muscle activity occurring before maximal body curvature. A negative phase-shift indicates maximal muscle activity occurring after maximal body curvature.

Figure 4.23 plots the average period of sinusoidal body curvature against the measured phase-shift for \textit{ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} worms. Cross-correlation analysis measured the mean period of sinusoidal body curvature as 3.09 ± 0.79 seconds with a mean phase shift between maximal muscle activity and maximal body curvature of +0.06 ± 0.14 seconds (+0.12 ± 0.30 radians). The values for measured phase shifts are small but the distribution is significantly different to 0 (p < 0.0001). Correcting each phase-shift value for the period of sinusoidal body curvature shows that the magnitude of the phase-shift is independent of the oscillation period (R = -0.11). This result suggests that maximal muscle activity does precede maximal body curvature by a small but significant time.

There was no significant difference between dorsal and ventral periods of sinusoidal body curvature and measured phase-shifts (mean ± stdev for dorsal period; 3.13 ± 0.82 seconds, ventral period; 3.04 ± 0.78 seconds, dorsal phase-shift; 0.04 ± 0.14 seconds, ventral phase-shift; 0.08 ± 0.13 seconds). There were no significant differences between anterior, mid-body and posterior periods of sinusoidal body curvature (mean ± stdev for anterior; 3.23 ± 0.80 seconds, mid-body; 3.02 ± 0.69 seconds, posterior; 3.01 ± 0.89 seconds, see Figure 4.24A). However, the phase-shift for posterior muscle was significantly greater than that for anterior and mid-body muscle (mean ± stdev for anterior; 0.03 ± 0.11 seconds, mid-body; 0.04 ± 0.15 seconds, posterior; 0.11 ± 0.15 seconds, see Figure 4.24B).
Figure 4.23: Scatter plot of phase-shift between maximal muscle activity and maximal body curvature for *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* crawling worms. Measurements combined for dorsal, ventral, anterior, mid-body and posterior body wall muscle, n = 96 measurements.
Figure 4.24: Phase shift and bending period measurements along the body for crawling worms. 32 measurements taken at each position along the worm body. A: Phase shift; B: Bending Period.
4.4.5 Calcium imaging from the body wall muscle of freely swimming wildtype worms constrained in the z-axis

I assayed muscle activity in swimming worms whilst minimizing movement in the z-axis by placing the worm between a microscope slide and a coverslip. Swimming movement was unhindered with C-shaped dorsal and ventral bends propagating from the head towards the tail over time in both experimental and control worms (see Figure 4.25). The rate of bend propagation along the body was faster for swimming than for crawling, indicated by the steeper gradient of dorsal and ventral bends on the curvature kymograph (see Figure 4.26).

The dorsal and ventral fluorescence ratios for experimental and control worms are shown in Figures 4.26C, D, G and H. For experimental worms, alternation in the fluorescence ratio between higher and lower values (i.e. between black and white) is observed along the entire length of body, although this is not as marked as in crawling animals. This is not observed in control worms. Combining the fluorescence ratio matrices with the curvature kymograph shows that for experimental worms, dorsal bends coincide with higher dorsal fluorescence (bright red stripes on kymograph, see Figure 4.26E) and ventral bends coincide with higher ventral fluorescence (bright blue stripes on kymograph, see Figure 4.26I). This is not observed in control worms.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

A

\[ l[ls131\mathcal{P}myo-3::GCaMP3-SL2-tagRFP-T] \]

Green Channel:
GCaMP3

Red Channel:
RFP

0.00s 0.05s 0.10s 0.15s 0.20s

0.25s 0.30s 0.35s 0.40s 0.45s

B

\[ l[ls132\mathcal{P}myo-3::GFP-SL2-tagRFP-T] \]

Green Channel:
GFP

Red Channel:
RFP

0.00s 0.05s 0.10s 0.15s 0.20s

0.25s 0.30s 0.35s 0.40s 0.45s
Figure 4.25: Unprocessed time lapse images from the EMCCD camera for \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} swimming worms and constrained in z-axis with coverslip. Upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.01 seconds and shown at 0.05 second intervals over a total time of 0.5 seconds. \textbf{A:} \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]}; \textbf{B:} \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]}. 
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

Body Curvature

Dorsal Fluorescence Ratio

Dorsal Fluorescence Ratio Overlaid on Curvature
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

Figure 4.26: Example curvature and fluorescence ratio matrices for *Ijs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *Ijs132[pmyo-3::GFP-SL2-tagRFP-T]* swimming worms constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for *Ijs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Curvature
Plotting dorsal and ventral fluorescence ratios with corresponding body curvature over time shows oscillations in fluorescence ratios corresponding with body bending for experimental worms, but not for control worms (see Figure 4.27). Oscillations in the fluorescence signal are more evident at the mid-body and posterior body of the worm compared to the head, likely due to the higher degree of motion of the head in all three axes causing correspondingly larger movement artefacts.

Analysis of the variance of the fluorescence signal in the green and red channel for swimming worms shows that the ventral and dorsal GCaMP3 variances are significantly greater than the corresponding ventral and dorsal RFP variances in experimental worms (mean variance ± stdev for ventral GCaMP3; 0.028 ± 0.010, ventral RFP; 0.011 ± 0.006, dorsal GCaMP3; 0.027 ± 0.019, dorsal RFP; 0.012 ± 0.012, see Figure 4.28). However, the magnitude of the variance in the GCaMP3 channels for swimming worms is smaller than the magnitude of the variance in the GCaMP3 channels for crawling worms (6.4 times smaller for ventral GCaMP3 fluorescence and 5.1 times smaller for dorsal GCaMP3 fluorescence).

The ventral and dorsal GFP variances are not significantly different to the ventral and dorsal RFP variances in control worms and they are comparable in size to the variance of the reference RFP channel in experimental worms (mean variance ± stdev for ventral GFP; 0.014 ± 0.005, ventral RFP; 0.012 ± 0.005, dorsal GFP; 0.013 ± 0.004, dorsal RFP 0.011 ± 0.006). Taken together, these results suggest that the GCaMP3 fluorescence is changing significantly with changes in calcium concentration within body wall muscle cells during swimming, but that the changes in calcium concentration are less than that observed during crawling locomotion.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

**Anterior Body**

![Graph A](image1)

A. Normalized fluorescence ratio and curvature for anterior body

**Mid-body**

![Graph C](image2)

C. Normalized fluorescence ratio and curvature for mid-body

**Posterior Body**

![Graph E](image3)

E. Normalized fluorescence ratio and curvature for posterior body

---

166
Figure 4.27: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljls132[pmyo-3::GFP-SL2-tagRFP-T]* swimming worms constrained in z-axis by coverslip. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. A: Anterior body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljls132[pmyo-3::GFP-SL2-tagRFP-T]*.

Figure 4.28: Variance for green and red channels in freely swimming *ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljls132[pmyo-3::GFP-SL2-tagRFP-T]* worms. The variance of the fluorescence signal is plotted for green and red channels, for ventral and dorsal muscle, for GCaMP3 experimental worms (left) and GFP control worms (right). Each point represents a single recording, n = 20 recordings for experimental worms and n = 20 recordings for control worms.
In experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.42 ± 0.18, mid-body; 0.58 ± 0.13, posterior body; 0.59 ± 0.11, see Figure 4.29). In contrast to this, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.04 ± 0.26, mid-body; -0.09 ± 0.18, posterior body; -0.06 ± 0.22).

In experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.44 ± 0.15, mid-body; -0.59 ± 0.15, posterior body; -0.57 ± 0.19). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.04 ± 0.24, mid-body; -0.05 ± 0.17, posterior body; 0.04 ± 0.14).

Whilst dorsal and ventral muscle activity display weak negative correlation with respect to each other (mean R value ± stdev for anterior body; -0.16 ± 0.26, mid-body; -0.34 ± 0.16, posterior body; -0.42 ± 0.14, see Figure 4.30) this is not significantly different from control worms. Dorsal and ventral fluorescence ratios in control worms display no correlation with respect to each other (mean R value ± stdev for anterior body; 0.20 ± 0.19, mid-body; 0.14 ± 0.11, posterior body; 0.13 ± 0.11). Taken together, these results suggest that muscle activity and body curvature display the same relationships to each other during swimming as they do during crawling.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

 ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]  ljls132[pmyo-3::GFP-SL2-tagRFP-T]

Anterior Body

A  Scatter plot of fluorescence ratio against curvature for anterior body

B  Scatter plot of fluorescence ratio against curvature for anterior body

Mid-body

C  Scatter plot of fluorescence ratio against curvature for mid-body

D  Scatter plot of fluorescence ratio against curvature for mid-body

Posterior Body

E  Scatter plot of fluorescence ratio against curvature for posterior body

F  Scatter plot of fluorescence ratio against curvature for posterior body
Figure 4.29: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljis132[pmyo-3::GFP-SL2-tagRFP-T]* swimming worms constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values and body curvature are plotted as one point. Blue points indicate dorsal ratio and curvature values and red points indicate ventral ratio and curvature values. A: Anterior body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Anterior Body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Mid-body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Mid-body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Posterior Body for *ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Posterior Body for *ljis132[pmyo-3::GFP-SL2-tagRFP-T]*.
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

\[ \text{ijis}[\text{pmyo-3::GCaMP3-SL2-tagRFP-T}] \quad \text{ijis}[\text{pmyo-3::GFP-SL2-tagRFP-T}] \]

Anterior Body

\[ \text{A} \quad \text{B} \]

Mid-body

\[ \text{C} \quad \text{D} \]

Posterior Body

\[ \text{E} \quad \text{F} \]
Figure 4.30: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljs132[pmyo-3::GFP-SL2-tagRFP-T]* swimming worms constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values are plotted against each other. **A**: Anterior body for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **B**: Anterior Body for *ljs132[pmyo-3::GFP-SL2-tagRFP-T]*; **C**: Mid-body for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **D**: Mid-body for *ljs132[pmyo-3::GFP-SL2-tagRFP-T]*; **E**: Posterior Body for *ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; **F**: Posterior Body for *ljs132[pmyo-3::GFP-SL2-tagRFP-T]*.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental and control worms are shown in the tables below (see Figures 4.31 and 4.32).
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

### *lJls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* worms

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dorsal Fluorescence Signal</th>
<th>Ventral Fluorescence Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
<td>Mid-body</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Min</strong></td>
<td>0.65 ± 0.06</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td><strong>Max</strong></td>
<td>1.42 ± 0.12</td>
<td>1.48 ± 0.14</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0.78 ± 0.16</td>
<td>0.85 ± 0.18</td>
</tr>
<tr>
<td><strong>StDev</strong></td>
<td>0.15 ± 0.03</td>
<td>0.17 ± 0.03</td>
</tr>
</tbody>
</table>

**Figure 4.31:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming *lJls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* worms. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 20 recordings from 12 worms.
### Dorsal Fluorescence Signal

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Min</td>
<td>0.71 ± 0.06</td>
<td>0.70 ± 0.06</td>
<td>0.71 ± 0.05</td>
<td>0.72 ± 0.05</td>
<td>0.69 ± 0.06</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>Max</td>
<td>1.42 ± 0.12</td>
<td>1.42 ± 0.12</td>
<td>1.41 ± 0.16</td>
<td>1.39 ± 0.10</td>
<td>1.48 ± 0.19</td>
<td>1.49 ± 0.17</td>
</tr>
<tr>
<td>Range</td>
<td>0.71 ± 0.17</td>
<td>0.72 ± 0.17</td>
<td>0.70 ± 0.19</td>
<td>0.67 ± 0.12</td>
<td>0.78 ± 0.23</td>
<td>0.79 ± 0.19</td>
</tr>
<tr>
<td>StDev</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.02</td>
</tr>
</tbody>
</table>

### Ventral Fluorescence Signal

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Min</td>
<td>0.71 ± 0.06</td>
<td>0.70 ± 0.06</td>
<td>0.71 ± 0.05</td>
<td>0.72 ± 0.05</td>
<td>0.69 ± 0.06</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>Max</td>
<td>1.42 ± 0.12</td>
<td>1.42 ± 0.12</td>
<td>1.41 ± 0.16</td>
<td>1.39 ± 0.10</td>
<td>1.48 ± 0.19</td>
<td>1.49 ± 0.17</td>
</tr>
<tr>
<td>Range</td>
<td>0.71 ± 0.17</td>
<td>0.72 ± 0.17</td>
<td>0.70 ± 0.19</td>
<td>0.67 ± 0.12</td>
<td>0.78 ± 0.23</td>
<td>0.79 ± 0.19</td>
</tr>
<tr>
<td>StDev</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 4.32: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} worms. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, \( n = 20 \) recordings from 12 worms.
For wildtype worms expressing GCaMP3 in body wall muscle, the normalized and averaged swimming fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.64 ± 0.07, a maximum value of 1.50 ± 0.15, a range of 0.86 ± 0.19 and a standard deviation of 0.16 ± 0.03. For control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.70 ± 0.06, a maximum value of 1.43 ± 0.14, a range of 0.73 ± 0.18 and a standard deviation of 0.12 ± 0.02.

Experimental swimming worms show a significantly larger range in the fluorescence signal, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal when compared to control worms. For experimental swimming worms, there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, or between anterior, mid-body and posterior regions, suggesting that body wall muscle activity is comparable on both sides of the worm and along the length of the worm body.

This result is similar to that for crawling worms, although the magnitude of the differences between experimental worms and control worms is smaller for swimming than for crawling. Taking these results together with the lower variances displayed in the GCaMP3 channels, suggests that the changes in calcium concentration in body wall muscle during swimming are less than that observed during crawling locomotion, and this correlates with the shallower C-shaped body bending and faster kinetics of swimming locomotion. However, the fluorescence decay time for GCaMP3 of approximately 600ms is comparable to the bending period during swimming, and hence the time during which calcium concentration should oscillate between maximum and minimum in the body wall muscle. As such, a lower variance in GCaMP3 fluorescence would also be observed if oscillations in calcium concentration are too fast to be detected by GCaMP3 and GCaMP3 fluorescence fails to return to baseline during each bending cycle.
4.4.6 Measuring the phase-shift between muscle activity and body curvature during swimming

Cross-correlations were measured between muscle activity and body curvature for anterior, mid-body and posterior body during swimming locomotion. As for crawling, the phase-shift for dorsal muscle activity was calculated as the displacement of the central maxima from a phase lag of 0. The phase-shift for ventral muscle activity was calculated as the displacement of the central minima from a phase lag of 0. A positive phase-shift indicates maximal muscle activity occurring before maximal body curvature. A negative phase-shift indicates maximal muscle activity occurring after maximal body curvature.

Figure 4.33 plots the average period of sinusoidal body curvature against the measured phase-shift for *C. elegans* worms. Cross-correlation analysis measured the mean period of sinusoidal body curvature as 0.98 ± 0.12 seconds with a mean phase shift between maximal muscle activity and maximal body curvature of -0.06 ± 0.10 seconds (-0.37 ± 0.66 radians). The values for measured phase shifts are small but the distribution is significantly different to 0 (p < 0.0001). Correcting each phase-shift value for the period of sinusoidal body curvature shows that the magnitude of the phase-shift is independent of the oscillation period (R = 0.0484). This result suggests that for swimming maximal muscle activity could lag behind maximal body curvature by a small but significant time. However, as discussed above, it is possible that the GCaMP3 sensor has kinetics that are too slow to detect the faster calcium oscillations during swimming. Furthermore, there is a delay between peak muscle calcium influx and peak GCaMP3 fluorescence which could underestimate the magnitude of the phase-shift and so place a true phase shift closer to 0.

There was no significant difference between dorsal and ventral periods of sinusoidal body curvature and measured phase-shifts (mean ± stdev for dorsal period; 0.99 ± 0.13 seconds, ventral period; 0.97 ± 0.12 seconds, dorsal phase-shift; -0.06 ± 0.08 seconds, ventral phase-shift; -0.06 ± 0.11 seconds). There were no significant differences between anterior, mid-body and posterior periods of sinusoidal body curvature (mean ± stdev for anterior; 0.98 ±
0.13 seconds, mid-body; 0.99 ± 0.12 seconds, posterior; 0.98 ± 0.12 seconds, see Figure 4.34A). However, the phase-shift for anterior muscle was significantly greater than that for mid-body and posterior muscle (mean ± stdev for anterior; -0.12 ± 0.08 seconds, mid-body; -0.03 ± 0.05 seconds, posterior; -0.02 ± 0.11 seconds, see Figure 4.34B).

![Scatter plot of phase shift between maximal calcium activity and maximal body curvature for body wall muscle](image)

**Figure 4.33**: Scatter plot of phase-shift between maximal muscle activity and maximal body curvature for *ljls131[myo-3::GCaMP3-SL2-tagRFP-T]* swimming worms. Measurements combined for dorsal, ventral, anterior, mid-body and posterior body wall muscle, n = 120 measurements.
Figure 4.34: Phase shift and bending period measurements along the body for swimming worms. 40 measurements taken at each position along the worm body. 

A: Phase shift; B: Bending Period.
4.5 Discussion

In this study I combine automated worm tracking with GCaMP3 calcium imaging to investigate the pattern of muscle activity in freely-moving wildtype worms during forward and backward crawling and during swimming.

4.5.1 GCaMP3 as a non-ratiometric calcium indicator in freely moving worms

I investigated the relationship between muscle activity and body curvature in worms freely-moving in x-, y- and z-axes. I showed that there was a strong correlation between the fluorescence in the calcium-insensitive RFP reference channel and body curvature during locomotion. Furthermore, there was a low variance for the fluorescence signal in the GCaMP3 channel, which weakly follows that of the corresponding RFP channel. I suggested that there might be significant movement of the worm body in the z-axis perpendicular to the imaging plane during free locomotion that prevents detection of the true GCaMP3 signal corresponding to changes in calcium concentration within body wall muscle. In support of this, minimizing movement within the z-axis by placing a coverslip over the worm removes the strong correlation between the fluorescence in the RFP reference channel and body curvature and allows detection of oscillations in GCaMP3 fluorescence on body bending. However, worms that are freely-crawling are likely restrained to the agar pad by a thin layer of water. It is possible that the refractive index at this water-air boundary is changing as the layer of water gets thinner and thicker during motion. As such the fluorescence would be diffracted by different amounts and this could also contribute to the observed fluorescence artefacts.

The results suggest that GCaMP3, as a non-ratiometric calcium indicator, is somewhat sensitive to movement artefacts within the z-axis. Moreover, using the ratio of the GCaMP3 signal to an RFP signal produced by a co-expressed RFP reference protein does not adequately compensate for such artefacts in true free-moving worms. For studies involving free-moving animals where significant z-axis movement is anticipated, the use of a
ratiometric calcium indicator would likely be preferred due to their lower sensitivity to movement artefacts and changes in focus. The results further highlight the importance of co-expressing non-ratiometric calcium indicators with a calcium insensitive reference fluorescent protein to allow for ratiometric measurements and the importance of performing control experiments using a calcium-insensitive fluorescent protein so that true calcium signals can be verified.

### 4.5.2 Calcium dynamics and muscle activity

On choosing which GECI to use, it is essential to evaluate a number of factors (Palmer and Tsien 2006; Tian, Hires et al. 2009). Often it is desirable to pick the indicator with the greatest dynamic range so that small rises in calcium concentration will be easily detectable over noise. Compared to other available FRET-based sensors, GCaMP3 displays much greater increases in fluorescence intensity on calcium changes in vitro (GCaMP3; 480 ± 50% for 10 APs (Tian, Hires et al. 2009), D3cpV; maximum fluorescence change 190%, TN-XXL maximum fluorescence change 150% (Hendel, Mank et al. 2008). A second consideration is to pick an indicator with an apparent affinity ($K_d$) for calcium that is appropriate for the changes in calcium concentration to be measured. A lower $K_d$ indicates a higher affinity for calcium. GCaMP3 has a lower $K_d$ than available FRET-based sensors ($K_d$ for GCaMP3; 540nM (Zhao, Araki et al. 2011), D3cpV; 660nM (Hendel, Mank et al. 2008) TN-XXL; 800nM (Mank, Santos et al. 2008). These two factors were the major reason to use GCaMP3 as the calcium sensor in this study.

A third consideration for the measurement of rapid neuronal calcium dynamics is to choose a sensor with fast response kinetics. The mean fluorescence rise times for GCaMP3, TN-XXL and D3cpV are similar (GCaMP3; 95 ± 27 ms, TN-XXL; 80 ± 18 ms, and D3cpV; 108 ± 26 ms, (Tian, Hires et al. 2009)). However, the fluorescence decay time of GCaMP3 (650 ± 230 ms) is significantly shorter than the FRET-based calcium indicators (TN-XXL; 1550 ± 640 ms, D3cpV; 9500 ± 3400 ms, (Tian, Hires et al. 2009)). These three factors make GCaMP3 a suitable choice of calcium indicator for this study. Although the fluorescence decay time for
Chapter IV: Imaging Muscle Activity in Freely Moving Wildtype Worms

YC2.0 is marginally faster than that for GCaMP3, GCaMP3 additionally displays a 3-fold increase in dynamic range and a 1.3-fold increase in calcium affinity (Tian, Hires et al. 2009). It was hoped that these improved properties would maximize signal-to-noise detection, and GCaMP3 did display over 5-fold change in fluorescence in body wall muscle during crawling.

Despite the improved kinetics of GCaMP3, the fluorescence response of GECIs remains slower relative to the rapid calcium transients that accompany channel openings and cell depolarization (Llinas 1982; Kotlikoff 2007). Actual cellular calcium influx therefore precedes observed changes in GCaMP3 fluorescence. As such, phase shift measurements between peak calcium signals and peak body curvature will be underestimated and larger than measured, though unlikely to reach the $\pi/4$ radians predicted by theoretical models.

4.5.3 Muscle activity in crawling worms

I investigated the relationship between muscle activity and body curvature in worms freely-crawling in x- and y- axes but constrained within the z-axis. I showed that dorsal muscle calcium activity is high during dorsal body bends and ventral muscle calcium activity is high during ventral body bends. Furthermore, dorsal muscle calcium activity is out-of-phase with that of ventral muscle calcium activity and these relationships apply along the length of the worm body and for both forward and backward movement. This observed pattern of muscle activity is consistent with preliminary results observed by Beth Chen (Chen 2007) PhD Thesis and strongly supports the cross-inhibition model of *C. elegans* locomotion, where dorsal and ventral muscle display alternating activities due to the inhibition and relaxation of muscles on one side of the body when the opposite side is active and contracted.

I investigated the relationship between maximal muscle activity and maximal body curvature. Cross-correlation analysis showed that maximal muscle activity does precede maximal body curvature by a small but significant time and that the magnitude of the phase-shift is independent of the oscillation period. This result agrees with previous studies where peak muscle activity either coincides with or slightly precedes peak body curvature.
but where phase-shift measurements were not taken (Chen 2007; Pierce-Shimomura, Chen et al. 2008).

The measured phase-shift is significantly smaller than that predicted by theoretical models (+0.12 ± 0.30 radians compared to \(\pi/2\) (1.57) radians). Such a small phase shift suggests that increasing muscle curvature requires increasing muscle activity. No significant difference was observed between dorsal and ventral phase-shifts, but the phase-shift for posterior muscle was significantly greater than that for anterior and mid-body muscle, suggesting that anterior muscles tend to be turned on later in their muscle contraction cycle than more posteriorly located muscle. Interestingly, this relationship has been observed for the longitudinal red muscle fibres of many fish species with a variety of body forms and swimming modes, including eels (*Anguilla rostrata* (Gillis 1998)) and swimming snakes (*Nerodia fasciata* and *Elaphe obsolete* (Jayne 1988)) that have an elongated body and display anguilliform locomotion similar to *C. elegans*. The relative timing of muscle activity to its contraction cycle is thought to shift along the body because the travelling wave of muscle activation travels faster than the mechanical wave of bending that it creates. This result suggests that such a longitudinal phase-shift in muscle activity could be a general feature of axial-based undulatory locomotion.

How such a phase-shift relates to muscle function along the body is not clear, but posterior muscle is also observed to display greater length changes and higher power production on contraction (Rome, Swank et al. 1993; Johnson, Syme et al. 1994; Jayne and Lauder 1995) and this has lead to the hypothesis that undulatory locomotion could be powered mainly *via* posterior musculature (Rome, Swank et al. 1993). An alternative hypothesis is that turning on posterior muscle earlier in the muscle contraction cycle acts to stiffen the posterior muscle allowing it to act as a “steering rudder”.
4.5.3 Muscle activity in swimming worms

I investigated the relationship between muscle activity and body curvature in swimming worms. I showed that the same relationships between muscle activity and curvature apply to swimming worms as for crawling worms; dorsal muscle calcium activity is high during dorsal body bends and ventral muscle calcium activity is high during ventral body bends. Dorsal and ventral muscle activity display weak negative correlation with respect to each other. Muscle activity propagates much faster along the body for swimming than for crawling and so body bending reflects the pattern of muscle activity in these two forms of locomotion.

The changes in the calcium concentration observed in body wall muscle during swimming are smaller than those observed during crawling (2.3-fold change in fluorescence signal with body bending on swimming compared to 5.6-fold on crawling). There are several possible explanations for this. It is possible that the faster kinetics and the lower body curvatures assumed during the swimming motion do not require the larger changes in calcium concentration as observed during crawling. In a less viscous environment, such as swimming in liquid, the forces exerted on the worm by the environment are smaller than when the worm is in a more viscous environment or when crawling. Under such conditions, the body wall muscles do not need to generate as much force to bend the worm body and so lower changes in calcium concentration may be required. To test this hypothesis, swimming worms could be imaged in environments of increasing viscosity and the magnitude of the body wall muscle calcium signal measured. It would be predicted that the magnitude of the calcium signal would increase with increasing viscosity.

It is also possible that the off kinetics of GCaMP3 are too slow to detect the oscillations in calcium concentration during swimming. The fluorescence decay time for GCaMP3 is approximately 600ms, which is comparable to the 980ms bending period during swimming, and hence the time during which calcium concentration should oscillate between maximum
and minimum in the body wall muscle. A lower variance in GCaMP3 fluorescence would then be explained if the fluorescence fails to return to baseline during each bending cycle.

No phase-shift between muscle activity and body curvature is predicted by theoretical models. Instead I detected a phase-shift that is small but significantly different to 0 (-0.37 ± 0.66 radians) and that suggests maximal curvature actually precedes maximal calcium signal. No significant difference was observed between dorsal and ventral phase-shifts but the phase-shift for anterior muscle was significantly greater than that for mid-body and posterior muscle. A possible explanation for this is that there is a delay between peak muscle calcium influx and peak GCaMP3 fluorescence which could result in underestimation of the magnitude of the phase-shift and so place a true phase-shift value for swimming closer to 0. A way to measure this delay would be to stimulate channelrhodopsin (a light-activated cation channel) expressed in body wall muscle and calculate the time between stimulation and the peak of GCaMP3 fluorescence. However, this experiment has some technical challenges as the excitation wavelengths for channelrhodopsin (at 473nm) and GCaMP3 (at 488nm) overlap significantly. One possible way around this is to try exciting channelrhodopsin at a high laser intensity and recording GCaMP3 fluorescence at lower laser intensity.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

Chapter V

Imaging Muscle Activity in Locomotion Mutants
5.1 Abstract

In this study, I combine automated worm tracking and GCaMP3 calcium imaging to investigate how mutations in genes affecting the function of the locomotion circuit change the pattern of muscle activity to generate a locomotion phenotype. I investigate the pattern of muscle activity that is responsible for the fainting phenotype observed in nca-1(gk9);nca-2(gk5), unc-79(e1068) and unc-80(e1069) mutant worms during crawling and swimming locomotion, as well as the pattern of muscle activity that results from motor neuron mis-wiring in vab-7(e1562), unc-4(e120) and unc-55(e402) mutant worms during crawling locomotion.

I show that the basic relationships between muscle activity and body curvature described for wildtype worms also apply to all locomotion mutants: dorsal muscle calcium signal correlates with dorsal bending, ventral muscle calcium signal correlates with ventral bending, and dorsal and ventral muscle display reciprocal activity. Dorsal and ventral muscle activity frequently display a strong hyperbolic relationship to each other suggesting that muscle activity on one side of the worm body does not increase until the muscle activity on the opposite side has decreased to very near its minimum value. This appears to be a shared feature of muscle calcium signal between strains that exhibit slow movement and significant pausing periods in their locomotion.

I show that fainting body postures result from calcium depletion and body wall muscle relaxation. All three fainter mutants display a calcium signal that is shifted toward a higher baseline and higher maximum value. Furthermore, areas of high calcium signal are observed to originate at the mid-body and tail and do not require propagation from the anterior body. These features of body wall muscle activity persist during both crawling and swimming locomotion in these mutants.
In contrast, *unc-55(e402)* and *unc-4(e120)* mutants frequently adopt fixed body postures where the dorsal calcium signal remains higher than the ventral calcium signal during dorsal bends and ventral calcium signal remains higher than dorsal calcium signal during ventral bends. This indicates that maintenance of fixed body posture in these mutants occurs actively through higher levels of calcium and muscle contraction.

For worms carrying the *vab-7(e1562)* mutation the dorsal muscle at the mid-body and posterior body often shows little change in fluorescence ratio with changes in curvature. A similar but less severe pattern of activity is observed in the ventral muscle of these mutants. This pattern of muscle activity likely results from two effects; the transformation of DB motor neurons to DA motor neurons and the abnormal patterning of posterior body wall muscle in these mutants.

Surprisingly, none of the mis-wiring mutants displayed an imbalance between dorsal and ventral body wall muscle calcium signal parameters. Whereas both *unc-4(e120)* and *vab-7(e1562)* mutants with mis-wired excitatory neurons displayed significantly greater range, maximum and standard deviation values for muscle calcium signals compared to wildtype worms, *unc-55(e402)* mutants with mis-wired inhibitory neurons displayed significantly lower minimum values for muscle calcium signals. Unlike for wildtype worms, for all mis-wiring mutants the range and maximum values of the muscle calcium signal increased significantly along the body.
5.2 Introduction

5.2.1 Imaging muscle activity in locomotion mutants

A major strength of C. elegans as a model organism for neurobiology research is the availability of a large number of genetic mutants for study. As of yet, there are no studies that investigate the pattern of muscle activity in freely-behaving locomotion mutants and compare the observed activity to that in wildtype worms. Such studies would give insight not just into the muscular basis of an abnormal locomotion phenotype, but would also contribute to understanding the importance of the wildtype pattern of muscle activity for generating efficient sinusoidal locomotion.

In this study, I combine automated worm tracking and GCaMP3 calcium imaging from the body wall muscle of several locomotion mutants to investigate how mutations in genes affecting the function of the locomotion circuit change the pattern of muscle activity to generate a locomotion phenotype. I investigated the pattern of muscle activity that is responsible for the fainting phenotype observed in \textit{nca-1(gk9);nca-2(gk5)}, \textit{unc-79(e1068)} and \textit{unc-80(e1069)} mutants, as well as the pattern of muscle activity that results from motor neuron mis-wiring in \textit{vab-7(e1562)}, \textit{unc-4(e120)} and \textit{unc-55(e402)} mutants. I compare the observed patterns of muscle activity in these mutants to that observed in wildtype worms described in Chapter IV, to help understand the molecular and muscular basis of these abnormal locomotion phenotypes.

5.2.2 Imaging muscle activity during crawling and swimming in the fainter mutants

Worms that carry mutations in the \textit{unc-79} or \textit{unc-80} genes or mutations in both the \textit{nca-1} and \textit{nca-2} genes display a “fainting” locomotion phenotype (Sedensky and Meneely 1987; Humphrey, Hamming et al. 2007). No calcium imaging studies have yet been carried out from the motor neurons or body wall muscle in these mutants during free movement to investigate the pattern of neuromuscular activity that is responsible for this fainting. I chose
to image muscle activity in this group of mutants during crawling and swimming locomotion, to investigate how these genetic mutations alter muscle activity from the wildtype pattern and contribute to the fainting phenotypes of these mutants.

5.2.3 Imaging muscle activity in motor neuron mis-wiring mutants

In Chapter III I chose to analyse the behavioural phenotypes of a group of locomotion mutants, with the aim of correlating observed movement defects to identified changes in their motor neuron wiring. In this study, I have chosen to take three of these mutants; \textit{vab-7}, \textit{unc-4} and \textit{unc-55}, which display very specific motor neuron mis-wiring and investigate how this changes the pattern of muscle activity in these worms. Such specific motor neuron mis-wiring allows the generation and testing of specific hypotheses on how muscle activity could be affected.

The \textit{unc-55} gene is required for VD neurons to make their normal synapses with ventral muscle (Walthall 1990). Previous phenotypic analysis of these mutants showed that they coil ventrally in response to head touch (Walthall 1990). I further observed that imbalanced D class motor neuron function in \textit{unc-55} worms reduces locomotion and increases body bending. These locomotion defects are hypothesized to be caused by less inhibition of ventral muscle and more inhibition of dorsal muscle on VD neuron mis-wiring, resulting in unbalanced body wall muscle excitation and coiling (Walthall 1990; Walthall and Plunkett 1995), though this is yet to be confirmed experimentally. As such, I decided to image muscle activity in these mutants during crawling locomotion to test this hypothesis.

In \textit{unc-4} mutants the VA neurons VA2 to VA10 receive synaptic input from the forward command interneurons that usually synapse onto B motor neurons (White, Southgate et al. 1992). As ventral muscle would continue to receive input from VB neurons during forward locomotion, ventral muscle in these mutants might display increased calcium activity compared to dorsal muscle during forward locomotion. I decided to test this hypothesis by imaging their muscle activity during forward crawling.
In *vab-7(e1562)* mutants DB neuron axons change polarity and project anteriorly along the DNC rather than posteriorly, so becoming morphologically identical to DA neurons. (Ahringer 1996; Esmaeili, Ross et al. 2002). If A and B class motor neurons do act as stretch receptors to modulate sinusoidal locomotion, this change in DB neuron axon polarity would be predicted to reduce dorsal muscle activity during forward locomotion. I decided to test this hypothesis by imaging muscle activity in these mutants during crawling locomotion.
5.3 Methods

5.3.1 Strains

Strains were maintained at 22°C, using standard methods (Brenner 1974). Strains used in this study include:

AQ2955 nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2956 nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2957 unc-79(e1068); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2958 unc-79(e1068); ljls132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2959 unc-80(e1069); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2960 unc-80(e1069); ljls132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2961 vab-7(e1562); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2962 vab-7(e1562); ljls132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2963 unc-4(e120); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2964 unc-4(e120); ljls132[pmyo-3::GFP-SL2-tagRFP-T]
AQ2965 unc-55(e402); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]IV
AQ2966 unc-55(e402); ljls132[pmyo-3::GFP-SL2-tagRFP-T]

Construction of the body wall muscle GCaMP3 and GFP constructs and the worm lines were carried out as detailed in Chapter III Methods 3.5.

5.3.2 Calcium Imaging Assays and Muscle Calcium Analysis

Calcium imaging assays and muscle calcium analysis was carried out as detailed in Chapter III Methods 3.10.
5.3.3 Statistical Analysis

Standard statistical tests were performed using Matlab functions. Correlation coefficients were calculated using the Matlab function `corrcoef`. A value of +1 indicates perfect positive correlation. A value of -1 indicates perfect negative correlation. A one-sample t-test performed a t-test of the null hypothesis that data are a random sample from a normal distribution with mean 0 and unknown variance, against the alternative that the mean is not 0. A Two-sample t-tests performed a t-test of the null hypothesis that two sets of data are independent random samples from normal distributions with equal means and equal but unknown variances, against the alternative that the means are not equal. A one-way analysis of variance (ANOVA) compared the distribution of mean values (one per individual worm) from each population of worms against that of each other population. Each ANOVA tested the null hypothesis that the mean of the mean values from each population were the same. Multiple comparison tests were then performed to indentify which pairs of means were significantly different. A significance level of $\alpha = 0.05$ was used.
5.4 Results

5.4.1 Imaging muscle activity in the fainter mutants during crawling

To investigate the pattern of muscle activity in the fainter mutants during crawling locomotion, I crossed the integrated \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} arrays into \textit{nca-1(gk9);nca-2(gk5), unc-79(e1068)} and \textit{unc-80(e1069)} worms. Figure 5.1 shows an example of the unprocessed time lapse images captured by the EMCCD camera for \textit{nca-1(gk9);nca-2(gk5)} mutants crawling between an NGM pad and coverslip during imaging and illustrates the decrease in muscle activity observed during episodes of fainting.

A

\textit{nca-1(gk9);nca-2(gk5); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]}

\begin{tabular}{cccccc}
0s & 1s & 2s & 3s & 4s & 5s \\
\end{tabular}

Green Channel: GCaMP3

Red Channel: RFP

B

\textit{nca-1(gk9);nca-2(gk5); ljs132[pmyo-3::GFP-SL2-tagRFP-T]}

\begin{tabular}{cccccc}
0s & 1s & 2s & 3s & 4s & 5s \\
\end{tabular}

Green Channel: GFP

Red Channel: RFP
Figure 5.1: Unprocessed time lapse images from the EMCCD camera for nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants crawling on an NGM pad. The upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and the lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.034 seconds and shown at 1 second intervals over a total time of 4 seconds. A: nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; B: nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant.

The curvature kymographs for both nca-1(gk9);nca-2(gk5) experimental and control worms show that full dorsal and ventral bends rarely propagate along the full length of the body in these worms (see Figure 5.2). Instead, body bends propagate more slowly along the body than in wildtype worms, and frequently these mutants will spontaneously stop moving and maintain a fixed body posture. These fainting episodes can last up to several hundred frames and are indicated by ‘F’ on the curvature kymographs, where dorsal and ventral curvature bands extend horizontally along the traces indicating that body curvature values are not changing over time. Body curvature often follows a zigzag pattern, which shows that worms stop moving during fainting episodes and then frequently perform a short, rapid reversing movement before moving forward and fainting again.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

*nca-1(gk9); nca-2(gk5); lin-131(pmyo-3::GCaMP3-SL2-tomRFP-T)*

Body Curvature

A

B

Dorsal Fluorescence Ratio

C

D

Ventral Fluorescence Ratio

E

F

195
Figure 5.2: Example crawling curvature and fluorescence ratio matrices for nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; B: Curvature kymograph for nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]; C: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; D: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]; E: Ventral fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; F: Ventral fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T].
During fainting episodes, lower muscle activity between the neck and tail is observed (see Figure 5.2C and E). The calcium signal decreases both on the dorsal and ventral sides of the worm simultaneously, shown by the pale red and blue portions in the fluorescence ratio matrix that extend between body points 30 and 100 (indicated by red rectangles). Fine foraging movements at the head and neck are maintained in these mutants during fainting and are indicated by the fast dorsal and ventral bending originating at the tip of the nose and propagating up to body point 20 on the curvature kymograph and fluorescence ratio matrix.

These mutants also show that muscle activity does not have to originate at the head and propagate along the body. Instead, areas of high calcium signal are also observed to originate at both the mid-body and the tail during forward and backward movement (indicated by black rectangles). Control worms display the same locomotion as described above, but without the observed changes in the fluorescence ratio.

Plotting the dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body, mid-body and posterior body shows oscillations in the fluorescence ratios corresponding with body bending for experimental worms along the length of their body, but not for control worms (see Figure 5.3).
Chapter V: Imaging Muscle Activity in Locomotion Mutants

\[
\text{nco-1(gk9);nco-2(gk5);} \\
\text{Ijs[pmyo-3::GCaMP3-SL2-tagRFP-T]}
\]

**Anterior Body**

A

\[
\text{Normalized fluorescence ratio and curvature for anterior body} \\
\text{Frontal Ratio} \\
\text{Dorsal Ratio} \\
\text{Curvature}
\]

B

\[
\text{Normalized fluorescence ratio and curvature for anterior body} \\
\text{GFP Ratio and Body Curvature} \\
\text{Frontal Ratio}
\]

**Mid-body**

C

\[
\text{Normalized fluorescence ratio and curvature for mid-body} \\
\text{Frontal Ratio} \\
\text{Dorsal Ratio} \\
\text{Curvature}
\]

D

\[
\text{Normalized fluorescence ratio and curvature for mid-body} \\
\text{GFP Ratio and Body Curvature} \\
\text{Frontal Ratio}
\]

**Posterior Body**

E

\[
\text{Normalized fluorescence ratio and curvature for posterior body} \\
\text{Frontal Ratio} \\
\text{Dorsal Ratio} \\
\text{Curvature}
\]

F

\[
\text{Normalized fluorescence ratio and curvature for posterior body} \\
\text{GFP Ratio and Body Curvature} \\
\text{Frontal Ratio}
\]
Figure 5.3: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for \textit{nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T]} crawling mutants. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. \textbf{A: Anterior body for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant}; \textbf{B: Anterior Body for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutant}; \textbf{C: Mid-body for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant}; \textbf{D: Mid-body for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutant}; \textbf{E: Posterior Body for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant}; \textbf{F: Posterior Body for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutant}.

Similar relationships between muscle activity and body curvature are observed in \textit{nca-1(gk9);nca-2(gk5)} mutants as in wildtype worms. In experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.60 ± 0.14, mid-body; 0.51 ±0.15, posterior body; 0.49 ± 0.18). In contrast, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.05 ± 0.22, mid-body; 0.02 ± 0.15, posterior body; -0.08 ± 0.37).

In experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.61 ± 0.13, mid-body; -0.54 ± 0.17, posterior body; -0.46 ± 0.19). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; 0.02 ± 0.28, mid-body; 0.04 ± 0.24, posterior body; 0.04 ± 0.25).
Dorsal and ventral muscle activity appears to be out-of-phase (see Figure 5.4). Quantifying this by calculating the correlation in calcium signal between dorsal and ventral muscles shows that the fluorescence ratios display a weak negative correlation with respect to each other along the length of the worm body (mean R value ± stdev for anterior body; -0.33 ± 0.14, mid-body; -0.33 ± 0.15, posterior body; -0.18 ± 0.27). In contrast to this, dorsal and ventral fluorescence ratios in control worms display no consistent correlation with respect to each other (mean R value ± stdev for anterior body; 0.22 ± 0.19, mid-body; -0.02 ± 0.25, posterior body; 0.09 ± 0.20).

Both the dorsal and ventral correlations between muscle activity and body curvature in nca-1(gk9);nca-2(gk5) mutants are significantly weaker than in wildtype worms. Plotting dorsal and ventral fluorescence ratios against each other shows a strong hyperbolic relationship between the calcium signal in these opposing muscles along the length of the mutant (see Figure 5.4), which is not observed in control worms and is unlike the more linear negative relationship observed in wildtype worms. Such a hyperbolic relationship suggests that muscle activity on one side of the worm body does not increase until the muscle activity on the opposite side has decreased to very near its minimum value. On ventral bending, the dorsal muscle adopts near-baseline activity and on dorsal bending, the ventral muscle adopts near-baseline activity.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

**A**

\[ nco-1(k9); nco-2(k5); flis131[pmyo-3::GCaMP3-SL2-tagRFP-T] \]

**B**

\[ nco-1(k9); nco-2(k5); flis132[pmyo-3::GFP-SL2-tagRFP-T] \]

**Anterior Body**

- Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

**Mid-body**

- Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

**Posterior Body**

- Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body
Figure 5.4: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for *nca-1*(gk9);*nca-2*(gk5); *ljls131*[pmyo-3::GCaMP3-SL2-tagRFP-T] and *nca-1*(gk9);*nca-2*(gk5); *ljls132*[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values are plotted against each other. A: Anterior body for *nca-1*(gk9);*nca-2*(gk5); *ljls131*[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; B: Anterior Body for *nca-1*(gk9);*nca-2*(gk5); *ljls132*[pmyo-3::GFP-SL2-tagRFP-T] mutant; C: Mid-body for *nca-1*(gk9);*nca-2*(gk5); *ljls131*[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; D: Mid-body for *nca-1*(gk9);*nca-2*(gk5); *ljls132*[pmyo-3::GFP-SL2-tagRFP-T] mutant; E: Posterior Body for *nca-1*(gk9);*nca-2*(gk5); *ljls131*[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; F: Posterior Body for *nca-1*(gk9);*nca-2*(gk5); *ljls132*[pmyo-3::GFP-SL2-tagRFP-T] mutant.
During fainting episodes, body curvature values remain fixed but dorsal and ventral fluorescence ratios decrease in experimental worms (see Figure 5.5). This decrease is not observed in control worms during fainting (see Figure 5.6). The RFP fluorescence in the red reference channel remains constant for both experimental and control worms, as does the GFP fluorescence in the green channel for control worms.

The GCaMP3 fluorescence in the green channel for experimental worms decreases over the duration of the fainting episode and remains at a low value until body curvature changes again (see Figure 5.5B, C, E and F). When the body is in a dorsal bend during fainting, ventral GCaMP3 fluorescence is already low at the beginning of the faint, and maintains a low value throughout the fainting period (see Figure 5.5B). Dorsal GCaMP3 fluorescence is initially high with the onset on the dorsal bend and then the fluorescence decreases to a baseline value during the fainting period (see Figure 5.5C).

When the body is in a ventral bend during fainting, ventral GCaMP3 fluorescence is initially high with the onset on the dorsal bend and then the fluorescence decreases to a baseline value during the fainting period (see Figure 5.5E). Dorsal GCaMP3 fluorescence is already low at the beginning of the faint, and maintains a low value throughout the fainting period (see Figure 5.5F). This indicates that the calcium concentration in both ventral and dorsal muscle cells is decreasing to a baseline level during fainting, regardless of how the body is curved. Therefore, rather than maintaining a fainting posture through high levels of calcium at curved body positions, this result suggests that the maintenance of fainting body posture in these mutants occurs through lowering levels of calcium and body wall muscle relaxation.
Figure 5.5: Example plots of dorsal and ventral muscle activity during fainting episodes for *nca-1(gk9);nca-2(gk5); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* crawling mutants. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio in Figures A and D. Green line plots GCaMP3 fluorescence and red line plots RFP fluorescence in Figures B, C, E and F. **A:** Ventral and dorsal fluorescence ratio; **B:** Ventral GCaMP3 and RFP fluorescence; **C:** Dorsal GCaMP3 and RFP fluorescence; **D:** Ventral and dorsal fluorescence ratio; **E:** Ventral GCaMP3 and RFP fluorescence; **F:** Dorsal GCaMP3 and RFP fluorescence.
Figure 5.6: Example plots of dorsal and ventral muscle activity during fainting episodes for *nca-1(gk9);nca-2(gk5); ljs132[pmyo-3::GFP-SL2-tagRFP-T]* crawling mutants. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio in Figures A and D. Green line plots GFP fluorescence and red line plots RFP fluorescence in Figures B, C, E and F. **A:** Ventral and dorsal fluorescence ratio; **B:** Ventral GFP and RFP fluorescence; **C:** Dorsal GFP and RFP fluorescence; **D:** Ventral and dorsal fluorescence ratio; **E:** Ventral GFP and RFP fluorescence; **F:** Dorsal GFP and RFP fluorescence.
Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for \( \text{nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} \) mutants are shown in the table below (see Figure 5.7). For these worms, the normalized and averaged crawling fluorescence signal has a mean value of \(1.00 \pm 0.00\), a minimum value of \(0.44 \pm 0.07\), a maximum value of \(2.87 \pm 0.48\), a range of \(2.44 \pm 0.51\) and a standard deviation of \(0.46 \pm 0.09\). For \(\text{nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]}\) control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of \(1.00 \pm 0.00\), a minimum value of \(0.70 \pm 0.05\), a maximum value of \(1.43 \pm 0.15\), a range of \(0.72 \pm 0.17\) and a standard deviation of \(0.12 \pm 0.02\).

Experimental worms show a significantly larger range in the fluorescence signal compared to control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. This result is expected as GFP and RFP fluorescence should not change on body curvature with changes in calcium concentration. For experimental worms, there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, or between anterior, mid-body and posterior regions, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm and along the length of the worm body.

Compared to wildtype N2 worms, \(\text{nca-1(gk9);nca-2(gk5)}\) mutants show significantly higher minimum \((p = 0.0096)\), maximum \((p < 0.0001)\), range \((p < 0.0001)\) and standard deviation \((p < 0.0001)\) of fluorescence signal, suggesting that the calcium signal in the body wall muscle of these mutants has a higher baseline and a greater range than in wildtype animals.
<table>
<thead>
<tr>
<th></th>
<th>Wildtype N2</th>
<th>nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dorsal Fluorescence Signal</td>
<td>Ventral Fluorescence Signal</td>
</tr>
<tr>
<td><strong>Parameter</strong></td>
<td><strong>Average</strong></td>
<td><strong>Anterior</strong></td>
</tr>
<tr>
<td>Mean</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Min</td>
<td>0.40 ± 0.08</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>Max</td>
<td>2.23 ± 0.44</td>
<td>2.62 ± 0.30</td>
</tr>
<tr>
<td>Range</td>
<td>1.83 ± 0.47</td>
<td>2.19 ± 0.30</td>
</tr>
<tr>
<td>StDev</td>
<td>0.38 ± 0.07</td>
<td>0.43 ± 0.08</td>
</tr>
</tbody>
</table>

Figure 5.7: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 14 worms. A ‘**’ indicates a statistically significant difference from wildtype N2 worms.
Worms carrying the *unc-79(e1068)* and *unc-80(e1069)* mutations display similar body curvature and muscle activity to *nca-1(gk9);nca-2(gk5)* double mutant worms.

Similar to *nca-1(gk9);nca-2(gk5)* mutants, the curvature kymographs for *unc-79(e1068)* and *unc-80(e1069)* experimental and control worms shows that full dorsal and ventral bends rarely propagate along the full length of the body in these mutants (see Figures 5.8 and 5.9). Instead, body bends propagate more slowly along the body than in wildtype worms, and frequently these mutants will spontaneously stop moving and faint, indicated by ‘F’ on the curvature kymographs.

During fainting episodes, lower muscle activity between the neck and tail is observed (see Figure 5.8 and 5.9). The calcium signal decreases both on the dorsal and ventral sides of the worm simultaneously, shown by the pale red and blue portions in the fluorescence ratio matrix. Fine foraging movements at the head and neck are maintained in these mutants during fainting and are indicated by the fast dorsal and ventral bending originating at the tip of the nose and propagating up to body point 20 on the curvature kymograph and fluorescence ratio matrix.

Similar to *nca-1(gk9);nca-2(gk5)* mutants, areas of high calcium signal are observed to originate at both the mid-body and the tail during forward and backward movement. Control worms display the same locomotion as described above, but without the observed changes in the fluorescence ratio.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]  unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]

Body Curvature

Dorsal Fluorescence Ratio

Ventral Fluorescence Ratio
Figure 5.8: Example crawling curvature and fluorescence ratio matrices for unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; B: Curvature kymograph for unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]; C: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; D: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]; E: Ventral fluorescence ratio matrix overlaid on curvature kymograph for unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; F: Ventral fluorescence ratio matrix overlaid on curvature kymograph for unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T].
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-80(e1069); jlj131[pmyo-3::GCaMP3-SL2-tagRFP-T]  unc-80(e1069); jlj132[pmyo-3::GFP-SL2-tagRFP-T]

Body Curvature

Dorsal Fluorescence Ratio

Ventral Fluorescence Ratio
Figure 5.9: Example crawling curvature and fluorescence ratio matrices for unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-80(e1069); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; B: Curvature kymograph for unc-80(e1069); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]; C: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; D: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for unc-80(e1069); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]; E: Ventral fluorescence ratio matrix overlaid on curvature kymograph for unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]; F: Ventral fluorescence ratio matrix overlaid on curvature kymograph for unc-80(e1069); ljIs132[pmyo-3::GFP-SL2-tagRFP-T].
Dorsal and ventral fluorescence ratios show oscillations corresponding with body bending for experimental worms along the length of their body, but not for control worms. Similar relationships between muscle activity and body curvature are observed in \textit{unc-79(e1068)} and \textit{unc-80(e1069)} mutants as in wildtype worms.

For \textit{unc-79(e1068)} experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body: 0.66 ± 0.07, mid-body: 0.53 ± 0.29, posterior body: 0.59 ± 0.25). In contrast, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body: 0.15 ± 0.34, mid-body: -0.16 ± 0.33, posterior body: 0.07 ± 0.26).

Similarly, for \textit{unc-80(e1069)} experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body: 0.67 ± 0.12, mid-body: 0.65 ± 0.11, posterior body: 0.63 ± 0.11). In contrast, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body: 0.03 ± 0.19, mid-body: -0.18 ± 0.14, posterior body: 0.15 ± 0.22).

In \textit{unc-79(e1068)} experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body: -0.65 ± 0.16, mid-body: -0.51 ± 0.21, posterior body: -0.48 ± 0.22). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body: 0.08 ± 0.27, mid-body: 0.11 ± 0.29, posterior body: 0.14 ± 0.27).

Similarly, in \textit{unc-80(e1069)} experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body: -0.71 ± 0.08, mid-body: -0.63 ± 0.16, posterior body: -0.61 ± 0.14). In contrast to this, the ventral fluorescence ratio in control worms
shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.16 ± 0.20, mid-body; -0.04 ± 0.21, posterior body; -0.01 ± 0.26).

A similar pattern of body curvature and muscle activity is observed in these mutants during fainting episodes as that observed in nca-1(gk9);nca-2(gk5) mutants. During fainting, the body curvature values for unc-79(e1068) and unc-80(e1069) mutants remain fixed but dorsal and ventral fluorescence ratios decrease in experimental worms. The GCaMP3 ratio for experimental worms decreases over the duration of the fainting episode and remains at a low value until body curvature changes again. The calcium concentration in both ventral and dorsal muscle cells decreases to a baseline level during fainting, regardless of how the body is curved. This decrease is not observed in control worms during fainting. This result suggests that the maintenance of fainting body posture occurs through depletion of calcium and muscle relaxation in all three classes of fainter mutant.

As for nca-1(gk9);nca-2(gk5) mutants, quantifying the correlation in calcium signal between dorsal and ventral muscles shows that the fluorescence ratios display a weak negative correlation with respect to each other along the length of the worm body for unc-79(e1068) experimental worms (mean R value ± stdev for anterior body; -0.36 ± 0.20, mid-body; -0.26 ± 0.25, posterior body; -0.31 ± 0.26) and for unc-80(e1069) experimental worms (mean R value ± stdev for anterior body; -0.50 ± 0.16, mid-body; -0.41 ± 0.19, posterior body; -0.37 ± 0.14). In contrast to this, dorsal and ventral fluorescence ratios in unc-79(e1068) and unc-80(e1069) control worms display no consistent correlation with respect to each other (mean R value ± stdev for unc-79(e1068) control worms anterior body; 0.24 ± 0.29, mid-body; -0.20 ± 0.21, posterior body; 0.22 ± 0.29, mean R value ± stdev for unc-80(e1069) control worms anterior body; 0.12 ± 0.16, mid-body; 0.01 ± 0.16, posterior body; 0.20 ± 0.19).

Furthermore, dorsal and ventral fluorescence ratios show a strong hyperbolic relationship to each other in these mutants which is not observed in control worms and is unlike the more linear negative relationship observed in wildtype worms. Such a hyperbolic relationship was also seen in nca-1(gk9);nca-2(gk5) mutants and suggests that this could be a shared feature
of muscle activity in the fainter mutants, where muscle activity on one side of the worm body does not increase until the muscle activity on the opposite side has decreased to very near its minimum value.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for \textit{unc-79(e1068); \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]}} mutants and \textit{unc-80 (e1069); \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]}} mutants are shown in the two tables below (see Figures 5.10 and 5.11).
## Figure 5.10: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling \textit{unc-79(e1068); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutants.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, \( n = 11 \) worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Min</strong></td>
<td>0.40 ± 0.08</td>
<td>0.52 ± 0.09</td>
<td>0.51 ± 0.13</td>
<td>0.49 ± 0.09</td>
<td>0.50 ± 0.10</td>
<td>0.51 ± 0.11</td>
<td>0.56 ± 0.10</td>
<td>*0.52 ± 0.10</td>
</tr>
<tr>
<td><strong>Max</strong></td>
<td>2.23 ± 0.44</td>
<td>2.20 ± 0.22</td>
<td>2.52 ± 0.46</td>
<td>2.79 ± 0.77</td>
<td>2.32 ± 0.37</td>
<td>2.37 ± 0.20</td>
<td>2.42 ± 0.64</td>
<td>*2.44 ± 0.44</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>1.83 ± 0.47</td>
<td>1.68 ± 0.28</td>
<td>2.01 ± 0.54</td>
<td>2.29 ± 0.79</td>
<td>1.81 ± 0.34</td>
<td>1.87 ± 0.25</td>
<td>1.86 ± 0.67</td>
<td>1.92 ± 0.48</td>
</tr>
<tr>
<td><strong>StDev</strong></td>
<td>0.38 ± 0.07</td>
<td>0.34 ± 0.05</td>
<td>0.40 ± 0.14</td>
<td>0.40 ± 0.12</td>
<td>0.37 ± 0.07</td>
<td>0.38 ± 0.10</td>
<td>0.33 ± 0.12</td>
<td>0.37 ± 0.10</td>
</tr>
</tbody>
</table>
### Table 5.11: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling *unc-80(e1069); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutants.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 12 worms. A '*' indicates a statistically significant difference from wildtype N2 worms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
<td>1.00 ±</td>
</tr>
<tr>
<td>Min</td>
<td>0.40 ±</td>
<td>0.48 ±</td>
<td>0.48 ±</td>
<td>0.47 ±</td>
<td>0.48 ±</td>
<td>0.49 ±</td>
<td>0.49 ±</td>
<td>*0.48 ±</td>
</tr>
<tr>
<td>Max</td>
<td>2.23 ±</td>
<td>2.42 ±</td>
<td>2.62 ±</td>
<td>2.61 ±</td>
<td>2.18 ±</td>
<td>2.67 ±</td>
<td>2.60 ±</td>
<td>*2.52 ±</td>
</tr>
<tr>
<td>Range</td>
<td>1.83 ±</td>
<td>1.94 ±</td>
<td>2.13 ±</td>
<td>2.14 ±</td>
<td>1.70 ±</td>
<td>2.18 ±</td>
<td>2.12 ±</td>
<td>*1.92 ±</td>
</tr>
<tr>
<td>StDev</td>
<td>0.38 ±</td>
<td>0.38 ±</td>
<td>0.43 ±</td>
<td>0.39 ±</td>
<td>0.36 ±</td>
<td>0.43 ±</td>
<td>0.40 ±</td>
<td>0.37 ±</td>
</tr>
</tbody>
</table>
For *unc-79(e1068)* experimental worms, the normalized and averaged crawling fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.52 \pm 0.10$, a maximum value of $2.44 \pm 0.44$, a range of $1.92 \pm 0.48$ and a standard deviation of $0.37 \pm 0.10$. For *unc-79(e1068)* control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.73 \pm 0.06$, a maximum value of $1.36 \pm 0.09$, a range of $0.63 \pm 0.13$ and a standard deviation of $0.11 \pm 0.03$.

For *unc-80(e1069)* experimental worms, the normalized and averaged crawling fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.48 \pm 0.11$, a maximum value of $2.52 \pm 0.43$, a range of $2.04 \pm 0.41$ and a standard deviation of $0.40 \pm 0.07$. For *unc-79(e1068)* control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.71 \pm 0.07$, a maximum value of $1.44 \pm 0.20$, a range of $0.73 \pm 0.25$ and a standard deviation of $0.11 \pm 0.03$.

Both *unc-79(1068)* and *unc-80(e1069)* experimental worms show a significantly larger range in the fluorescence signal compared to control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. This result is expected as GFP and RFP fluorescence should not change on body curvature with changes in calcium concentration.

For *unc-79(e1068)* experimental worms there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, or between anterior, mid-body and posterior regions, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm and along the length of the worm body.

For *unc-80(e1069)* experimental worms there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm. However, the range of
the fluorescence signal and its maximum value and standard deviation is significantly higher at the mid-body and tail compared to the anterior body of the worm.

Compared to wildtype N2 worms, \textit{unc-79(e1068)} mutants show comparable values for the range of the fluorescence signal ($p = 0.2920$) and the standard deviation of the signal ($p = 0.6859$), but similar to \textit{nca-1(gk9);nca-2(gk5)} mutants they do display significantly higher minimum fluorescence values ($p < 0.0001$) and maximum fluorescence values ($p = 0.0142$). These results suggest that wildtype worms and \textit{unc-79(e1068)} mutants display similar distribution parameters in their body wall muscle calcium signal but that in \textit{unc-79(e1068)} mutants, the calcium signal is shifted toward a higher baseline and higher maximum value.

Compared to wildtype N2 worms, \textit{unc-80(e1069)} mutants also show a significantly higher minimum ($p < 0.0001$), maximum ($p = 0.0001$) and range ($p = 0.0049$) of fluorescence signal, although the standard deviation is not significantly different ($p = 0.0824$). This result suggests that the calcium signal in the body wall muscle of these mutants has a higher baseline and a greater range than in wildtype animals.
5.4.2 Imaging muscle activity in fainter mutants during swimming

I investigated the pattern of muscle activity in the fainter mutants during swimming. Figure 5.12 shows an example of the unprocessed time lapse images captured by the EMCCD camera for *nca-1(gk9);nca-2(gk5)* mutants swimming in M9 buffer during imaging and illustrates the decreased locomotion and muscle activity of these mutants when in a liquid environment.
Figure 5.12: Unprocessed time lapse images from the EMCCD camera for \( nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] \) and \( nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] \) mutants swimming in M9 buffer. The upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and the lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.01 seconds and shown at 0.05 second intervals over a total time of 0.45 seconds. \( \text{A: } nca-1(gk9);nca-2(gk5); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] \) mutant; \( \text{B: } nca-1(gk9);nca-2(gk5); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] \) mutant.
Similar to \textit{nca-1(gk9);nca-2(gk5)} crawling mutants, the curvature kymographs for both \textit{nca-1(gk9);nca-2(gk5)} experimental and control swimming mutants shows that full dorsal and ventral bends rarely propagate along the full length of the body in these worms (see Figure 5.13). As observed on a solid substrate, these mutants are capable of swimming but will frequently stop moving and maintain a fixed body posture. These fainting episodes can last up to several hundred frames and are indicated by ‘F’ on the curvature kymographs, where dorsal and ventral curvature bands extend horizontally along the traces.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

\[ nco-1(gk9);nco-2(gk5); \]
\[ l/jls131[pmypo-3::GCaMP3-SL2-tagRFP-T] \]

Body Curvature

Dorsal Fluorescence Ratio

Ventral Fluorescence Ratio
Figure 5.13: Example swimming curvature and fluorescence ratio matrices for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Curvature kymograph for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]; B: Curvature kymograph for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T]; C: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]; D: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T]; E: Ventral fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]; F: Ventral fluorescence ratio matrix overlaid on curvature kymograph for nca-1(gk9);nca-2(gk5); ljls132[pmyo-3::GFP-SL2-tagRFP-T].
Similar to crawling fainting episodes, swimming fainting episodes are characterized by lower muscle activity between the neck and tail (see Figures 5.13C and E). The calcium signal decreases both on the dorsal and ventral sides of the worm simultaneously, shown by the light red and blue portions in the fluorescence ratio matrix that between body points 60 and 100. However, undulatory movements are somewhat maintained at the head and neck when the mid-body and tail remain in a fixed posture. These dorsal and ventral bends are associated with changes in calcium concentration in body wall muscle and usually extend up to half the body length but frequently fail to propagate fully to the tail. The posterior half of the worm often adopts a fixed posture for an extended time period. Similar to crawling, it is frequently observed that areas of high calcium signal can originate at the mid-body and tail during swimming locomotion. Control worms display the same locomotion, although some changes in ratio are observed in the control fluorescence ratio matrices. This is likely due to increased movement artefacts in the z-axis with faster swimming locomotion compared to crawling.

Plotting the dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body, mid-body and posterior body shows oscillations in the fluorescence ratios corresponding with body bending for experimental worms, which is more prominent for anterior and mid-body muscle than for posterior body muscle (see Figure 5.14). Where body curvature values remain fixed for extend time periods, dorsal and ventral fluorescence ratios also remain at low values until body curvature changes again.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

\( nco-1(ek9); nco-2(ek5); \)
\( /\!\!LJls131[pmyo-3::GCaMP3-SL2-tagRFP-T] \)

\( nco-1(ek9); nco-2(ek5); \)
\( /\!\!LJls132[pmyo-3::GFP-SL2-tagRFP-T] \)

**Anterior Body**

A

**Mid-body**

C

**Posterior Body**

E
Figure 5.14: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for \textit{nca-1(gk9);nca-2(gk5); ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{nca-1(gk9);nca-2(gk5); ljis132[pmyo-3::GFP-SL2-tagRFP-T]} swimming mutants. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. \textbf{A:} Anterior body for \textit{nca-1(gk9);nca-2(gk5); ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; \textbf{B:} Anterior Body for \textit{nca-1(gk9);nca-2(gk5); ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; \textbf{C:} Mid-body for \textit{nca-1(gk9);nca-2(gk5); ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; \textbf{D:} Mid-body for \textit{nca-1(gk9);nca-2(gk5); ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; \textbf{E:} Posterior Body for \textit{nca-1(gk9);nca-2(gk5); ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; \textbf{F:} Posterior Body for \textit{nca-1(gk9);nca-2(gk5); ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutant.
Similar relationships between muscle activity and body curvature are observed in *nca-1(gk9);nca-2(gk5)* swimming mutants as in wildtype swimming worms. In experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.51 ± 0.15, mid-body; 0.60 ± 0.09, posterior body; 0.48 ± 0.25). In contrast to this, the dorsal fluorescence ratio in control worms shows no consistent correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.05 ± 0.17, mid-body; -0.15 ± 0.14, posterior body; -0.04 ± 0.33).

In experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.53 ± 0.14, mid-body; -0.60 ± 0.19, posterior body; -0.37 ± 0.12). In contrast to this, the ventral fluorescence ratio in control worms shows no consistent correlation with the curvature of the worm body (mean R value ± stdev for anterior body; 0.01 ± 0.12, mid-body; -0.01 ± 0.25, posterior body; 0.06 ± 0.24).

Dorsal and ventral muscle activity display very weak negative correlation with each other in experimental worms (mean R value ± stdev for experimental worms; anterior body; -0.17 ± 0.25, mid-body; -0.20 ± 0.24, posterior body; -0.09 ± 0.22, for control worms; anterior body; 0.17 ± 0.23, mid-body; 0.13 ± 0.18, posterior body; 0.18 ± 0.17). Such weak correlations between dorsal and ventral muscle activity in these mutants compared to wildtype worms could be due to the simultaneous reduction in dorsal and ventral fluorescence ratios during fainting.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for *nca-1(gk9);nca-2(gk5); ljjIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* swimming mutants are shown in the table below (see Figure 5.15).
<table>
<thead>
<tr>
<th></th>
<th>Wildtype N2</th>
<th>nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
<td><strong>Average</strong></td>
<td><strong>Anterior</strong></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Min</strong></td>
<td>0.64 ± 0.07</td>
<td>0.65 ± 0.07</td>
</tr>
<tr>
<td><strong>Max</strong></td>
<td>1.50 ± 0.15</td>
<td>1.52 ± 0.18</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0.86 ± 0.19</td>
<td>0.88 ± 0.22</td>
</tr>
<tr>
<td><strong>StDev</strong></td>
<td>0.16 ± 0.03</td>
<td>0.17 ± 0.04</td>
</tr>
</tbody>
</table>

**Figure 5.15:** Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming *nca-1(gk9);nca-2(gk5); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutants. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 10 worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

For these mutants, the normalized and averaged swimming fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.64 ± 0.08, a maximum value of 1.61 ± 0.27, a range of 0.97 ± 0.31 and a standard deviation of 0.18 ± 0.04. For *nca-1(gk9);nca-2(gk5)* control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal during swimming has a mean value of 1.00 ± 0.00, a minimum value of 0.70 ± 0.10, a maximum value of 1.41 ± 0.16, a range of 0.71 ± 0.23 and a standard deviation of 0.12 ± 0.03.

Experimental worms do show a larger range in the fluorescence signal compared to control worms, with a lower minimum value, a higher maximum value and a larger standard deviation in the signal, but this difference does not reach statistical significance. This suggests that, similar to wildtype swimming worms, *nca-1(gk9);nca-2(gk5)* swimming mutants display a smaller muscle calcium signal than *nca-1(gk9);nca-2(gk5)* crawling mutants. For experimental worms, there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, or between anterior, mid-body and posterior regions, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm and along the length of the worm body.

Compared to wildtype swimming worms, *nca-1(gk9);nca-2(gk5)* swimming mutants show no significant difference in the minimum fluorescence values (p = 0.6186). However, they do show a significantly larger range in fluorescence signal (p = 0.0014), with a higher maximum fluorescence value (p = 0.0005) and a greater standard deviation (p = 0.0005). This result is the same as that seen between wildtype crawling worms and *nca-1(gk9);nca-2(gk5)* crawling mutants, except that a higher minimum fluorescence value is not observed for *nca-1(gk9);nca-2(gk5)* swimming mutants (p = 0.3946). This result suggests that the main features of body wall muscle calcium signal observed in the fainter mutants during crawling locomotion also persists during swimming locomotion.
Worms carrying the *unc-79(e1068)* and *unc-80(e1069)* mutations display similar body curvature and muscle activity to *nca-1(gk9);nca-2(gk5)* double mutant worms during swimming locomotion.

Similar to *nca-1(gk9);nca-2(gk5)* swimming worms, the curvature kymographs for both *unc-79(e1068)* and *unc-80(e1069)* experimental and control worms shows that full dorsal and ventral bends rarely propagate along the full length of the body in these mutants (see Figure 5.16 and Figure 5.17). As observed on a solid substrate, these mutants are capable of swimming but will frequently stop moving and maintain a fixed body posture. These fainting episodes can last up to several hundred frames and are indicated by ‘F’ on the curvature kymographs, where dorsal and ventral curvature bands extend horizontally along the traces.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-79(e1068); jls131[pmyo-3::GCaMP3-SL2-tagRFP-T]  
unc-79(e1068); jls132[pmyo-3::GFP-SL2-tagRFP-T]

Body Curvature

Dorsal Fluorescence Ratio

Ventral Fluorescence Ratio
Chapter V: Imaging Muscle Activity in Locomotion Mutants

Figure 5.16: Example swimming curvature and fluorescence ratio matrices for *unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]* mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. 

A: Curvature kymograph for *unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; B: Curvature kymograph for *unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]*; C: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for *unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; D: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for *unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]*; E: Ventral fluorescence ratio matrix overlaid on curvature kymograph for *unc-79(e1068); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*; F: Ventral fluorescence ratio matrix overlaid on curvature kymograph for *unc-79(e1068); ljs132[pmyo-3::GFP-SL2-tagRFP-T]*.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-80[e1069]; ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T] unc-80[e1069]; ljs132[pmyo-3::GFP-SL2-tagRFP-T]

Body Curvature

A

Kymograph of Body Curvature

B

Kymograph of Body Curvature

Dorsal Fluorescence Ratio

C

Dorsal GCaMP3/RFP ratio overlaid on body curvature

D

Dorsal GFP/RFP ratio overlaid on body curvature

Ventral Fluorescence Ratio

E

Ventral GCaMP3/RFP ratio overlaid on body curvature

F

Ventral GFP/RFP ratio overlaid on body curvature
Figure 5.17: Example swimming curvature and fluorescence ratio matrices for \textit{unc-80(e1069); \textit{lj}is131[\textit{pmyo-3::GCanP3-SL2-tagRFP-T]} and \textit{unc-80(e1069); \textit{lj}is132[\textit{pmyo-3::GFP-SL2-tagRFP-T}] mutants constrained in z-axis by coverslip.} Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. 

\textbf{A: Curvature kymograph for \textit{unc-80(e1069); \textit{lj}is131[\textit{pmyo-3::GCanP3-SL2-tagRFP-T]}; B: Curvature kymograph for \textit{unc-80(e1069); \textit{lj}is132[\textit{pmyo-3::GFP-SL2-tagRFP-T}]; C: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for \textit{unc-80(e1069); \textit{lj}is131[\textit{pmyo-3::GCanP3-SL2-tagRFP-T}]; D: Dorsal fluorescence ratio matrix overlaid on curvature kymograph for \textit{unc-80(e1069); \textit{lj}is132[\textit{pmyo-3::GFP-SL2-tagRFP-T}]; E: Ventral fluorescence ratio matrix overlaid on curvature kymograph for \textit{unc-80(e1069); \textit{lj}is131[\textit{pmyo-3::GCanP3-SL2-tagRFP-T}]; F: Ventral fluorescence ratio matrix overlaid on curvature kymograph for \textit{unc-80(e1069); \textit{lj}is132[\textit{pmyo-3::GFP-SL2-tagRFP-T}].}

Similar to \textit{nca-1(gk9);nca-2(gk5)} mutants, swimming fainting episodes in \textit{unc-79(e1068)} and \textit{unc-80(e1069)} mutants are characterized by lower muscle activity between the neck and tail (see Figures 5.16C and E and Figures 5.17C and E). The calcium signal decreases both on the dorsal and ventral sides of the worm simultaneously, shown by the light red and blue portions in the fluorescence ratio matrix that between body points 60 and 100. Undulatory movements are somewhat maintained at the head and neck even when the mid-body and tail remain in a fixed posture. These dorsal and ventral bends are associated with changes in calcium concentration in body wall muscle and usually extend up to half the body length but frequently fail to propagate fully to the tail. The posterior half of the worm often adopts a fixed posture for an extended time period. As with crawling, areas of high calcium signal are observed to originate at the mid-body and tail during swimming locomotion. Control worms display the same locomotion but are not correlated with ratio changes in the control fluorescence ratio matrices.
Dorsal and ventral fluorescence ratios show oscillations corresponding with body bending for experimental worms. Where body curvature values remain fixed for extend time periods, dorsal and ventral fluorescence ratios also remain fixed at low values until body curvature changes again. Similar relationships between muscle activity and body curvature are observed in \textit{unc-79(e1068)} and \textit{unc-80(e1069)} swimming mutants as in \textit{nca-1(gk9);nca-2(gk5)} swimming worms and wildtype swimming mutants.

For \textit{unc-79(e1068)} experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean $R$ value ± stdev for anterior body: 0.39 ± 0.11, mid-body: 0.44 ± 0.14, posterior body: 0.36 ± 0.19). In contrast to this, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean $R$ value ± stdev for anterior body: -0.05 ± 0.16, mid-body: 0.00 ± 0.26, posterior body: -0.08 ± 0.24). For \textit{unc-79(e1068)} experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean $R$ value ± stdev for anterior body: -0.27 ± 0.17, mid-body: -0.37 ± 0.27, posterior body: -0.29 ± 0.28). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean $R$ value ± stdev for anterior body: -0.07 ± 0.21, mid-body: 0.07 ± 0.17, posterior body: 0.02 ± 0.27).

For \textit{unc-80(e1069)} experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean $R$ value ± stdev for anterior body: 0.42 ± 0.17, mid-body: 0.55 ± 0.15, posterior body: 0.46 ± 0.17). In contrast to this, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean $R$ value ± stdev for anterior body: -0.08 ± 0.15, mid-body: -0.07 ± 0.25, posterior body: 0.01 ± 0.22). For \textit{unc-80(e1069)} experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean $R$ value ± stdev for anterior body: -0.46 ± 0.14, mid-body: -0.58 ± 0.08, posterior body: -0.43 ± 0.18). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the
curvature of the worm body (mean R value ± stdev for anterior body; 0.02 ± 0.23, mid-body; 0.04 ± 0.22, posterior body; 0.03 ± 0.25).

Dorsal and ventral muscle activity display no consistent correlation with each other in unc-79(e1068) and unc-80(e1069) experimental worms (mean R value ± stdev for unc-79(e1068) worms; anterior body; 0.26 ± 0.22, mid-body; 0.17 ± 0.23, posterior body; 0.00 ± 0.27, for unc-80(e1069) worms; anterior body; -0.07 ± 0.22, mid-body; -0.21 ± 0.25, posterior body; -0.14 ± 0.22). Such correlations between dorsal and ventral muscle activity in these mutants compared to the moderate negative correlations observed in wildtype worms could be due to the simultaneous reduction in dorsal and ventral fluorescence ratios during fainting.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for unc-79(e1068) and unc-80(e1069) swimming mutants are shown in the two tables below (see Figures 5.18 and 5.19).
Chapter V: Imaging Muscle Activity in Locomotion Mutants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dorsal Fluorescence Signal</th>
<th>Ventral Fluorescence Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.64 ± 0.07</td>
<td>0.60 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>0.58 ± 0.11</td>
<td>0.59 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>0.62 ± 0.10</td>
<td>0.63 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>0.56 ± 0.20</td>
<td>0.60 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.50 ± 0.15</td>
<td>1.68 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>1.62 ± 0.26</td>
<td>1.71 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>1.66 ± 0.43</td>
<td>1.72 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>1.66 ± 0.23</td>
<td>1.67 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.86 ± 0.19</td>
<td>1.08 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>1.03 ± 0.30</td>
<td>1.12 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>1.04 ± 0.48</td>
<td>1.08 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>1.10 ± 0.36</td>
<td>1.08 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>StDev</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.16 ± 0.03</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.20 ± 0.06</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.20 ± 0.09</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.19 ± 0.05</td>
<td>0.20 ± 0.07</td>
</tr>
</tbody>
</table>

Figure 5.18: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming *unc-79(e1068); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutants.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 11 worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
Figure 5.19: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for swimming *unc-80(e1069); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutants.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 12 worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
For *unc-79(e1068)* experimental worms, the normalized and averaged swimming fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.60 \pm 0.12$, a maximum value of $1.67 \pm 0.35$, a range of $1.08 \pm 0.42$ and a standard deviation of $0.20 \pm 0.07$. For *unc-79(e1068)* control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal during swimming has a mean value of $1.00 \pm 0.00$, a minimum value of $0.69 \pm 0.11$, a maximum value of $1.50 \pm 0.33$, a range of $0.80 \pm 0.41$ and a standard deviation of $0.13 \pm 0.05$.

For *unc-80(e1069)* experimental worms, the normalized and averaged swimming fluorescence signal has a mean value of $1.00 \pm 0.00$, a minimum value of $0.57 \pm 0.14$, a maximum value of $1.71 \pm 0.31$, a range of $1.14 \pm 0.40$ and a standard deviation of $0.20 \pm 0.05$. For *unc-80(e1069)* control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal during swimming has a mean value of $1.00 \pm 0.00$, a minimum value of $0.73 \pm 0.07$, a maximum value of $1.42 \pm 0.18$, a range of $0.69 \pm 0.22$ and a standard deviation of $0.12 \pm 0.03$.

For *unc-79(e1068)* experimental worms, there is a larger range in the fluorescence signal compared to control worms, with a lower minimum value, a higher maximum value and a larger standard deviation in the signal, but this difference does not reach statistical significance. For experimental worms, there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, or between anterior, mid-body and posterior regions, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm and along the length of the worm body.

For *unc-80(e1069)* experimental worms there is also a significantly larger range in the fluorescence signal compared to control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. There is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle suggesting that body wall muscle activity is comparable on both sides of the worm. However, the fluorescence signal has a significantly higher maximum
value \( (p = 0.0172) \) and a significantly greater range \( (p = 0.0361) \) at posterior body and a significantly greater standard deviation at the mid-body and posterior body compared to the anterior body \( (p = 0.0109) \).

Compared to wildtype swimming worms, the body wall muscle fluorescence signals in \textit{unc-79(e1068)} and \textit{unc-80(e1069)} swimming mutants both show a significantly larger range in fluorescence signal \( (p \text{ values} < 0.0001) \), with a higher maximum fluorescence value \( (p \text{ values} < 0.0001) \), a lower minimum fluorescence value \( (p = 0.0009 \text{ for } \textit{unc-79(e1068)} \text{ worms and } p < 0.0001 \text{ for } \textit{unc-80(e1069)} \text{ worms}) \) and a greater standard deviation \( (p < 0.0001) \). Taken together, these result suggests that the features of body wall muscle calcium signal observed in fainter mutants during crawling locomotion, also persists during swimming locomotion.

There could be several possible explanations for the larger calcium signals observed. It is possible that fainting results in the adaptation of body wall muscle to lower levels of acetylcholine in these mutants. As a result, periods of wildtype-like locomotion could cause body wall muscle to be more excitable and show higher calcium signals than in wildtype worms. Alternatively, the slower oscillations in the calcium signal in these mutants could give GCaMP3 more time to bind calcium and increase fluorescence than in faster-oscillating wildtype muscle.
5.4.3 Imaging muscle activity in unc-55(e402) mutants during crawling

To investigate the pattern of muscle activity in the unc-55(e402) mutant during locomotion and test whether VD neuron mis-wiring results in imbalanced activity between dorsal and ventral muscle, I crossed the integrated *ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* arrays into these mutants. Figure 5.20 shows an example of the unprocessed time lapse images captured by the EMCCD camera for unc-55(e402) mutants crawling between an NGM pad and coverslip during imaging.

![Image of time lapse images for unc-55(e402) mutants with GCaMP3 and GFP channels](image-url)
Figure 5.20: Unprocessed time lapse images from the EMCCD camera for unc-55(e402); \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and unc-55(e402); \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} mutants crawling on an NGM pad. The upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and the lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.034 seconds and shown at 1 second intervals over a total time of 4 seconds. \textbf{A:} unc-55(e402); \textit{ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; \textbf{B:} unc-55(e402); \textit{ljls132[pmyo-3::GFP-SL2-tagRFP-T]} mutant.

Curvature kymographs for both experimental and control worms shows that full dorsal and ventral bends propagate very slowly and unsmoothly along the body in unc-55(e402) mutants compared to wildtype worms (see Figure 5.21). Frequently the mutants adopt a fixed posture in which body curvature changes little over a time period. Similar to the fainter mutants, the fine foraging movements at the head and neck are maintained in these mutants and are indicated by the fast dorsal and ventral bending originating at the tip of the nose and propagating up to body point 20 on the curvature kymograph and fluorescence ratio matrix.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-55(e402); ljs131[pmyo-3::GCaMP3::SL2:tagRFP-T]  unc-55(e402); ljs132[pmyo-3::GFP::SL2:tagRFP-T]

Body Curvature

Dorsal Fluorescence Ratio

Ventral Fluorescence Ratio
Figure 5.21: Example crawling curvature and fluorescence ratio matrices for \(unc-55(e402); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]\) and \(unc-55(e402); ljls132[pmyo-3::GFP-SL2-tagRFP-T]\) mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Body curvature kymograph for \(unc-55(e402); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]\) mutant; B: Body curvature kymograph for \(unc-55(e402); ljls132[pmyo-3::GFP-SL2-tagRFP-T]\) mutant; C: Dorsal fluorescence ratio matrix overlaid on body curvature kymograph for \(unc-55(e402); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]\) mutant; D: Dorsal fluorescence ratio matrix overlaid on body curvature kymograph for \(unc-55(e402); ljls132[pmyo-3::GFP-SL2-tagRFP-T]\) mutant, E: Ventral fluorescence ratio matrix overlaid on body curvature kymograph for \(unc-55(e402); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T]\) mutant; F: Ventral fluorescence ratio matrix overlaid on body curvature kymograph for \(unc-55(e402); ljls132[pmyo-3::GFP-SL2-tagRFP-T]\) mutant.

Plotting the dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body, mid-body and posterior body shows oscillations in the fluorescence ratios corresponding with body bending for experimental worms along the length of their body, but not for control worms (see Figure 5.22).
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-55(e402); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T] unc-55(e402); ljs132[pmyo-3::GFP-SL2-tagRFP-T]

Anterior Body

A

Normalized fluorescence ratios and curvature for anterior body

B

Normalized fluorescence ratios and curvature for anterior body

Mid-body

C

Normalized fluorescence ratios and curvature for mid-body

D

Normalized fluorescence ratios and curvature for mid-body

Posterior Body

E

Normalized fluorescence ratios and curvature for posterior body

F

Normalized fluorescence ratios and curvature for posterior body
Figure 5.22: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for *unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* crawling mutants constrained in z-axis by coverslip. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. 

A: Anterior body for *unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutant; B: Anterior Body for *unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* mutant; C: Mid-body for *unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutant; D: Mid-body for *unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* mutant; E: Posterior Body for *unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutant; F: Posterior Body for *unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]* mutant.
Similar relationships between muscle activity and body curvature are observed in *unc-55(e402)* mutants as in wildtype worms. In experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.59 ± 0.21, mid-body; 0.68 ± 0.13, posterior body; 0.65 ± 0.18). In contrast, the dorsal fluorescence ratio in control worms shows no consistent correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.13 ± 0.25, mid-body; -0.14 ± 0.28, posterior body; 0.12 ± 0.20).

In experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.59 ± 0.18, mid-body; -0.63 ± 0.22, posterior body; -0.68 ± 0.16). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; 0.03 ± 0.24, mid-body; 0.04 ± 0.21, posterior body; -0.01 ± 0.33).

Dorsal and ventral muscle activity also appears to be out-of-phase with each other (see Figure 5.23). Quantifying this by calculating the correlation in calcium signal between dorsal and ventral muscles shows that the fluorescence ratios display a moderate negative correlation with respect to each other along the length of the worm body (mean R value ± stdev for anterior body; -0.30 ± 0.32, mid-body; -0.49 ± 0.18, posterior body; -0.47 ± 0.12). In contrast to this, dorsal and ventral fluorescence ratios in control worms display no consistent correlation with respect to each other (mean R value ± stdev for anterior body; 0.20 ± 0.18, mid-body; -0.02 ± 0.23, posterior body; 0.08 ± 0.16).

Similar to the fainter mutants, plotting dorsal and ventral fluorescence ratios against each other shows a strong hyperbolic relationship between the calcium signal in these opposing muscles along the length of these mutants (see Figure 5.23). This result suggests that muscle activity on one side of the worm body does not increase until the muscle activity on the opposite side has decreased to very near its minimum value, and appears to be a shared feature between mutants that have disrupted locomotion with pausing periods.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

unc-55(e402); jlls131[pgmyo-3::GCaMP3-SL2:tagRFP-T] unc-55(e402); jlls132[pgmyo-3::GFP-SL2:tagRFP-T]

**Anterior Body**

A

Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

B

Scatter plot of dorsal against ventral normalized fluorescence ratio for anterior body

**Mid-body**

C

Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

D

Scatter plot of dorsal against ventral normalized fluorescence ratio for mid-body

**Posterior Body**

E

Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body

F

Scatter plot of dorsal against ventral normalized fluorescence ratio for posterior body
Figure 5.23: Example scatter plots of dorsal fluorescence ratio values against ventral fluorescence ratio values for \textit{unc-55(e402)}; \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{unc-55(e402)}; \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} crawling mutants constrained in z-axis by coverslip. For each frame in a recording, the dorsal and ventral fluorescence ratio values are plotted against each other. A: Anterior body for \textit{unc-55(e402)}; \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; B: Anterior Body for \textit{unc-55(e402)}; \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; C: Mid-body for \textit{unc-55(e402)}; \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; D: Mid-body for \textit{unc-55(e402)}; \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; E: Posterior Body for \textit{unc-55(e402)}; \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; F: Posterior Body for \textit{unc-55(e402)}; \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant.

During episodes where body posture remains fixed, dorsal fluorescence ratios remain higher than ventral fluorescence ratios during dorsal bends (see Figures 5.24) and ventral fluorescence ratios remain higher than dorsal fluorescence ratios during ventral bends (see Figures 5.25). This imbalance between dorsal and ventral fluorescence ratios is not observed in control worms (see Figures 5.24D and F and Figures 5.25D and E) and, in contrast to fainting postures, indicates that the maintenance of these fixed body postures occurs actively through higher levels of calcium and muscle contraction.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

**unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]**

**Fluorescence Ratio**

**Ventral Channels**

**Dorsal Channels**

**unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]**

Figure 5.24: Example plots of dorsal and ventral muscle activity during a fixed dorsal bend for **unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]** and **unc-55(e402); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]** mutants. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio in Figures A and D. Green line plots GCaMP3 fluorescence and red line plots RFP fluorescence in Figures B, C, E and F. **A**: Ventral and dorsal fluorescence ratio; **B**: Ventral GCaMP3 and RFP fluorescence; **C**: Dorsal GCaMP3 and RFP fluorescence; **D**: Ventral and dorsal fluorescence ratio; **E**: Ventral GFP and RFP fluorescence; **F**: Dorsal GFP and RFP fluorescence.
Figure 5.25: Example plots of dorsal and ventral muscle activity during a fixed ventral bend *unc-55(e402); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* and *unc-55(e402); ljs132[pmyo-3::GFP-SL2-tagRFP-T]* mutants. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio in Figures A and D. Green line plots GCaMP3 fluorescence and red line plots RFP fluorescence in Figures B, C, E and F. A: Ventral and dorsal fluorescence ratio; B: Ventral GCaMP3 and RFP fluorescence; C: Dorsal GCaMP3 and RFP fluorescence; D: Ventral and dorsal fluorescence ratio; E: Ventral GFP and RFP fluorescence; F: Dorsal GFP and RFP fluorescence.
Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for \( \text{unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} \) mutants are shown in the table below (see Figure 5.26). For \( \text{unc-55(e402)} \) mutants expressing GCaMP3 in body wall muscle, the normalized and averaged crawling fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.37 ± 0.07, a maximum value of 2.19 ± 0.37, a range of 1.82 ± 0.38 and a standard deviation of 0.44 ± 0.08. For \( \text{unc-55(e402)} \) control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.67 ± 0.09, a maximum value of 1.47 ± 0.21, a range of 0.80 ± 0.27 and a standard deviation of 0.14 ± 0.03.

Experimental worms show a significantly larger range in the fluorescence signal compared to control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. This result is expected as GFP and RFP fluorescence should not change on body curvature with changes in calcium concentration. Compared to wildtype N2 worms, \( \text{unc-55(e402)} \) mutants show significantly lower minimum (\( p = 0.0052 \)) and greater standard deviation (\( p < 0.0001 \)) of fluorescence signal although the maximum of the signal and the range of the signal is comparable (\( p \) values for maximum; 0.5093, range; \( p = 0.8827 \)).

For experimental worms, there is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm and along the length of the worm body. Ventral muscle was not observed to have greater calcium activity than dorsal muscle. This result does not support the hypothesis that VD neuron mis-wiring results in an imbalance of muscle activity. However, the range of the fluorescence signal and its maximum value and standard deviation are significantly greater for the mid-body and posterior body of the worm than for the anterior (\( p \) values for range; 0.0001, maximum; \( p = 0.0002 \), standard deviation; \( p < 0.0001 \)). Such a result was not observed in wildtype worms.
and could suggest that regulation of muscle activity by cross-inhibition plays an increasingly important role down the length of the worm body.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Min</strong></td>
<td>0.40 ± 0.08</td>
<td>0.39 ± 0.06</td>
<td>0.38 ± 0.06</td>
<td>0.36 ± 0.07</td>
<td>0.38 ± 0.07</td>
<td>0.38 ± 0.10</td>
<td>0.33 ± 0.06</td>
<td>*0.37 ± 0.07</td>
</tr>
<tr>
<td><strong>Max</strong></td>
<td>2.23 ± 0.44</td>
<td>1.89 ± 0.38</td>
<td>2.32 ± 0.41</td>
<td>2.22 ± 0.37</td>
<td>2.01 ± 0.31</td>
<td>2.23 ± 0.30</td>
<td>2.46 ± 0.42</td>
<td>2.19 ± 0.37</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>1.83 ± 0.47</td>
<td>1.50 ± 0.43</td>
<td>1.94 ± 0.41</td>
<td>1.85 ± 0.37</td>
<td>1.63 ± 0.35</td>
<td>1.85 ± 0.32</td>
<td>2.13 ± 0.43</td>
<td>1.82 ± 0.38</td>
</tr>
<tr>
<td><strong>StDev</strong></td>
<td>0.38 ± 0.07</td>
<td>0.36 ± 0.08</td>
<td>0.47 ± 0.10</td>
<td>0.45 ± 0.07</td>
<td>0.40 ± 0.08</td>
<td>0.43 ± 0.07</td>
<td>0.52 ± 0.08</td>
<td>*0.44 ± 0.08</td>
</tr>
</tbody>
</table>

Figure 5.26: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling \textit{unc-55(e402); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutants. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, \(n = 15\) worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
5.4.4 Imaging muscle activity in \textit{unc-4(e120)} mutants during crawling

To investigate the pattern of muscle activity in the \textit{unc-4(e120)} mutant during locomotion and test whether VA motor neuron mis-wiring results in increased ventral muscle calcium activity compared to dorsal muscle during forward locomotion and reduced activity during backward locomotion, I crossed the integrated \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} arrays into these mutants. Figure 5.27 shows an example of the unprocessed time lapse images captured by the EMCCD camera for \textit{unc-4(e120)} mutants crawling between an NGM pad and coverslip during imaging.
Figure 5.27: Unprocessed time lapse images from the EMCCD camera for *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants crawling on an NGM pad. The upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and the lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.034 seconds and shown at 1 second intervals over a total time of 4 seconds. A: *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; B: *unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant.

Curvature kymographs for both experimental and control worms show that full dorsal and ventral bends can propagate backward along the body in *unc-4(e402) forward crawling mutants (see Figure 5.28). During reversals, body curvature frequently becomes fixed into a posture which changes little over a time period, before forward crawling is resumed, indicated by ‘R’ on the curvature kymographs. Similar to the fainter mutants and *unc-55(e402) mutants, the fine foraging movements at the head and neck are maintained in *unc-4(e120) mutants and are indicated by the fast dorsal and ventral bending originating at the tip of the nose and propagating up to body point 10 on the curvature kymograph and fluorescence ratio matrix.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

*unc-4(e120); ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]*  *unc-4(e120); ljs132[pmyo-3::GFP-SL2-tagRFP-T]*

**Body Curvature**

A. Kymograph of Body Curvature

B. Kymograph of Body Curvature

**Dorsal Fluorescence Ratio**

C. Dorsal GCaMP3/RFP ratio overlaid on body curvature

D. Dorsal GFP/RFP ratio overlaid on body curvature

**Ventral Fluorescence Ratio**

E. Ventral GCaMP3/RFP ratio overlaid on body curvature

F. Ventral GFP/RFP ratio overlaid on body curvature
Chapter V: Imaging Muscle Activity in Locomotion Mutants

**Figure 5.28:** Example crawling curvature and fluorescence ratio matrices for *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and *unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. **A:** Body curvature kymograph for *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; **B:** Body curvature kymograph for *unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant; **C:** Dorsal fluorescence ratio matrix overlaid on body curvature kymograph for *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; **D:** Dorsal fluorescence ratio matrix overlaid on body curvature kymograph for *unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant; **E:** Ventral fluorescence ratio matrix overlaid on body curvature kymograph for *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; **F:** Ventral fluorescence ratio matrix overlaid on body curvature kymograph for *unc-4(e120); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant.

Plotting the dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body, mid-body and posterior body shows oscillations in the fluorescence ratios corresponding with body bending for experimental worms along the length of their body, but not for control worms (see Figure 5.29).
Figure 5.29: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for unc-4(e120); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] and unc-4(e120); ljls132[pmyo-3::GFP-SL2-tagRFP-T] crawling mutants constrained in z-axis by coverslip. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. A: Anterior body for unc-4(e120); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; B: Anterior Body for unc-4(e120); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutant; C: Mid-body for unc-4(e120); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; D: Mid-body for unc-4(e120); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutant; E: Posterior Body for unc-4(e120); ljls131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; F: Posterior Body for unc-4(e120); ljls132[pmyo-3::GFP-SL2-tagRFP-T] mutant.

Similar relationships between muscle activity and body curvature are observed in unc-4(e120) mutants as in wildtype worms. In experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.75 ± 0.10, mid-body; 0.57 ± 0.33, posterior body; 0.70 ± 0.16). In contrast, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.00 ± 0.31, mid-body; -0.03 ± 0.27, posterior body; 0.02 ± 0.32).

In experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.78 ± 0.05, mid-body; -0.69 ± 0.23, posterior body; -0.70 ± 0.12). In contrast to this, the ventral fluorescence ratio in control worms shows no consistent correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.17 ± 0.33, mid-body; -0.04 ± 0.20, posterior body; 0.02 ± 0.24).

Dorsal and ventral muscle activity appears to be out-of-phase. Quantifying this by calculating the correlation in calcium signal between dorsal and ventral muscles shows that the fluorescence ratios display a moderate negative correlation with respect to each other along the length of the worm body (mean R value ± stdev for anterior body; -0.56 ± 0.10,
mid-body; -0.37 ± 0.29, posterior body; -0.48 ± 0.18). In contrast to this, dorsal and ventral fluorescence ratios in control worms display weak positive correlation with respect to each other (mean R value ± stdev for anterior body; 0.23 ± 0.24, mid-body; 0.13 ± 0.24, posterior body; 0.08 ± 0.21).

During periods of attempted backward locomotion, unc-4(e120) mutants often adopt a fixed body posture which changes little over time. During this time, ventral muscle calcium signal is observed to remain high during ventral body bends and dorsal muscle calcium signal is observed to remain high during dorsal bends. This result is the same as that observed for unc-55(e402) mutants. In disagreement with my original hypothesis, ventral muscle displays robust calcium activity during periods of attempted backward locomotion.

Similar to the fainter mutants and unc-55(e402) mutants, dorsal and ventral fluorescence ratios show a strong hyperbolic relationship to each other in these opposing muscles which becomes more prominent along the length of worm. This result suggests that muscle activity on one side of the worm body does not increase until the muscle activity on the opposite side has decreased to very near its minimum value, and highlights this result as a shared feature between mutants that show significant pausing periods in their locomotion.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants are shown in the table below (see Figure 5.30).
### Chapter V: Imaging Muscle Activity in Locomotion Mutants

#### Figure 5.30: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling *unc-4(e120); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]* mutants. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 12 worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Min</strong></td>
<td>0.40 ± 0.08</td>
<td>0.45 ± 0.08</td>
<td>0.42 ± 0.08</td>
<td>0.40 ± 0.09</td>
<td>0.44 ± 0.07</td>
<td>0.38 ± 0.08</td>
<td>0.38 ± 0.09</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td><strong>Max</strong></td>
<td>2.23 ± 0.44</td>
<td>2.23 ± 0.32</td>
<td>2.52 ± 0.39</td>
<td>2.70 ± 0.34</td>
<td>2.10 ± 0.42</td>
<td>2.43 ± 0.52</td>
<td>2.42 ± 0.39</td>
<td><em>2.40 ± 0.40</em></td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>1.83 ± 0.47</td>
<td>1.78 ± 0.35</td>
<td>2.09 ± 0.38</td>
<td>2.30 ± 0.35</td>
<td>1.66 ± 0.44</td>
<td>2.05 ± 0.52</td>
<td>2.04 ± 0.37</td>
<td><em>1.99 ± 0.40</em></td>
</tr>
<tr>
<td><strong>StDev</strong></td>
<td>0.38 ± 0.07</td>
<td>0.40 ± 0.09</td>
<td>0.48 ± 0.10</td>
<td>0.52 ± 0.12</td>
<td>0.38 ± 0.09</td>
<td>0.49 ± 0.07</td>
<td>0.48 ± 0.12</td>
<td>0.46 ± 0.10</td>
</tr>
</tbody>
</table>
For *unc-4(e120)* mutants expressing GCaMP3 in body wall muscle, the normalized and averaged crawling fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.41 ± 0.08, a maximum value of 2.40 ± 0.40, a range of 1.99 ± 0.40 and a standard deviation of 0.46 ± 0.10. For *unc-4(e120)* control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.68 ± 0.07, a maximum value of 1.45 ± 0.14, a range of 0.77 ± 0.19 and a standard deviation of 0.12 ± 0.02.

Experimental worms show a significantly larger range in the fluorescence signal compared to control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. This result is expected as GFP and RFP fluorescence should not change on body curvature with changes in calcium concentration. Compared to wildtype N2 worms, *unc-4(e120)* mutants show a comparable minimum fluorescence signal (*p* = 0.6043) but a significantly greater range in their fluorescence signal (*p* = 0.0428), with a higher maximum fluorescence signal (*p* = 0.0268) and a larger standard deviation (*p* < 0.0001).

There is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm body. There is no significant difference in minimum fluorescence values along the body, although the range of the fluorescence signal is significantly greater at the mid-body and posterior body compared to anterior body (*p* = 0.0006), with a higher maximum fluorescence signal (*p* = 0.0028) and greater standard deviation (*p* = 0.0007).
5.4.4 Imaging muscle activity in \textit{vab-7(e1562)} mutants during crawling

To investigate the pattern of muscle activity in the \textit{vab-7(e1562)} mutant during locomotion and test whether DB motor neuron mis-wiring results in reduced dorsal muscle activity during locomotion, I crossed the integrated \textit{ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{ljs132[pmyo-3::GFP-SL2-tagRFP-T]} arrays into these mutants. Figure 5.31 shows an example of the unprocessed time lapse images captured by the EMCCD camera for \textit{vab-7(e1562)} mutants crawling between an NGM pad and coverslip during imaging.
Figure 5.31: Unprocessed time lapse images from the EMCCD camera for \textit{vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} mutants crawling on an NGM pad. The upper channel is the green channel (detecting GCaMP3 in experimental worms and GFP in control worms) and the lower channel is the red channel (detecting RFP in both experimental and control worms). Image sequences are taken with an exposure time of 0.034 seconds and shown at 1 second intervals over a total time of 4 seconds. \textbf{A:} \textit{vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; \textbf{B:} \textit{vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant.

Curvature kymographs for both experimental and control worms shows that full dorsal and ventral bends do propagate along the body in \textit{vab-7(e1562)} mutants, though these worms appear to switch frequently between forward and backward locomotion but rarely propagate full forward travelling waves during reversing (see Figure 5.32). Similar to the previous mutants studied, fine foraging movements at the head and neck are maintained in these worms and are indicated by the fast dorsal and ventral bending originating at the tip of the nose and propagating up to body point 20 on the curvature kymograph and fluorescence ratio matrix.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

vab-7[e1562]; ljs131[pmyo-3::GCaMP3-SL2-tagRFP-T] vab-7[e1562]; ljs132[pmyo-3::GFP-SL2-tagRFP-T]

Body Curvature

A

B

Dorsal Fluorescence Ratio

C

D

Ventral Fluorescence Ratio

E

F
Figure 5.32: Example crawling curvature and fluorescence ratio matrices for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] and vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutants constrained in z-axis by coverslip. Curvature kymographs plot the midline curvature along the body over the duration of the recording, with point 1 corresponding to the head and point 100 corresponding to the tail. Red (positive) curvature indicates dorsal bending and blue (negative) curvature indicates ventral bending. Fluorescence ratio matrices for each side of the worm plot high calcium levels in black and low calcium levels in white. A: Body curvature kymograph for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; B: Body curvature kymograph for vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant; C: Dorsal fluorescence ratio matrix overlaid on body curvature kymograph for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; D: Dorsal fluorescence ratio matrix overlaid on body curvature kymograph for vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant, E: Ventral fluorescence ratio matrix overlaid on body curvature kymograph for vab-7(e1562); ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T] mutant; F: Ventral fluorescence ratio matrix overlaid body curvature kymograph for vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T] mutant.

Plotting the dorsal and ventral fluorescence ratios with corresponding body curvature over time for anterior body, mid-body and posterior body shows oscillations in the fluorescence ratios corresponding with body bending for experimental worms along the length of their body, but not for control worms (see Figure 5.33). Noticeably, for mid-body and posterior body, the dorsal fluorescence ratio often remains low even during dorsal bends (see Figure 5.33C and E). This is also observed for ventral muscle, but to a lesser extent.
Chapter V: Imaging Muscle Activity in Locomotion Mutants

**Anterior Body**

A. Normalized fluorescence ratios and curvature for anterior body

**Mid-body**

C. Normalized fluorescence ratios and curvature for mid-body

**Posterior Body**

E. Normalized fluorescence ratios and curvature for posterior body

vab-7(e1562); jis131[pmyo-3::GCaMP3-SL2-tagRFP-T] vab-7(e1562); jis132[pmyo-3::GFP-SL2-tagRFP-T]
Figure 5.33: Example dorsal and ventral fluorescence ratios plotted with body curvature over time for \textit{vab-7(e1562)}; \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{vab-7(e1562); ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} mutants constrained in z-axis by coverslip. Black line plots body curvature, red line plots ventral muscle fluorescence ratio and blue line plots dorsal muscle fluorescence ratio. 

A: Anterior body for \textit{vab-7(e1562)}; \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; B: Anterior Body for \textit{vab-7(e1562)}; \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; C: Mid-body for \textit{vab-7(e1562)}; \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; D: Mid-body for \textit{vab-7(e1562)}; \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; E: Posterior Body for \textit{vab-7(e1562)}; \textit{ljIs131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; F: Posterior Body for \textit{vab-7(e1562)}; \textit{ljIs132[pmyo-3::GFP-SL2-tagRFP-T]} mutant.

Similar relationships between muscle activity and body curvature are observed in \textit{vab-7(e1562)} mutants as in wildtype worms. In experimental worms the dorsal fluorescence ratio shows a moderate positive correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; 0.63 ± 0.16, mid-body; 0.46 ± 0.24, posterior body; 0.33 ± 0.23). In contrast, the dorsal fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; -0.07 ± 0.25, mid-body; -0.11 ± 0.38, posterior body; 0.00 ± 0.28).

In experimental worms the ventral fluorescence ratio shows a moderate negative correlation with positive (dorsal) curvature along the length of the worm (mean R value ± stdev for anterior body; -0.68 ± 0.11, mid-body; -0.60 ± 0.15, posterior body; -0.47 ± 0.20). In contrast to this, the ventral fluorescence ratio in control worms shows no correlation with the curvature of the worm body (mean R value ± stdev for anterior body; 0.04 ± 0.33, mid-body; -0.00 ± 0.26, posterior body; 0.00 ± 0.23).

However, the strength of the correlation between muscle activity and body curvature decreases along the length of the worm. This decrease is greater for dorsal muscle than for ventral muscle. The strength of the correlation also decreases for the out-of-phase relationship between dorsal and ventral muscle activity. Dorsal and ventral muscle activity
display a moderate negative correlation with respect to each other for anterior and mid-body muscle but not for posterior muscle (mean R value ± stdev for anterior body; -0.51 ± 0.19, mid-body; -0.45 ± 0.29, posterior body; -0.15 ± 0.30). In contrast to this, dorsal and ventral fluorescence ratios in control worms display no consistent correlation with respect to each other (mean R value ± stdev for anterior body; 0.14 ± 0.27, mid-body; 0.02 ± 0.27, posterior body; 0.08 ± 0.16).

Similar to the previous mutant worms studied, plotting dorsal and ventral fluorescence ratios against each other shows a hyperbolic relationship between the calcium signal in these opposing muscles for mid-body and posterior body, although this appears weaker in \textit{vab-7(e1562)} mutants. Plotting the fluorescence ratio against curvature shows that for the mid-body and posterior body, dorsal muscle frequently shows little change in fluorescence ratio with changes in curvature (see Figure 5.34C and E), with dorsal fluorescence remaining low even during dorsal bending. This relationship was observed in 6 out of the 11 \textit{vab-7(e1562)} mutants imaged. The same relationship was observed with ventral fluorescence ratios and ventral bending but to a lesser extent (4 out of 11 \textit{vab-7(e1562)} mutants).
Chapter V: Imaging Muscle Activity in Locomotion Mutants

Anterior Body

Scatter plot of fluorescence ratio against curvature for anterior body

Mid-body

Scatter plot of fluorescence ratio against curvature for mid-body

Posterior Body

Scatter plot of fluorescence ratio against curvature for posterior body
Figure 5.34: Example scatter plots of dorsal and ventral fluorescence ratios against curvature for \textit{vab-7(e1562)}; \textit{ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} and \textit{vab-7(e1562)}; \textit{ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutants. For each frame in a recording, the dorsal and ventral fluorescence ratio values and body curvature are plotted as one point. Blue points indicate dorsal ratio and curvature values and red points indicate ventral ratio and curvature values. A: Anterior body for \textit{vab-7(e1562)}; \textit{ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; B: Anterior Body for \textit{vab-7(e1562)}; \textit{ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; C: Mid-body for \textit{vab-7(e1562)}; \textit{ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; D: Mid-body for \textit{vab-7(e1562)}; \textit{ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutant; E: Posterior Body for \textit{vab-7(e1562)}; \textit{ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutant; F: Posterior Body for \textit{vab-7(e1562)}; \textit{ljis132[pmyo-3::GFP-SL2-tagRFP-T]} mutant.

Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for \textit{vab-7(e1562)}; \textit{ljis131[pmyo-3::GCaMP3-SL2-tagRFP-T]} mutants are shown in the table below (see Figure 5.35).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Anterior</th>
<th>Mid-body</th>
<th>Posterior</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Min</td>
<td>0.40 ± 0.08</td>
<td>0.40 ± 0.08</td>
<td>0.39 ± 0.09</td>
<td>0.41 ± 0.11</td>
<td>0.39 ± 0.09</td>
<td>0.41 ± 0.10</td>
<td>0.41 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>2.23 ± 0.44</td>
<td>2.12 ± 0.29</td>
<td>2.69 ± 0.76</td>
<td>2.71 ± 0.67</td>
<td>2.31 ± 0.35</td>
<td>2.46 ± 0.64</td>
<td>2.61 ± 0.76</td>
<td>*2.48 ± 0.58</td>
</tr>
<tr>
<td>Range</td>
<td>1.83 ± 0.47</td>
<td>1.72 ± 0.32</td>
<td>2.30 ± 0.72</td>
<td>2.31 ± 0.66</td>
<td>1.92 ± 0.35</td>
<td>2.07 ± 0.58</td>
<td>2.20 ± 0.73</td>
<td>*2.09 ± 0.56</td>
</tr>
<tr>
<td>StDev</td>
<td>0.38 ± 0.07</td>
<td>0.40 ± 0.10</td>
<td>0.50 ± 0.11</td>
<td>0.47 ± 0.15</td>
<td>0.45 ± 0.09</td>
<td>0.47 ± 0.11</td>
<td>0.46 ± 0.12</td>
<td>*0.46 ± 0.11</td>
</tr>
</tbody>
</table>

Figure 5.35: Summary of measured parameters for dorsal and ventral fluorescence ratio signal for crawling vab-7(e1562); ljls[pmyo-3::GCaMP3-SL2-tagRFP-T] mutants. Dorsal and ventral fluorescence ratios are normalized to their mean fluorescence value and the mean, minimum, maximum, range, variance and standard deviation in the fluorescence signals for experimental worms are shown as the mean ± stdev, n = 11 worms. A ‘*’ indicates a statistically significant difference from wildtype N2 worms.
For \textit{vab-7(e1562)} mutants expressing GCaMP3 in body wall muscle, the normalized and averaged crawling fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.40 ± 0.09, a maximum value of 2.48 ± 0.58, a range of 2.09 ± 0.56 and a standard deviation of 0.46 ± 0.11. For \textit{vab-7(e1562)} control worms expressing GFP in body wall muscle, the normalized and averaged fluorescence signal has a mean value of 1.00 ± 0.00, a minimum value of 0.70 ± 0.08, a maximum value of 1.42 ± 0.12, a range of 0.72 ± 0.17 and a standard deviation of 0.12 ± 0.03.

As expected, \textit{vab-7(e1562)} experimental worms show a significantly larger range in the fluorescence signal compared to \textit{vab-7(e1562)} control worms, with a significantly lower minimum value, a significantly higher maximum value and a significantly larger standard deviation in the signal. Compared to wildtype N2 worms, \textit{vab-7(e1562)} mutants show a comparable minimum fluorescence signal (p = 0.6790) but a significantly greater range in their fluorescence signal (p = 0.0063), with a higher maximum fluorescence signal (p = 0.0074) and a larger standard deviation (p < 0.0001).

There is no significant difference in any of the signal parameters between dorsal and ventral body wall muscle, suggesting that the patterns of body wall muscle activity are comparable on both sides of the worm body. There is no significant difference in minimum fluorescence values along the body, although the range of the fluorescence signal is significantly greater at the posterior body compared to the anterior and mid-body (p = 0.0350), with a higher maximum fluorescence signal (p = 0.0387), although the standard deviations of the fluorescence signals are comparable along the worm body (p = 0.2050).
5.5 Discussion

5.5.1 Basic relationships between muscle activity and body curvature are conserved in locomotion mutants

In this study, I combined automated worm tracking and GCaMP3 calcium imaging from the body wall muscle of several locomotion mutants to investigate how mutations in genes affecting the function of the locomotion circuit change the pattern of muscle activity to generate a locomotion phenotype. I investigated the pattern of muscle activity that is responsible for the fainting phenotype observed in nca-1(gk9);nca-2(gk5), unc-79(e1068) and unc-80(e1069) mutants during crawling and swimming locomotion, as well as the pattern of muscle activity that results from motor neuron mis-wiring in vab-7(e1562), unc-4(e120) and unc-55(e402) mutants during crawling locomotion.

The basic relationships between muscle activity and body curvature observed in wildtype N2 worms are conserved for all mutant strains imaged: dorsal muscle calcium signal correlates with dorsal bending, ventral muscle calcium signal correlates with ventral bending, and dorsal and ventral muscle display reciprocal activity. Muscle activity and body curvature is the final output of the C. elegans locomotion circuit. Despite a variety of genetic mutations affecting the activity of the locomotion circuit, these results demonstrate that body wall muscle activity robustly determines body curvature in C. elegans.

Dorsal and ventral muscle activity frequently display a strong hyperbolic relationship to each other in these locomotion mutants suggesting that muscle activity on one side of the worm body does not increase until the muscle activity on the opposite side has decreased to very near its minimum value. This result suggests that, unlike that observed in wildtype N2 worms, the dorsal and ventral muscles of these mutants are rarely co-active even at very low levels of calcium activity. This appears to be a shared feature of muscle calcium signals between mutants that show slow movement and significant pausing periods in their locomotion.
5.5.2 Muscle activity in the fainter mutants

Imaging body wall muscle activity in the three classes of fainter mutants shows that during crawling and swimming fainting episodes, body wall muscle calcium signal decreases to a baseline level on both the dorsal and ventral sides of the worm regardless of how the body is curved. This suggests that rather than maintaining a fainting posture through high levels of calcium at curved body positions, the maintenance of fainting body posture in these mutants occurs through depletion of calcium and body wall muscle relaxation. In agreement with these results, electrophysiological studies show reduced spontaneous and evoked currents in the body wall muscle of nca-1(gk9); nca-2(gk5) and unc-80(e1068) mutants (Yeh, Ng et al. 2008). However, it is unclear how these worms sustain a curved posture during the calcium depletion because the hydrostatic skeleton should make them stretch out and straighten when the body wall muscle is inactive.

These mutants are capable of bouts of wildtype-like crawling and swimming locomotion. Quantitative analysis of the GCaMP3 calcium signal from these mutants during crawling shows that they display a significantly higher minimum and maximum GCaMP3 fluorescence compared to wildtype N2 worms. Furthermore, during swimming locomotion, the fainter mutants display a significantly greater range, standard deviation and maximum GCaMP3 fluorescence compared to wildtype N2 swimming worms. Taken together, these results suggest that the muscle calcium signal in the fainter mutants is shifted toward a higher baseline and higher maximum value than in wildtype worms.

Cholinergic transmission at the C. elegans neuromuscular junction is mediated by nicotinic acetylcholine receptors (nAChRs) expressed postsynaptically on body wall muscle (Lewis, Wu et al. 1980; Avery and Horvitz 1990). These ligand-gated receptors are activated by their endogenous ligand acetylcholine, as well as the exogenous ligand nicotine. nAChRs are required for worms to exhibit behavioural sensitization to nicotine, whereby repeated intermittent exposure to nicotine evokes robust stimulation of locomotion (Feng, Li et al. 2006). It is possible that frequent fainting results in the intermittent exposure and
sensitization of body wall muscle to acetylcholine. As a result, periods of wildtype-like locomotion could cause body wall muscle to respond to neurotransmitter release with a greater extent of depolarization and calcium influx in the fainter mutants. Optogenetic experiments in presynaptic transmission mutants show similar findings, where body-wall muscle was more excitable than in wildtype worms (Liewald, Brauner et al. 2008).

However, during swimming locomotion *nca-1(gk9);nca-2(gk5)* mutants display a comparable minimum fluorescence to swimming wildtype worms, whereas *unc-79(e1068)* and *unc-80(e1069)* mutants display a significantly reduced minimum fluorescence compared to swimming wildtype worms. It is possible that the smaller changes in calcium concentration during swimming compared to crawling, coupled with calcium depletion from body wall muscles during fainting episodes, contributes to this observed lower minimum fluorescence in these mutants during swimming.

The *nca-1(gk9), nca-2(gk5), unc-79(e1068)* and *unc-80(e1069)* genes encode voltage-insensitive cation leak channel subunits that are expressed in the axon regions of cholinergic motor neurons (Yeh, Ng et al. 2008). Therefore, the pattern of body wall muscle activity observed in the fainter mutants reflects an underlying abnormal pattern of motor neuron activity. Calcium imaging in the hermaphrodite–specific egg-laying neuron (HSNs) cell body and processes of *nca-1(gk9); nca-2(gk5) and unc-80(e1068)* mutants shows that these mutants have decreased synaptic spike frequency and the initiation of calcium transients is disrupted (Yeh, Ng et al. 2008). However, no studies have yet investigated the activity of the A and B motor neurons in these mutants. The results suggest that during fainting episodes, the A and B classes of motor neurons reduce the release of acetylcholine onto body wall muscle. To test this hypothesis it would be necessary to image A and B class motor neuron activity during fainting episodes in these mutants.

In all three fainter mutants areas of high calcium signal are observed to originate independently at the mid-body and tail and do not require propagation from the anterior body. This result suggests that *C. elegans* has some local control of muscle activity and body
curvature along its length. These genes could have a role in the proper propagation of calcium signals from the head of the worm along the body that normally activate the motor circuit, or they could be involved in sensing anterior bending, which results in motor neuron activation occurring randomly in the mutants.

5.5.3 Muscle activity in the mis-wiring mutants

In disagreement to my original hypotheses, none of the mis-wiring mutants imaged displayed an imbalance between dorsal and ventral body wall muscle calcium signal for any of the signal parameters measured (minimum, maximum, range and standard deviation of signal). These results suggests that despite defects in motor neuron wiring, muscle activity remains constrained within values that are comparable along each side of the worm. It is possible that during development, body wall muscle exhibits mechanisms to compensate for the motor neuron mis-wiring and abnormal of neurotransmitter release, to maintain a wildtype-like pattern of muscle activity.

However, the mis-wiring mutants do display significant changes in some calcium signal parameters compared to wildtype worms. Both *unc-4(e120)* and *vab-7(e1562)* mutants, which have mis-wired excitatory neurons (White, Southgate et al. 1992; Ahringer 1996; Esmaeili, Ross et al. 2002), displayed significantly greater range, maximum and standard deviation values for muscle calcium signals compared to wildtype worms. In contrast, *unc-55(e402)* mutants, which have mis-wired inhibitory neurons (Walthall 1990; Walthall and Plunkett 1995), displayed significantly lower minimum values for muscle calcium signals. Unlike for wildtype worms, for all mis-wiring mutants the range and maximum values of the muscle calcium signal increased significantly along the body. Therefore, mis-wiring of motor neurons within the *C. elegans* locomotion circuit does result in altered body wall muscle activity.

Worms carrying the *unc-55(e402)* and *unc-4(e120)* mutations frequently adopt a fixed body posture where the dorsal calcium signal remains higher than the ventral calcium signal
during dorsal bends and the ventral calcium signal remains higher than dorsal calcium signal during ventral bends. For *unc-55(e402)* mutants this occurs during forward locomotion but for *unc-4(e120)* mutants this occurs during periods of attempted backward locomotion. In contrast to the fainter mutants, this indicates that the maintenance of fixed body posture in these worms occurs actively through higher levels of calcium and muscle contraction.

For worms carrying the *vab-7(e1562)* mutation the dorsal muscle at the mid-body and posterior body often shows little change in fluorescence ratio with changes in curvature. Instead, dorsal fluorescence remains low even during dorsal bending and does not consistently oscillate out-of-phase with the ventral fluorescence ratio. A similar pattern of activity is observed in ventral muscle but less frequently. The strength of the correlation between muscle activity and body curvature decreases along the length of the worm and this decrease is greater for dorsal muscle than for ventral muscle. The strength of the correlation also decreases for the out-of-phase relationship between dorsal and ventral muscle activity. It is possible that the transformation of DB motor neurons to DA motor neurons does result in reduced dorsal muscle activity, but with periods of normal dorsal muscle activity giving calcium signal parameters that are comparable to those of ventral muscle in these mutants.

However, the *vab-7(e1562)* gene is also known to function in the development of posterior muscle and epidermis (Ahringer 1996). Dorsal posterior muscle and, to a lesser extent, ventral posterior muscle express the *vab-7* gene. In *vab-7(e1562)* mutants the dorsal and ventral posterior muscle rows do not form properly and are often positioned together laterally. As such, this makes it hard to separate the effect of motor neuron mis-wiring from the effect of abnormal muscle patterning in these mutants. Even so, these worms are observed to curl ventrally during forward locomotion but can coordinate backward movement, suggesting at least some of the observed movement defects have a neural origin.
5.5.4 Relating muscle activity to body posture

In Chapter III I showed that all three fainter mutants display significantly reduced body curvature along their lengths. In contrast, *unc-4(e120)* mutants display a significantly greater body curvature at their head, mid-body, hips and tail than wildtype worms, and *unc-55(e402)* and *vab-7(e1562)* mutants also display a significant increase in body curvature at their mid-body. It is possible that differences in the levels of body wall muscle calcium and contraction observed in the mutants in this study contribute to differences in their body posture and body curvature.

For example, depletion of body wall muscle calcium and relaxation of these muscles during fainting episodes could underlie the adoption of a typical body posture in the fainter mutants, characterized by reduced body curvature.

In contrast, the *unc-4(e120)* and *vab-7(e1562)* mutants display significantly greater range, maximum and standard deviation values for muscle calcium signals compared to wildtype worms. Furthermore, *unc-55(e402)* and *unc-4(e120)* mutants frequently adopt fixed body postures where a high level of calcium and body wall muscle contraction is sustained. Such high and sustained levels of calcium and muscle contraction could contribute to the increased body curvature observed in these mutants.
Chapter VI

Conclusions
6.1 Conclusions

In the previous chapters I have described my PhD work investigating the activity of the locomotion circuit in the nematode worm *C. elegans*. I described the development of a calcium imaging worm tracker and the generation of transgenic lines required for imaging muscle and motor neuron activity within the locomotion circuit. I have presented and discussed the results from analyzing the locomotion phenotypes of wildtype worms and several mutant strains, and the results from imaging body wall muscle activity in these worms. These experiments aimed to give insight into the molecular and cellular basis of locomotion in *C. elegans*.

6.2 Development of the calcium imaging worm tracker

I built a tracking microscope to allow simultaneous whole-worm tracking and imaging of neuromuscular calcium activity. The Schafer lab Worm Tracker 2.0 developed by E. Yemini and T. Jucikas (MRC LMB, UK, http://www.mrc-lmb.cam.ac.uk/wormtracker/, unpublished) was successfully modified for calcium imaging through the addition of an EMCCD camera, beam splitter and magnification lens. Replacement of the original linear actuators with linear slides that have increased load capacity allows the entire dual camera setup to be moved to follow the worm.

Basing the design of the calcium imaging worm tracker on Worm Tracker 2.0 generated several major technical problems that had to be overcome. Worm tracking for Worm Tracker 2.0 occurs through movement of the tracking camera rather than the worm plate. It was thought that such a design to keep the worm stationary would prevent behavioural responses to stage movements and accelerations, and would also aid imaging in liquid environments. Therefore, this design was applied to the calcium imaging tracker. However, for calcium imaging, this required movement of the entire calcium imaging optical pathway, including the EMCCD camera, beam splitter, tube lens and objective lens, which resulted in severe vibrations on stage movement.
Chapter VI: Conclusions

Vibrations were successfully eliminated by reducing the speed and acceleration parameters on the worm tracking software, by physically separating the worm plate holder from the moving stage and by stabilizing each optical component for calcium imaging through a scaffolding system. The current software settings move the stage very slowly so that there are no longer blurred frames which need to be discarded.

Initially I tried using blue and yellow LEDs to provide fluorescence excitation, but I found the yellow lighting was of insufficient power to excite the red reference fluorescence. The LEDs were swapped with 150mW lasers, providing excitation at 488nm (blue) and 561nm (yellow-green). Each laser beam was free-space coupled into a 3.5μm diameter single-mode fibre optic cable but the very small cable diameter makes coupling unstable during stage movement and so the laser illumination requires daily calibration.

If the system were to be replicated, I would build a design that either moves the worm plate and keeps both the tracking and calcium imaging optical pathways stationary, or I would keep the worm plate stationary and fold the optical pathway for the calcium imaging back on itself so that it does not form a long and unstable tower structure. Additionally, I would use fibre-coupled lasers to avoid loss of coupling efficiency on stage movement and daily calibration of the laser illumination. The current design of the calcium imaging tracker is one that has allowed the technical problems described above to be overcome.

6.3 GCaMP3 as a genetically encoded calcium sensor in C. elegans

In making the transgenic lines required for calcium imaging from the body wall muscles and motor neurons, I decided to use the recently-developed GECI GCaMP3 (Tian, Hires et al. 2009). The main reasons for this choice were its increased dynamic range, higher affinity for calcium and significantly shorter fluorescence decay time than other available calcium sensors (Hendel, Mank et al. 2008; Tian, Hires et al. 2009). I found that GCaMP3 displayed greater than 5-fold change in fluorescence during crawling locomotion and it has a
sufficiently high baseline fluorescence to allow the continuous tracking of muscle and motor neurons even when they are at their lowest levels of calcium activity.

However, the use of this sensor did cause several technical problems. My results from body wall muscle imaging suggest that GCaMP3, as a non-ratiometric calcium indicator, is sensitive to movement artefacts within the z-axis. Such movement impacts on the fluorescence signal and using the ratio of the GCaMP3 signal to an RFP signal produced by a co-expressed RFP reference protein does not adequately compensate for such movements in true unconstrained, free-moving worms.

For studies involving free-moving animals where significant motion artefacts are anticipated a FRET-based ratiometric calcium indicator would likely be preferred over non-ratiometric GECIs such as GCaMP3 due to their lower sensitivity to movement artefacts and changes in focus. Where z-axis movement artefacts can be limited and non-ratiometric GECIs can be used, it is essential to co-express such non-ratiometric calcium indicators with a calcium insensitive reference fluorescent protein to allow for ratiometric measurements, and to perform control experiments using a calcium-insensitive fluorescent protein so that true calcium signals can be verified.

Furthermore, I found that there were severe incompatibilities between several promoters and GCaMP3 expression in selected neurons. Despite several attempts, involving changing the vector backbone and the red reference fluorescence protein, I was unable to express GCaMP3 in the A class motor neurons using the unc-4 promoter, in the AVA, AVD and AVE backward command interneurons and in the PVC forward command interneuron using the nmr-1 promoter (Brockie, Mellem et al. 2001), in only the DD motor neurons using the flip-13 promoter (Von Stetina, Watson et al. 2007), or in only the VB motor neurons using the ceh-12 promoter (Von Stetina, Fox et al. 2007).
When screening for F1s after microinjection of such constructs I repeatedly observed fluorescent eggs that failed to hatch and larvae with abnormal development, even when injecting at low DNA concentrations. This suggests that GCaMP3 expression could be toxic to some neurons. Consistent with this hypothesis I also frequently obtained expression of tag-RFP but not expression of GCaMP3. It is possible that these classes of motor neurons are particularly sensitive to the expression of calcium sensors, which if too high, can significantly buffer cellular calcium levels (Hires, Tian et al. 2008). However, these neurons are usually not essential for the survival of the worm. The promoters could express GCaMP3 in other tissues during development or in the germline, where specific concentrations of calcium are essential and disturbed by the calcium buffering effect of the sensor. The focus to produce GECIs with higher calcium affinities to allow single action potential detection likely exacerbates this problem.

6.4 Calcium imaging of motor neuron activity

The GCaMP3 calcium signals so far obtained from D class motor neurons are noisy and variable. There could be several explanations for this observation. Firstly, as a result of the need to keep the whole worm in the field of view for body wall muscle imaging, the lower magnification of the imaging setup compared to similar setups that have been used to investigate neuron activity during worm locomotion ((Ben Arous, Tanizawa et al. 2010; Haspel, O'Donovan et al. 2010; Faumont, Rondeau et al. 2011; Kawano, Po et al. 2011)) could result in lower signal-to-noise when trying to image from smaller motor neurons.

Secondly, despite constraining worm movement within the z-axis using a coverslip, the small physical size of the motor neurons might still allow them to move a sufficiently large amount within the z-axis during body bending to impact on the GCaMP3 calcium signal.

Thirdly, the GCaMP3 expression levels in the motor neuron lines may not be optimal to give a high calcium signal in the motor neurons. As mentioned above, I noted that some neurons are especially sensitive to the expression of calcium sensors, which if too high, can
significantly buffer calcium transients (Hires, Tian et al. 2008). The optimal GECI concentration is therefore a balance between signal-to-noise and buffering. It is possible that the GCaMP3 expression in these lines it too high to obtain an acceptable signal-to-noise ratio.

I am currently working on optimizing the detection of GCaMP3 signals from motor neurons. It is not possible to increase the calcium imaging magnification in the current design of the setup. Therefore, improvements will necessarily come from reducing z-axis motion artefacts during the imaging assays, improving the detection and measurement of fluorescence signals in the neuron calcium imaging analysis or by optimizing GCaMP3 expression in these neurons. Additionally, I have started the construction of motor neuron lines using a new GCaMP3 version 7.112 that has a lower calcium affinity but similar kinetics to GCaMP3 (Eric Schreiter, Janelia Farm Research Campus GECI Project, unpublished).

6.5 Correlating locomotion behaviour with patterns of body wall muscle activity

I used the Schafer lab Worm Tracker 2.0 developed by E. Yemini and T. Jucikas (MRC LMB, UK, http://www.mrc-lmb.cam.ac.uk/wormtracker/, unpublished) to assess the locomotion phenotypes of wildtype N2 worms and several mutant lines. I then used my calcium imaging tracking setup to investigate the pattern of muscle activity in these worms.

My results showed that during forward and backward crawling and during swimming, wildtype dorsal muscle calcium signal correlates with dorsal bending, ventral muscle calcium signal correlates with ventral bending and dorsal and ventral muscle activity is out-of-phase. This result strongly supports the cross-inhibition model of C. elegans locomotion, where dorsal and ventral muscle display alternating activities due to the inhibition and relaxation of muscles on one side of the body when the opposite side is active and contracted.

I further showed that mutants with abnormal locomotion behaviour display altered patterns of body wall muscle activity. It appears that differences in the levels of body wall muscle
calcium and contraction observed in these mutants contribute to differences in their body posture and reduce the overall efficiency of locomotion.

The fainting body postures displayed by nca-1; nca-2, unc-79 and unc-80 mutants result from calcium depletion and body wall muscle relaxation. This pattern of muscle activity could underlie the adoption of a typical body posture in the fainter mutants, characterized by reduced body curvature. Furthermore, all three fainter mutants display a calcium signal that is shifted toward a higher baseline and higher maximum value, and areas of high calcium signal are frequently observed to originate at the mid-body and tail and do not require propagation from the anterior body.

These results suggest that the nca-1, nca-2, unc-79 and unc-80 genes are critically required for the maintenance of wildtype levels of body wall muscle calcium and contraction. As these genes are expressed in the cholinergic motor neurons the pattern of body wall muscle activity observed in the fainter mutants reflects an underlying abnormal pattern of motor neuron activity and acetylcholine release. It is unclear how these worms retain a curved body posture during calcium depletion because the hydrostatic skeleton should make them straighten when the body wall muscle is inactive. Body wall muscle in these mutants could undergo compensatory mechanisms that allow it to exhibit greater excitability to lower neurotransmitter release.

It is possible that these genes have a role in the proper propagation of calcium signals from the head of the worm along the body that normally activate the motor circuit, or they could be involved in sensing anterior bending, which results in motor neuron activation occurring randomly in the mutants. To address these questions, muscle activity could be imaged in microfluidic channels to induce bending. The hypothesis would be that inducing bending does not effectively cause calcium transients as it does in wildtype worms (Wen, Q et al., unpublished). It may be possible to distinguish between defects in bend sensing or propagation because a propagation defect would probably not impact calcium induced right at the point of bending but would prevent it from activating muscle calcium further away. It
would also be interesting to image cholinergic motor neuron activity during fainting episodes in these mutants.

In contrast, \textit{unc-55(e402)} and \textit{unc-4(e120)} mutants frequently adopt fixed body postures through the maintenance of higher levels of calcium and muscle contraction, which could contribute to the increased body curvature and pausing episodes observed in these mutants. For \textit{unc-55(e402)} mutants with imbalanced D class motor neuron function this occurs during forward locomotion, but for \textit{unc-4(e120)} mutants with mis-wired A class backward motor neurons this occurs during periods of attempted backward locomotion. These results show that correct motor neuron wiring is essential for the generation of wildtype patterns of body wall muscle calcium activity and contraction which allow the proper coordination of body bending and efficient forward and backward locomotion.

The above results suggest a general relationship whereby the strength of muscle activity correlates with the degree of body curvature. Indeed, I observed that the changes in the calcium concentration during swimming are smaller than those observed during crawling (2.3-fold change in fluorescence signal with body bending on swimming compared to 5.6-fold on crawling) and is correlated with the shallower body bending for this form of locomotion. To test this hypothesis, swimming worms could be imaged in environments of increasing viscosity and the magnitude of the body wall muscle calcium signal measured. It would be predicted that the magnitude of the calcium signal would increase with increasing viscosity. If improvements to calcium imaging are successful, it would be interesting to see whether the relationships observed between body wall muscle activity and body curvature are also observed for motor neurons.

However, it is also possible that the off kinetics of GCaMP3 are too slow to detect the oscillations in calcium concentration during swimming. A lower variance in GCaMP3 fluorescence would then be explained if the fluorescence fails to return to baseline during each bending cycle. This could be tested by comparing these results to those using calcium dyes or GECIs with faster kinetics.


Rankin, C. H. (2002). "From gene to identified neuron to behaviour in Caenorhabditis
Richmond, J. E. and K. S. Broadie (2002). "The synaptic vesicle cycle: exocytosis and
endocytosis in Drosophila and C-elegans." Current Opinion in Neurobiology 12(5):
499-507.
Richmond, J. E. and E. M. Jorgensen (1999). "One GABA and two acetylcholine receptors
function at the C. elegans neuromuscular junction." Nat Neurosci 2(9): 791-797.
Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.
343.
classes similarities and differences between in Caenorhabditis elegans."
Shreffler, W., T. Magardino, et al. (1995). "The Unc-8 and Sup-40 Genes Regulate Ion-
Channel Function in Caenorhabditis-Elegans Motorneurons." Genetics 139(3): 1261-
1272.
537-548.
Nematode Panagrellus-Redivivus - Description and Comparison with Those of
Sulston, J. E. (1976). "Post-Embryonic Development in Ventral Cord of Caenorhabditis-
Elegans." Philosophical Transactions of the Royal Society of London Series B-
demonstrates a specific role for the MEC-4 channel in the process of gentle touch


