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Optical imaging of neuronal population dynamics in the leech central nervous system

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Briggman, Kevin L.

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Optical Imaging of Neuronal Population Dynamics
in the Leech Central Nervous System

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in Biology

by

Kevin L. Briggman

Committee in charge:

Professor William B. Kristan Jr., Chair
Professor Henry D. I. Abarbanel
Professor Marla B. Feller
Professor David Kleinfeld
Professor Nicholas C. Spitzer

2005
The dissertation of Kevin L. Briggman is approved, and it is acceptable in quality and form for publication on microfilm:

\[\text{Signed signatures}\]

Chair

University of California, San Diego

2005
To Mom and Dad
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This dissertation is organized into three chapters (Chapters 2, 3, & 4), with an introductory chapter (Chapter 1). Chapter 3, in full, was published in *Science*, volume 307, pages 896-901. It is printed here with permission of the publisher, the American Association for the Advancement of Science (Washington, DC). Permission to use this paper in the dissertation was obtained from the co-authors, Henry D. I. Abarbanel and William B. Kristan, Jr. Chapter 4 will be submitted to the journal *Nature Neuroscience*, published by Nature Publishing Group (New York, NY). Permission to use this paper in the dissertation was obtained from the co-author, William B. Kristan, Jr. The dissertation author was the primary investigator in both papers.
CURRICULUM VITA

Education

Ph.D., December 2005. University of California, San Diego, La Jolla, CA. Computational Neurobiology Program; Division of Biological Sciences Advisor: William B. Kristan Jr., Ph.D.

B.S., May 2000. Case Western Reserve University, Cleveland, OH. Degree in Electrical Engineering and Applied Physics Advisor: Hillel J. Chiel, Ph.D.

Teaching

2003/2004 Neural Systems & Behavior Course Teaching Assistant Marine Biological Laboratory, Woods Hole, MA

Publications


Funding and Awards

- Grass Fellowship in Neurobiology (Marine Biological Laboratory, Woods Hole, MA; 2005)
ABSTRACT OF THE DISSERTATION

Optical Imaging of Neuronal Population Dynamics
in the Leech Central Nervous System

by

Kevin L. Briggman
Doctor of Philosophy in Biology
University of California, San Diego, 2005
Professor William B. Kristan Jr., Chair

The focus of my dissertation centers on how networks of neurons interact to generate behaviors in the medicinal leech. I used voltage-sensitive dye imaging to simultaneously record from large populations of individual neurons to ask three related questions: 1) When and in what neurons is the decision made to either swim or crawl in response to a sensory stimulus? 2) Can single neurons influence the decision between swimming and crawling? 3) Do the swimming and crawling central pattern generators (CPGs) exist as distinct neuronal circuits, or do they share some of the same circuitry in a multifunctional manner? The first question was addressed by analyzing population recordings of the time between when a stimulus is delivered and when the choice between swimming and crawling is made. The result of the analysis highlighted populations of neurons that discriminated between the two behaviors earlier than any single neuron. The second question was addressed by attempting to manipulate candidate decision-making neurons in this population. We found a neuron, cell 208, which was sufficient to bias the
choice between swimming and crawling with intracellular current injection. Thus, cell 208 participates in the decision-making process. The third question was motivated by the need to understand how the two CPGs are organized. By recording ongoing swimming and crawling in the same preparation, we discovered that twice as many cells in a ganglion oscillated with crawling compared to swimming. Of the cells that oscillated with swimming, a large percentage also oscillated with crawling. We characterized two previously unidentified interneurons, one that has a multifunctional role in both swimming and crawling and one that is dedicated to crawling.
CHAPTER 1

Introduction

Advances in recording technologies over the last decade have opened up the possibility of monitoring the activity of large populations of neurons simultaneously. Both optical (Baker et al., 2005) and extracellular (Buzsaki, 2004) techniques can now be used to achieve recordings at the level of single cells and single trials. This level of detail is necessary in order to analyze networks of neurons as dynamical systems; averaging over space or time likely obscures much of the dynamics. The use of optical recording techniques is particularly attractive because it allows the experimenter to identify the very neurons that are recorded.

Invertebrate nervous systems are well suited to take advantage of optical techniques due to the stereotyped positions of neurons within ganglia. The two most commonly recorded optical signals are intracellular calcium transients and membrane potentials. While fluorescent calcium indicators offer large signal-to-noise ratios (Ikegaya et al., 2005), they only indirectly report the electrical activity of neurons. Membrane potential indicators (voltage-sensitive dyes; VSDs) are capable of directly measuring both depolarizations and hyperpolarizations of neurons (Gonzalez et al., 1999). The power of single cell resolution VSD imaging of neuronal populations was notably demonstrated in the abdominal ganglion of *Aplysia californica* (Wu et al., 1994; Tsau et al., 1994). Prior to these studies, the extent to which a sensory stimulus activates populations of neurons in this ganglion was unknown. While pairwise intracellular recordings are useful for characterizing synaptic connections between neurons, they provide only a narrow view on the function of networks as a whole.
Given the ability to record from many neurons simultaneously, it is equally important to develop and apply appropriate multidimensional analysis techniques. Existing analysis techniques can loosely be divided into linear correlation-based techniques and nonlinear techniques that take advantage of information contained in higher order statistics, such as information theoretic approaches (Shamir & Sompolinsky, 2004). For the time being, the analysis of population recordings as systems has largely relied on linear techniques. Studies have looked for correlations in space (across cells) and time, and ideally, both.

Neuronal population recordings generally result in a data matrix of the activity of many cells across time (Fig 1.1A, i). The goal is to relate the activity of the population to a stimulus or behavior (Fig 1.1A, ii). Analysis is usually performed in two steps: first the relevant dynamics (Fig 1.1A, iv) are extracted (Fig 1.1A, iii) from the data matrix; the dynamics are then related to features of the stimulus or behavior (Fig 1.1A, v). Often the second step involves using the neural dynamics to classify or discriminate a feature (Fig 1.1A, vi).

Some of the commonly used extraction techniques (Fig 1.1B) and recent applications include: embedding techniques such as locally linear embedding (LLE; Roweis & Saul, 2000; Stopfer et al., 2003), principal component analysis (PCA; Chapin & Nicolelis, 1999; Gervasoni et al., 2004), independent component analysis (ICA; Hyvarinen & Oja, 2000; Makeig et al., 2004), information theoretic techniques (Schnitzer & Meister, 2003; Sharpee et al., 2004) and traditional cross-correlation techniques in the time and frequency domains. Classification/discrimination techniques (Fig 1.1C; Duda et al., 2000) and recent applications include: cluster-based algorithms (Fee et al., 1996; Fellous et al., 2004; Matsumoto et al., 2005), factor analysis (FA; Friedrich & Laurent, 2001), linear discriminant analysis (LDA; Averbeck et al., 2003; Laubach 2004), support-vector machines (Vapnik, 1998; Fdez Galan et al., 2004), information theoretic approaches (Paninski et al., 2004), and peri-stimulus time histogram based methods (PSTH; Foffani
Figure 1.1. A) A generalized schematic for the analysis of neuronal population recordings. The goal is to relate a data matrix (i) containing the activity patterns of many neurons to a stimulus of behavior (ii). The first step involves extracting (iii) the relevant dynamics (iv) from the dataset. A second step attempts to classify or discriminate (vi) features of the stimulus or behavior (v). B) A large variety of extraction techniques exist; some of the popular techniques involve embedding techniques, component analyses, and joint cross-correlations between cells. C) Many classification/discrimination techniques are currently used including: different types of cluster analysis, factor analysis, discriminant analysis, and many other correlation-based methods. See text for recent examples of the use of both extraction and classification/discrimination techniques.
& Moxon, 2004; Gerstein & Kirkland, 2001). These lists are representative and the techniques used for the two analysis steps are, of course, not mutually exclusive. Many of the techniques are rooted in multivariate statistics (Mardia et al., 1980) and have been applied in many different fields of study (Heberger et al., 2003). Figure 1.1 emphasizes that though the combination of techniques may differ, researchers are generally interested in the same goal of relating population dynamics to a stimulus or behavior.

The aim of this dissertation was to use the recently developed imaging technique (Cacciatore et al., 1999; Gonzalez et al., 1999) of a fluorescence resonance energy transfer (FRET) based VSD to answer biological questions in the central nervous system (CNS) of the medicinal leech. The experiments described here were interdisciplinary in that they combined aspects of engineering, multidimensional data analysis, and optical and electrophysiological experimental techniques. Chapter 2 focuses on improvements I developed for the VSD technique that allowed me to make the recordings presented in Chapters 3 and 4. The primary improvement was the construction of a device to perform full-field alternating frame ratiometric imaging. This improvement roughly doubled the signal-to-noise ratio of the optical signals and helped reduce photobleaching and motion artifacts. The second improvement was the development of a custom image acquisition system. Precise control over the shutters and CCD camera allowed me to extend the duration of imaging trials from 10 seconds to 60 seconds. This improvement was necessary in order to image slow rhythmic behaviors like crawling.

The experimental work in the dissertation was largely driven by the observation that, in response to the same sensory stimulus, swimming and crawling are probabilistically elicited. This interested me because it meant that these behaviors are not simply
deterministic, reflexive responses to a stimulus. We termed the selection of a motor pattern “decision-making”, however it is also commonly referred to as “behavioral choice” in invertebrate systems (Kovac & Davis, 1977; Shaw & Kristan, 1997). I was interested in the dynamics of the CNS between when the stimulus was delivered and the onset of the motor patterns. It was presumably in this time period that the animal decided to swim or crawl. Chapter 3 is the result of experiments in which I imaged the ventral surface of segmental ganglia during this decision period. I used principal component analysis (PCA) and linear discriminant analysis (LDA) to visualize the temporal trajectories of the activity of population of neurons and to determine which neurons helped discriminate between swimming and crawling. The principal findings were that a population of neurons discriminates earlier than any single cell and that a neuron in the population, cell 208, is sufficient to bias the choice towards swimming or crawling when intracellurally stimulated.

While these experiments provided a clue about the distributed nature of the decision-making process, they did not determine the mechanism by which cell 208 biases the choice. There were two possible approaches to address this: 1) identify postsynaptic follower cells of cell 208 using the technique of Taylor et al., 2003, or 2) determine how the circuitry of the central pattern generators (CPGs) underlying swimming and crawling interacts, if at all. I chose the latter approach because, while there was some evidence for overlap between the two CPGs, the circuitry of the crawling CPG is unknown. For example, cell 208 oscillates with both swimming (Weeks, 1982) and crawling (Baader, 1997), but its role in the generation of crawling has not been studied. I believe it is critical to understand how both rhythmic behaviors are generated in order to better understand the behavioral choice mechanism.
Chapter 4 focuses on the multifunctional overlap between the two CPGs. Recording from populations of neurons simultaneously allowed me to sample a large percentage (~90%) of the total number of neurons in a segmental ganglion. This approach revealed the number and distribution of neurons that oscillated with each behavior as well as the distribution of the overlap between neurons that oscillated with both behaviors. I used a coherence-based approach (Taylor et al., 2003) to quantify the phase relationships among neurons. Phase maps provided a global picture of the dynamics during the two behaviors; they also serve as guides to identify interesting neurons. I focused on two previously unidentified interneurons, cell 255 and cell 257. I classified cell 255 as a multifunctional interneuron based on its ability to perturb both rhythms. Cell 257, on the other hand, is dedicated to crawling because it is hyperpolarized during swimming. It was surprising that the two CPGs overlap by such a large degree. In addition, I view the generation of the phase maps and the characterization of these two cells as a first step in elucidating the CPG for crawling and the identification of additional elements of the decision-making circuitry.
CHAPTER 2

Technique Development

Voltage-Sensitive Dye Imaging

The primary technology used for the experiments described in the following chapters was voltage-sensitive dye (VSD) imaging. The development of FRET-based VSDs with high sensitivity (Gonzalez et al., 1999) introduced the possibility of monitoring subthreshold membrane potential fluctuations of large populations of neurons with single cell resolution (Cacciatore et al., 1999). The dyes have been used successfully to identify follower cells of important command cells in the leech nervous system (Taylor et al., 2003). This technique involved stimulating a presynaptic cell at a fixed frequency (1 Hz) and calculating the coherence of imaged cells at the same frequency. There are, however, several limitations to the use of these dyes. I will first discuss the limitations and then the improvements we developed to overcome some of these limitations.

Technical Limitations

Photobleaching

The VSD we used is susceptible to strong photobleaching. Empirically, this is seen as an initially rapid decrease in fluorescence followed by a slower prolonged decrease. For the follower experiments described above, this effect was not a problem because the signals were high pass filtered and analyzed at the driving frequency. Photobleaching is a serious problem when one is interested in signals that are changing on the same timescale as the bleaching time constant. In this case, there is no obvious way to filter signals to remove the bleaching artifact.
Phototoxicity

The phototoxicity experienced by cells is proportional to the intensity and the duration of exposure to the excitation light source. Practically, this limited the total imaging time to approximately 300 s. when imaging at 10 Hz. Unfortunately, this imaging time had to be split into 10 s. trials to allow cells to recover from phototoxic damage in between trials. This limitation prevents imaging of dynamics with time scales longer than 10 s.

Sensitivity

The sensitivity of the FRET-based VSD is higher than other types of VSDs, typically 0.5-1% per 10 mV based on the coumarin emission wavelength (Taylor et al., 2003). However, these sensitivities were measured using a 40x water immersion objective, in which less than half a ganglion was visible in the field of view. The signal-to-noise ratio was therefore improved due to the spatial averaging of many pixels per cell. The desire to image the entire surface of a ganglion requires a 20x objective, which reduces the SNR ratio per cell. Thus, an increase in sensitivity is desirable. The FRET-based VSD is inherently a ratiometric indicator (Gonzalez et al., 1999), so imaging both emission wavelengths could approximately double the sensitivity.

Motion Artifacts

The isolated ventral nerve cord of the leech unfortunately contains muscles in the connective nerves. These muscles are active following nerve shocks and during ongoing rhythmic behaviors. While pinning down small strips of tissue across the cord minimizes some motion artifact (Taylor et al., 2003), there still exists the danger that slow motion artifacts be mistaken as neural signals.
Technical Improvements

The limitations described above prevented the goal of imaging the whole surface of a ganglion for longer than 10 s. without filtering the optical signals. A solution to the limitations of photobleaching, sensitivity, and motion artifact is to ratio the two emission wavelengths. Common mode signals occurring in both emission wavelengths will be eliminated. This overcomes the photobleaching problem, assuming there is no differential bleaching between the two fluorophores. It also protects against motion artifacts, since any motion would cause a common change in the fluorescence emitted at both wavelengths. Finally, ratiometric imaging improves the signal to noise ratio by combining the sensitivities of both emission wavelengths.

Currently, the most common method for ratioing brightfield images with a CCD camera is to divide the imaging area of the CCD in half. The two emission wavelengths are then imaged simultaneously onto the two halves. This has the advantage of simultaneous recording of the two signals. The main disadvantage is that the field of view is cut in half. This solution was undesirable because the goal was to record from an entire ganglion. The other option, instead of dividing space, is to divide time between the emission wavelengths. In this scheme, alternating frames capture alternating wavelengths. This allows one to take advantage of the full field of view and resolution of the CCD camera. The disadvantage is that the time resolution is halved for a given frame rate. This solution is acceptable given the slow time constant (~500 ms) of the VSD we used.

Unfortunately, no commercially available device exists to perform alternating frame ratioing (AFR). We therefore designed and built a novel device to achieve AFR. The design schematic for the device is shown in Figure 2.1. This basic idea was to split the emission wavelengths into two optical paths. Each path contains a small diameter rapid shutter to control which wavelength is imaged onto the CCD camera. Alternating the
Fig. 2.1. A schematic of the alternating frame ratioing device. Unfiltered emission light is collimated with a plano-convex singlet lens of equal focal length to the tube lens. The collimated emission light is then split into two optical paths with a 515 nm dichroic mirror. Along one path the light passes a 560LP filter (oxonol), along the other path the light is bandpass filtered with a 460BP filter (coumarin). Each path contains a small diameter (2mm) shutter that can be rapidly (<200 µs) opened and closed. The light in each path is focused through the shutters with achromatic doublet lenses. High-reflectance mirrors in each path steer the light in each path. The two paths are recombined with a 515 nm dichroic and then imaged onto a CCD camera.
opening and closing of these shutters for each frame achieves AFR. The stack of images is then divided into two stacks, one for each wavelength. The frame rate of the camera was usually set to 20 Hz, which, after ratioing, yields 10 Hz ratioed signals.

An example of signals recorded with this technique is shown in Figure 2.2. The ratio of the two emission wavelengths demonstrates the correction of the photobleaching effect (there was minimal differential bleaching). There was no obvious motion artifact in this example, but motion artifacts are also corrected with this technique. These two benefits of AFR allow unfiltered, raw optical signals to be used in the analysis. Finally, the increased sensitivity can be seen in the ratioed signal compared to either emission wavelength alone. Typical sensitivities using AFR were measured to be 1.5-2% per 10 mV (at 1 Hz), approximately double the sensitivity without ratioing. This allowed imaging to be performed with a 20x objective and signals to be recorded from small diameter cells from the entire surface of a ganglion.

Finally, the limitation of phototoxicity prevented continuous imaging of behaviors lasting longer than 10 s. While this is suitable to imaging swimming in the leech (~1 Hz), it prevented imaging of slower behaviors like crawling (0.05-0.1 Hz). To address this limitation, we developed custom acquisition software in Matlab. Integrating this software with the AFR device and the CCD camera allowed for precise control of frame acquisition sequences. The strategy was to image at slower frame rates (2 Hz) for longer periods of time (up to 60 s. continuously). We found that rapidly shuttering the excitation light on and off, yielding ‘snapshots’ at 2 Hz, prevented any measurable phototoxicity during the course of a 60 s. trial. An example of a 60 s. imaging trial during crawling is shown in Figure 2.3.

In summary, the development of a device to ratiometrically record from full frame fields of view has extended the use of the FRET-based VSDs. This device largely eliminates problems associated with photobleaching correction and motion artifacts.
Fig. 2.2 An example of signals obtained from ratiometric imaging of a single cell. The two emission wavelengths (coumarin and oxonol) are divided to yield the ratioed signal. All signals are expressed as percent changes in fluorescence. A simultaneous intracellular recording from the imaged neuron in the bottom panel demonstrates the high sensitivity of the dyes.
It also approximately doubles the sensitivity of the optical signals. Integrating this device into a fully customizable acquisition system allowed us to extend the time of continuous acquisition to 60 s. and to record crawling rhythms optically.
**Fig. 2.3** An example of a 60 second trial imaged at 2 Hz. Intracellular voltage (black) traces from two cells are overlaid with the simultaneously recorded raw optical (red) signals. There was no measurable phototoxicity during trials of this duration.
CHAPTER 3

Optical Imaging of Neuronal Populations During Decision-Making

Abstract

We investigated decision-making in the leech nervous system by stimulating identical sensory inputs that sometimes elicit crawling and other times swimming. Neuronal populations were monitored with voltage-sensitive dyes following each stimulus. By quantifying the discrimination time of each neuron, we found single neurons that discriminate prior to the two behaviors. We used principal component analysis and linear discriminant analysis to find populations of neurons that discriminated earlier than any single neuron. The analysis highlighted a neuron, cell 208. Hyperpolarizing cell 208 during a stimulus biases the leech to swim; depolarizing it biases the leech to crawl or to delay swimming.

Introduction

Understanding the mechanisms of behavioral choice would be a major step in bringing together neuroscience, psychology, and ethology (Glimcher 2003). Research into decision-making has used several different strategies. One very productive approach is to have an animal make a sensory discrimination between very similar stimuli while recording the activity of neurons in various parts of the primate nervous system (Shadlen & Newsome 1996, Glimcher 2001, Schall 2000, Shadlen & Newsome 2001, Gold & Shadlen 2000, Platt & Glimcher 1999, Romo & Salinas 2001). A second approach uses choice competition: presenting an animal with two stimuli that produce mutually exclusive behaviors (choices), to see which behavior predominates (Kovac & Davis 1977). This has led to the notion that behavioral choices are hierarchical. The neuronal mechanism
originally proposed to underlie behavioral hierarchies was inhibitory interactions among the neurons responsible for triggering the different behaviors (Kovac & Davis 1980). Later work has found neurons that elicit one behavior are often activated during other, sometimes conflicting, behaviors (Esch & Kristan 2002, Popescu & Frost 2002). Among other things, this observation suggests that individual decision-making neurons can be multiplexed—they contribute to choosing more than one behavior—and that they trigger behaviors by being active with other combinations of neurons.

We used a third approach to study decision-making: choice variability. We presented a nervous system with identical stimuli that repeatedly produce two different, mutually exclusive behaviors with roughly equal probabilities. This approach allowed us to focus on neurons involved in decision-making that are downstream from neurons used to make sensory discriminations.

We used the isolated central nervous system (CNS) of the medicinal leech. Motor neuron activity patterns characteristic of swimming (Kristan & Calabrese 1976) and crawling (Eisenhart et al. 2000) can be elicited from isolated preparations by electrically stimulating peripheral nerves. Such sensory stimulation activates mechanosensory neurons in patterns that mimic touching the leech’s skin (Kristan 1982). Stimulating the same kinds of mechanosensory neurons in different locations on the leech produce characteristic behaviors like swimming or crawling (Kristan et al. 1982, Cacciatore et al. 2001). We follow the terminology proposed by Schall (Schall 2001), referring to the different behavioral outputs as choices and the process leading up to a choice as decision-making.

Previously, recording from neurons intracellularly one at a time, then stimulating them to determine their effect on the initiation of behavior has successfully uncovered interneurons that activate swimming (Weeks & Kristan 1978, Brodfuehrer & Burns 1995), crawling (Esch et al. 2002), and shortening (Shaw & Kristan 1997). However, to explore how decisions are made by populations of neurons (Esch & Kristan 2002), we needed to
record from many neurons at once (Zochowski et al. 2000). We therefore used voltage-sensitive dyes (Gonzalez et al. 1999, Cacciatore et al. 1999) that allow us to record simultaneously from many neurons in a midbody segmental ganglion at a resolution better than 5 mV (Taylor et al. 2003).

**Results**

**The leech CNS makes behavioral choices**

The isolated leech CNS consists of a nerve cord connecting 21 segmental ganglia plus a head and tail brain (Fig. 3.1A). This preparation generates motor patterns that are recognizable as behaviors observed in intact leeches, including swimming and crawling. We exposed one ganglion between G7 and G10 for voltage-sensitive dye imaging. We also recorded extracellularly from at least two peripheral nerves (DP nerves) using suction electrodes. These electrodes were used to both stimulate and record. A train of electrical pulses to a DP nerve mimics a touch to the body wall in an intact leech (Kristan 1982) and can elicit both swimming and crawling (Fig. 3.1B). By stimulating DP nerves between G13 and G16 we evoked swimming in about half of the trials (blue) and crawling in the other half (red).

Each trial lasted 60 seconds, with an inter-trial interval of three minutes. While the nerve activity was recorded for the entire trial, the ganglion was imaged during only the initial 10 seconds (Fig. 3.1B, green bar). We imaged the neurons on the ventral surface of a midbody segmental ganglion (Fig. 3.1C). In each preparation, we were able to resolve 130-150 of the ~160 known neurons (Muller et al. 1981) on the ventral surface. The FRET-based voltage-sensitive dye we used (Gonzalez et al. 1999, Cacciatore et al. 1999) is very sensitive to small membrane potential fluctuations (Fig. 3.1D).
**Fig. 3.1.** Recording behavioral choices in the isolated leech CNS.  

**A)** Schematic of the sites of recording and stimulation. A midbody (G7-G10) ganglion was imaged with a voltage-sensitive dye (VSD). Suction electrodes were used to record from and stimulate dorsal posterior (DP) nerves (DP13-DP15).  

**B)** Eight sequential 60-second trials demonstrate inter-trial variability. The stimulus (2-3 V, 10 ms pulses at 15 Hz) lasted 300 ms (small black bar). A ganglion was imaged during the initial 10 seconds of each trial (green bar). Trials are color-coded by behavior; swimming (blue, ~1-2 Hz bursts) or crawling (red, ~0.05-0.1 Hz bursts).  

**C)** The population of neurons (143 in this example) from which we recorded on the ventral surface of a ganglion.  

**D)** We averaged the pixels from each neuron to produce a time-varying record of the percentage change in the fluorescence signal (measured as dF/F). The top two traces are the signals from the two VSD molecules, coumarin and oxonol. The largest and least noisy signal is the ratio of these two signals (third trace); it was used for all further analysis. The simultaneous intracellular recording (bottom trace) demonstrates the high sensitivity of the dye (15 – 20% / 100mV).  

**E, F)** Raw optical data from two trials, one which elicited swimming (E) and the other crawling (F). The optical signal for each neuron is plotted versus time. Color encodes the percent change in fluorescence (dF/F): positive changes (red) correspond to relative depolarization and negative changes (blue) correspond to relative hyperpolarization. The panels below each raster plot are simultaneous DP nerve recordings. Vertical black lines indicate the onset and duration of the stimulus.
For comparison, we show the raw data from a swim trial and a crawl trial (Fig. 3.1E, F). Almost every neuron was activated immediately after each stimulus. There was a clear difference in the activity of many neurons once the motor pattern was apparent in the nerve recordings, usually after about 4 seconds. These are presumably the central pattern generating or motor neurons that generate either swimming or crawling. We were more interested in activity differences prior to this time, between the stimulus and 4 seconds, when the decision between the two behaviors was made.

**Discrimination by single neurons**

The activity patterns of a subpopulation of single neurons were able to discriminate swimming trials from crawling trials. Of the neurons that responded to the stimulus, we observed four classes of responses: non-discriminating cells (ND cells), early discriminating cells (ED cells), late discriminating cells (LD cells), and transiently discriminating cells (TD cells) (Fig. 3.2A). We quantified the earliest discrimination time ($t_{SC}$) by performing a sliding window analysis of variance (ANOVA) for each cell (Fig. 3.2B).

Neurons were ordered by their earliest discrimination times (Fig. 3.2C). The number of cells that discriminated at some point in time (ED, LD, or TD cells) ranged between 50 – 75% across experiments. We also performed an ANOVA on the nerve recordings (see Materials and Methods) to determine the time ($t_{NERVE}$) at which we could discriminate the behaviors based on motor neuron activity (green line, Figs. 3.2C and 3.2D). We were most interested in cells that discriminate prior to $t_{NERVE}$ as these cells are predictive of the behaviors and are candidate decision-making neurons. A histogram of the earliest $t_{SC}$ for each cell shows that 17 cells (mean +/- SD = 19 +/- 6 cells; n = 6) discriminated prior to $t_{NERVE}$ (Fig. 3.2D).
Fig. 3.2. Single cell discrimination. A) Examples of the 4 classes of discrimination responses observed. Each graph plots overlapping raw fluorescence traces from 5 swimming (blue) and 5 crawling (red) trials for a single cell. In this and all subsequent plots the gray shaded region denotes the time and duration of the stimulus. B) A sliding window ANOVA was used to quantify the discrimination times for each cell. The earliest discrimination time ($t_{SC}$) was the time at which the swimming and crawling trajectories significantly diverged (black arrow). C) A raster plot of the ANOVA results from all of the cells from one experiment. Black indicates non-significant times and white indicates significantly different times ($p < 10^{-6}$). The discrimination time based on the nerve recording of the behavior is shown ($t_{NERVE}$, green line). D) A histogram of the earliest $t_{SC}$ from the raster plot in C. 17 neurons produced significantly different trajectories prior to $t_{NERVE}$. 
Discrimination by populations of neurons

We view the nervous system of the leech as a dynamical system (Kaplan & Glass 1995, Abarbanel 1996). Abstractly, the behavioral state of the nervous system at any instant is a point in the phase space of this system (Fig. 3.3A). Each axis represents a variable that measures the temporal evolution of trajectories in this space (Broomhead & King 1986, Broomhead et al. 1987). For us, these variables are linear combinations of observed neurons. As in the single cell analysis, we labeled the time at which the trajectories have significantly diverged as the discrimination time. The decision-making period must occur at or prior to this time. We recognize that the eventual choice may depend on the behavioral state prior to stimulation, but we did not address this issue in these experiments.

The single cell discrimination analysis gave us an idea of the number of potential candidate decision-making cells. However, the purpose of simultaneously recording populations of neurons was to look at dynamic interactions among them. Using the multiple recordings, we asked whether a linear combination of neurons could discriminate earlier in time than any single neuron. To address this question, we used two analysis techniques in conjunction: Linear Discriminant Analysis (LDA) and Principal Component Analysis (PCA) (see Materials and Methods, Duda et al. 2000, Mardia et al. 1980). LDA seeks a line in the multidimensional phase space of a system such that grouped data points projected onto the line are maximally separated (i.e., the distributions of swimming versus crawling data points along the line are maximally separated). The slope of this line indicates the relative contribution of each of the variables to this separation. The goal is to find the time at which the swimming and crawling data projected onto a linear discriminant are significantly separated. This technique is susceptible to overfitting when applied in a high dimensional space with a limited amount of data. To overcome this problem, one may either increase the number of samples or decrease the dimensionality of the system.
Phototoxicity limits the amount of data we can collect, so we instead used PCA to reduce the dimensionality of our datasets. PCA rotates the axes of our N-dimensional data so that the first few axes point in the directions of maximal covariance. These new directions are the PCs. A neuron whose activity was very different between swimming and crawling trials will have a large variance across trials. This neuron would then contribute strongly to one of the first few PCs.

**PCA identifies dimensions that separate behaviors**

For the experiment shown in Fig. 3.3C, we performed PCA on a dataset containing 143 neurons (dimensions) measured across 14 trials. We show the first three principal components (PCs; Fig. 3.3B). The bar graphs represent the dimensionless contribution of each neuron to each PC. Values close to zero indicate a small contribution, while high positive or negative values indicate a large contribution. The first 3 PCs typically account for 60-80% of the overall variance in a dataset. The remaining 140 PCs are ignored here for visualization purposes. Effectively, we have reduced the dimensionality of the dataset from 143 (neurons) to 3 (linear combinations of neurons, the PCs).

We plotted the data 3-dimensionally, using the first 3 PCs as the axes (Fig. 3.3C). The trajectories all start in one region and then diverge toward two different regions of the space. From this plot, we extract two features: 1) The separation between the trajectories is an objective measure of decision-making, and 2) The neurons that contribute most this separation (i.e., have large positive or negative values) are most likely to be responsible for making the choice.

Occasionally, we observed a trial such as the one colored green in Fig. 3.3C. This trial would have normally been classified as a crawling trial based on the nerve recording.
Fig. 3.3. Population discrimination. A) A schematic of our conceptual framework. The state of the leech CNS at any instant is a point in a d-dimensional phase space (d = 3 in the drawing). The temporal evolution of its state defines a trajectory in this space. Prior to stimulation, the state of the nervous system is in a rest region. Upon stimulation, the trajectories diverge toward either a swimming or crawling region based on which choice was made. B) The first 3 principal components (PCs) from a single experiment. Each PC is a linear combination of the observed neurons in the N-dimensional dataset (N = 143 in this experiment). C) The same data as in B, projected onto the 3 PC axes. Each trajectory represents a trial (swimming blue, crawling red). The average swimming and crawling trajectories are in bold. Black dots indicate 1-second intervals along the average trajectories. The right panel plots the simultaneous DP nerve recording for each trial. The black arrow illustrates the direction of a linear discriminant estimated for a 500 ms time bin in the PC space. The direction of the linear discriminant is a weighted vector sum of the 3 PCs with weights X, Y and Z. D) The resulting linear discriminant, a linear combination of the 3 PCs. This direction is similar to PC3 in panel B. E) The linear discriminant weightings for each of the neurons projected onto a map of the ganglion. Red indicates large magnitude (both positive and negative) contributions to the linear discriminant direction; blue indicates small contributions.
However, the trajectory in the phase space initially diverges in the direction of the swimming trajectories. After a delay, the trajectory turns and moves toward the direction of the crawling trajectories. While trials such as this were rare (2/60 trials from 6 experiments), we interpret this as evidence that decision-making is a dynamical process: the leech nervous system can start to make a decision and subsequently change to an alternative choice.

**LDA finds neuronal populations that discriminate**

Having reduced the dimensionality of the dataset with PCA, we asked: 1) At what time did the swimming and crawling trajectories significantly diverge, and 2) Which neurons are responsible for the separation at this time? We thus divided the temporal trajectories into time bins and estimated the linear discriminant for each bin. We then performed an ANOVA on the data in each respective time bin projected onto each linear discriminant. Therefore, each time bin has an associated p value. The time at which this p value became significant is denoted $t_{LDA}$. The time bin width and the number of PCs were varied to find the optimal $t_{LDA}$ for each dataset (Fig. 3.5). The combination of PCA and LDA found linear combinations of neurons that could discriminate the behaviors earlier than any single neuron. While each of the PCs we discarded did not explain much of the total variance, it is possible that we lost discrimination information by not using them. Thus, while the linear discriminants we found performed well (i.e., discriminated at an early $t_{LDA}$), an earlier $t_{LDA}$ may have been found had we been able to use all of the PCs. This illustrates the inherent tradeoff between the dimensionality of a system and the amount of data required to adequately sample a high-dimensional space.

We show an estimated linear discriminant in Fig. 3D using 3 PCs. Neurons contributing strongly to the linear discriminant were those that best helped discriminate between swimming and crawling at $t_{LDA}$. In this case PC3 had the largest weighting, so the linear discriminant direction is similar to PC3.
We wished to visualize the spatial locations of the neurons with large magnitude contributions to the linear discriminant. We used color to encode the magnitude of each neuron and projected the colors onto a map of the ganglion (Fig. 3.3E). This spatial ganglion map was used as a guide for identifying candidate decision-making neurons.

**Discrimination of single neurons versus neuronal populations**

We compared the discrimination times of the earliest single cell \(t_{ESC}\) and the linear discriminant \(t_{LDA}\) from 6 experiments (Fig. 3.4A). In all experiments \(t_{LDA}\) occurred prior to \(t_{ESC}\). Also plotted are all of the \(t_{SC}\) times (black lines) that occurred prior to the \(t_{NERVE}\) times (green lines).

The neuronal populations contributing highly to the linear discriminant were generally different than the neurons with early single cell discrimination times (Fig. 3.4B, C). Single cell discrimination times \(t_{SC}\) in Fig. 3.4C are colored-coded with yellow representing the earliest \(t_{SC}\) times and red representing later \(t_{SC}\) times. The ganglion maps from all experiments are shown in Figs. 3.6 and 3.7.

**Cell 208 biases decisions**

The analyses described to this point were performed within 15 minutes during an ongoing experiment, so the ganglion maps were used to identify candidate decision-making neurons. To test whether these neurons were sufficient to influence decision-making individually, we passed polarizing current into each of them during the nerve shock. We impaled each candidate neuron and injected hyperpolarizing or depolarizing current before and after the nerve shock. None of the neurons with early single cell discrimination times (Fig. 3.4C) significantly affected the elicited behaviors (33 neurons from 6 preparations). When we tested neurons contributing strongly to the linear discriminant (17 neurons from 6 preparations), we found a neuron, cell 208, that can selectively bias the decision to swim or to crawl (labeled with an arrow in Fig. 3.4B and
Fig. 3.4. Comparing single cell discrimination to population discrimination. A) The distribution of discrimination times from 6 experiments. Discrimination times for the LDA ($t_{\text{LDA}}$, red), single cells ($t_{\text{SC}}$, black), earliest single cells ($t_{\text{ESC}}$, cyan) and nerve recordings ($t_{\text{NERVE}}$, green) are shown. The average difference between $t_{\text{LDA}}$ and $t_{\text{ESC}}$ was 290 +/- 60 ms (mean +/- SEM). Single cell discrimination times occurring later than the $t_{\text{NERVE}}$ for each experiment are omitted. B) The color-coded linear discriminant weightings from a single experiment (color-coding as in Fig. 3E). The black arrow indicates cell 208. C) The color-coded single cell discrimination times ($t_{\text{SC}}$) from the same experiment. Yellow represents the earliest discrimination times and red represents later discrimination times. D) The result from one experiment. Cell 208 was intracellularly impaled and alternately depolarized (+1.5 nA) or hyperpolarized (-1.5 nA) for the initial 10 seconds of each trial (black bar). The membrane resistance of cell 208 at rest was approximately 50 MΩ. A nerve shock was delivered for 300 ms as in Fig. 1B. In the 4 trials in which cell 208 was hyperpolarized (blue), the preparation swam within 10 seconds. In the 4 remaining trials in which cell 208 was depolarized (red), the preparation either produced the crawling motor pattern or it delayed swimming until the intracellular stimulation ended. E) A contingency table summarizing the pooled results from 5 preparations. The values in parentheses are the expected counts if the observed behavior did not depend on the stimulus condition.
When depolarized or hyperpolarized alone, this neuron did not initiate any behaviors. However, when stimulated during a nerve shock, cell 208 biased the decision towards swimming or crawling (Fig. 3.4D). With cell 208 hyperpolarized, the nerve shock reliably evoked swimming (blue trials); with it depolarized, the nerve shock evoked crawling or delayed swimming (red trials). In 5 preparations, the correlation between the level of current injection and the observed behavior (Fig. 3.4E) was significant ($p < 0.01$; Fisher’s Exact Test). We conclude that this neuron plays a role in decision-making. The neuron was labeled with an intracellular dye in all experiments and identified as cell 208 based on its morphology and electrophysiological properties (Weeks 1982).

**Discussion**

One of the central questions we attempted to address is whether decision-making is performed by single neurons or by neuronal populations (Kristan & Shaw 1997). One extreme proposal is multiple competing circuits in which decision-making neurons for one behavior act by inhibiting the other behaviors, so that only one behavior occurs at a time (Kovac & Davis 1977, Kovac & Davis 1980). At the other extreme would be the complete sharing of decision-making neurons by two or more behaviors, with the dynamics of the network determining which behavior is chosen. In the leech, we hypothesize a middle ground in which decision-making neurons are partially shared, the dynamics of neuronal populations can determine choices, but individual neurons in these populations can profoundly influence decision-making. This view is supported by our results that 1) neuronal populations discriminated between swimming and crawling earlier in time than did single neurons; 2) none of the single neurons that discriminated early were able to bias the decision; and 3) while cell 208 is able to bias the decision, it is part of a population defined by the linear discriminant.

The finding that a linear combination of neurons discriminates early in time shows that there is sufficient information in a population of neurons for an experimenter to predict the
ultimate choice. However, this result demonstrates only that the activity pattern of this network correlates with the eventual choice. To show that this information is used during decision-making, we needed to manipulate the network. Ideally, we would like to selectively hyperpolarize or depolarize a population of individual cells; however, we were technologically limited to stimulating one or two cells at a time.

Although we have not exhaustively tested all candidate neurons detected, most of the neurons tested were not able to bias the behavioral choice. However, we were able to reliably bias the choice with one of the linear discriminant candidates, cell 208. A relatively large depolarization or hyperpolarization of cell 208 biased the choice towards crawling or swimming, respectively. While this result was statistically significant, cell 208 did not determine the choice on all trials. This less than perfect control by cell 208 can probably be credited to the cell 208 homologs in each of the 20 other ganglia (Weeks 1982) that we did not stimulate.

Cell 208 has previously been described as a central pattern generating-like neuron (Weeks 1982), but a unique one that connects the swim-initiating network to the swim central pattern generating circuit. It has not been shown to trigger behaviors in intact isolated nerve cords. Our results suggest that it is also part of a decision-making circuit, although we do not yet know its role in this circuit or the mechanism by which it biases the system. While it is possible that cell 208 is driven by a higher-order decision-making neuron, we have shown that it alone is capable of biasing the entire system.

Most recent research about decision-making has focused on value-based choices in which there is always a right and wrong answer (Shadlen & Newsome 1996, Glimcher 2001, Schall 2000, Shadlen & Newsome 2001, Gold & Shadlen 2000, Platt & Glimcher 1999, Romo & Salinas 2001). We propose that the term decision-making should refer to a spectrum of goal-driven behaviors. At the most complex level are conscious, introspective choices that incorporate expected reward (Glimcher 2003). At the simplest
level are reproducible, predictable reflexes. There is a large area of involuntary, subconscious decision-making that has been neglected. For example, suppose your goal is to walk down the street. Which foot do you lead with? This is a choice that is made without conscious effort and will vary from time to time. While this choice is goal-directed, there is no correct or incorrect choice. In our reduced preparation, there is no obvious value associated with swimming versus crawling, although both choices would achieve the goal of escaping from a stimulus.

Why then does the leech, a relatively simple nervous system, not respond in a predictable manner? We hypothesize two possibilities: 1) The choice depends on the rest state prior to each stimulus, or 2) The state is reset upon stimulation and then diverges stochastically due to noise in the system. The leech may provide a system in which to resolve this important question.

**Material and Methods**

**Preparation**

Animals were adult *Hirudo medicinalis* (2-5 gm), the European medicinal leech, maintained in artificial pond water at 15°C. To reliably evoke swimming and crawling, all experiments were performed between the months of December – June. We dissected out the full nerve cord, including the head brain, all 21 segmental ganglia, and the tail brain. We removed the blood sinus around the ganglion to be imaged, between G7 and G10. We dissected away the connective tissue sheath from the ventral surface of the exposed ganglion. We prepared at least two dorsal posterior (DP) nerves, chosen from between G13 and G16, for extracellular recording and stimulation. A motor neuron in this nerve bursts during both the dorsal contractile phase of swimming (at ~1 Hz) and the contractile phase of crawling (at ~0.05 Hz), so the nerve serves as a monitor of which locomotory behavior is elicited. The purpose of recording with two suction electrodes was to confirm
the propagation of behaviors down the nerve cord. One of these nerves was stimulated with 10 ms electrical pulses delivered at 15Hz, with an amplitude of 2-3 V. The duration of the stimulus train was adjusted empirically to generate crawling and swimming responses; they were generally 300 +/- 100 ms long. In all experiments, we stabilized the imaged ganglion by pinning small latex strips across the adjacent nerve cord to minimize motion artifact (Cacciatore et al. 1999). The preparation was maintained in a chamber filled with ~10 ml of room temperature leech saline, consisting of (in mM): 115 NaCl, 4KCl, 1.8CaCl₂, 1.5 MgCl₂, 10 dextrose, 4.6 Tris maleate, and 5.4 Tris base, pH 7.4.

**Staining with FRET dyes**

We first stained the ganglion to be imaged with the FRET donor, N-(6-chloro-7-hydroxycoumarin-3-carbonyl)-dimyristoylphosphatidylethanolamine (CC2-DMPE), a coumarin-labeled phospholipid. (Gonzalez et al. 1999). We made a 30 µM staining solution from 3 µl of 10 mM CC2-DMPE in DMSO, 1.5 ul of 20 mg/ml pluronic F-127 in DMSO, and 1 ml of saline. This solution was then serially diluted to a 10 µM final staining solution. We pinned out the nerve cord in a Sylgard-coated dish, placed a small plastic cylinder over the exposed ganglion, and sealed it with petroleum jelly to make a watertight chamber. We then replaced the saline in the chamber with the staining solution and stained the ganglion for 30 min. During the staining, we constantly recirculated the staining solution using a peristaltic pump. After 30 min, the staining solution was removed and the tissue was washed several times with fresh saline.

We next stained the whole nerve cord with the FRET acceptor, bis(1,3-diethylthiobarbiturate)-trimethine oxonol [DiSBAC₃(3)], an oxonol dye (Gonzalez et al. 1999). We made a 15 µM staining solution from 9.6 µl of 15.67 mM DiSBAC₃(3) in DMSO and 10 ml saline. This solution was then bath-sonicated for at least 3 min. We replaced the saline with this solution and left it on for 30 min. At the end of 30 min., the oxonol
solution quenched the coumarin emission by approximately 50%. We then removed the oxonol solution and replaced the bath with fresh saline.

**Electrophysiology and cell fills**

We recorded intracellularly from cells using 40-60 MΩ glass microelectrodes filled with 1M potassium acetate, using an AxoClamp 2A amplifier. For cell 208, we measured membrane resistances of approximately 50 MΩ, meaning that our current injections resulted in membrane potential changes of about +/- 75 mV from rest. We recorded extracellularly using suction electrodes and a four-channel differential amplifier. We digitized all electrical data at 1 kHz using a 16-bit analog-to-digital board and custom Matlab software.

We filled individual candidate decision-making neurons using Alexa 488 dextran (10,000 MW). Microelectrodes were back-filled using concentrations of 25 mg/ml Alexa 488. Cells were then impaled, and dye was iontophoretically injected. Dye was allowed to diffuse for ~ 1 hr, and tissue was fixed overnight, dehydrated, cleared, and mounted on a slide for viewing. Fills were imaged using a confocal microscope. The dye was excited by the 488 nm emission line of a KrAr laser. Candidate neurons were compared, based on morphology and electrophysiological properties, to previously identified leech neurons.

**Optical recording**

We acquired fluorescence images using an upright microscope. We used a 20x, 0.5 numerical aperture (NA), water-immersion objective. For epi-illumination we used a tungsten halogen lamp in a standard housing, powered by a low-ripple power supply. We filtered the excitation light at the coumarin excitation band with a 405 ± 15 nm bandpass filter. Emitted light was filtered with a custom designed wavelength switching device. This device split the emitted light into two optical paths with a 430 nm dichroic mirror. The light was filtered in each path at either the coumarin emission wavelength (460 ± 25
nm) or the oxonol emission wavelength (560LP). Optical shutters in each path were alternately opened or closed, allowing only one emission band to pass per frame. Following the shutters, the two paths were recombined (430 nm dichroic) and imaged onto a CCD camera. We used a cooled CCD camera operated in frame-transfer mode to acquire the optical data at a resolution of 256 x 256 pixels. Images were acquired at a frame rate of 40 Hz. Since every other frame captured one of the two emission wavelengths, the effective frame rate was 20 Hz. Imaging data were acquired using custom software written in Matlab. We synchronized the optical and electrical recordings by feeding the frame timing signals emitted by the camera into the A-to-D board, along with all of the electrophysiology signals.

Analysis

In a typical experiment, we imaged 10 – 20 trials, performed the following analysis and then intracellularly impaled candidate decision-making neurons. After acquiring the data, we analyzed them using Matlab. We outlined the images of individual somata manually using a custom-made graphic user interface. All pixels within each cellular outline were then averaged in each frame, yielding two raw fluorescence signals for each cell, one for the coumarin emission wavelength \( F_{CC2}(t) \) and one for the oxonol emission wavelength \( F_{OX}(t) \). The noise in these single-cell signals was shot-noise dominated. The raw fluorescence signals were divided, \( F_{CC2}(t) / F_{OX}(t) \), to yield a ratioed fluorescence signal \( F_R(t) \). Ratioing improved the signal-to-noise ratio of the signals and corrected both bleaching and motion artifacts. Signals were then normalized by the average baseline fluorescence before a stimulus was delivered (the first second of each trial) and expressed as a percent change in fluorescence (dF/F) from this baseline. We measured sensitivities of \( F_R(t) \) in the range of 15-20%/100 mV for 1 Hz square-wave voltage signals with a 10 mV amplitude, centered around a baseline voltage of -55 mV.
Principal component analysis (PCA) and linear discriminant analysis (LDA) were performed using Matlab toolboxes and custom code. Prior to performing any analysis, each trial was classified manually as either a swim or crawl trial. We were aware of the possibility of behavioral misclassification, but the difference between the motor pattern for swimming and crawling is very obvious. The periods of the motor bursts for the two behaviors do not overlap.

ANOVA

For the single neuron analysis, we performed a sliding window ANOVA using a 300 ms bin width. The earliest discrimination time for each neuron occurred when the p value first fell below a threshold ($p < 10^{-6}$). We chose a small p value threshold to increase our confidence in the discrimination time. We performed this same analysis on rectified, low-pass filtered (300 ms time constant) versions of the DP nerve recordings to determine the nerve discrimination time.

Principal Component Analysis

For the PCA, we organized the optical data from an experiment into an $[MP] \times N$ matrix, where $M$ is the number of trials, $P$ is the number of frames per trial, and $N$ is the number of neurons imaged. Each column of this matrix was normalized by the standard deviation of the data in that column. To visualize the data in a 3-dimensional principal component space we deconcatenated each trial from the PCA score matrix and plotted each trial separately. See additional supplemental materials for more details.

Linear Discriminant Analysis

A LDA was performed at each time point in the principal component space using a sliding window of variable width and variable number of PC dimensions. We chose the
linear discriminant that resulted in the earliest discrimination time (see Fig. 3.5 for the procedure). See additional supplemental materials for more details.
Fig 3.5. Linear discriminant analysis discrimination time. Linear discriminants were estimated by moving a sliding window along the data in the PC space. We varied the time bin width and number of PCs. At each time bin, we performed an ANOVA of the grouped data within the time bin, projected onto the linear discriminant, and derived a p value. The earliest time at which the p value became significant (p < 10^{-6}) is termed $t_{LDA}$ and plotted for each combination of bin width and number of PCs. We used the linear discriminant with the earliest $t_{LDA}$ for subsequent analysis. For the experiment shown, the linear discriminant was estimated using a 500 ms window and 12 PCs (white asterisk) as this combination led to the earliest $t_{LDA}$. We randomly shuffled trials to confirm that using up to 20 PCs with at least 10 trials per experiment did not lead to overfitting (i.e., the shuffled data projected onto the estimated linear discriminants was not significantly separable into two groups at any point in time).
Fig 3.6. Single cell discrimination ganglion maps. The ganglion maps from 6 experiments are shown. Neurons with single cell discrimination times ($t_{SC}$) that occurred prior to $t_{NERVE}$ for each experiment are colored (mean +/- SD = 17 +/- 6 cells for 6 experiments). Yellow indicates early discrimination times and red indicates later discrimination times.
Fig. 3.7. Linear discriminant analysis ganglion maps. The ganglion maps from 6 experiments are shown. The maps are from the same experiments as in Fig. S2. Cells are color-coded based on the magnitude of the contribution to the linear discriminant direction. Red and yellow represent large magnitude contributions, blue represents small contributions. The black arrows indicate cell 208 in each experiment.
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CHAPTER 4

Optical Imaging of the Multifunctional Overlap Between Two Central Pattern Generators

Abstract

Central pattern generators (CPG) control both swimming and crawling in the medicinal leech. To investigate whether these two CPGs are dedicated or multifunctional, we used voltage-sensitive dye image to record from ~90% of the neurons in a segmental ganglion. By eliciting swimming and crawling in the same preparation, we were able to explicitly measure neurons that participated in either of the two rhythms, or both. More than twice as many cells oscillated with crawling compared to swimming. Surprisingly, 93% of the cells that oscillated with swimming also oscillated with crawling. We then recorded from two previously unidentified interneurons based on the imaging results, cell 255 and cell 257. Cell 255 is a multifunctional interneuron that oscillates with and can perturb both rhythms. Cell 257 is an interneuron dedicated to crawling. The imaging results combined with the characterization of these two cells demonstrates that the swimming and crawling networks are driven by both multifunctional and dedicated circuitry.

Introduction

Central pattern generators (CPGs) have been shown to be the computational kernel underlying many rhythmic behaviors in both vertebrate and invertebrate nervous systems (Kiehn & Butt, 2003; Marder & Calabrese, 1996; Marder et al., 2005; Stein, 2005). These CPGs drive motor neurons to activate muscles in precise spatiotemporal patterns. In the case where the same muscles are used to generate multiple, mutually exclusive rhythmic behaviors, two models have been proposed for the organization of the underlying CPGs:
dedicated circuitry or reorganizing circuitry (Morton & Chiel, 1994). Dedicated circuitry refers to an architecture in which anatomically distinct neuron populations are used exclusively to generate behaviors. Such an organization has been observed in locusts, in which two different interneuron pools that control walking and flight converge on the same (bifunctional) muscles (Ramirez & Pearson, 1988). *In vivo* calcium imaging experiments in zebrafish spinal cord have also demonstrated that different interneurons are used for swimming and escape behaviors, suggesting that dedicated circuitry is used by vertebrates (Ritter et al., 2001).

In contrast, the concept of reorganizing (or multifunctional) circuitry is that there exists some degree of overlap between multiple CPGs. This type of organization has been shown to be a common feature across many invertebrate nervous systems (Weimann & Marder, 1994; Kristan & Shaw, 1997; Popsecu & Frost, 2002; Jing et al., 2004). Multifunctional circuitry in vertebrate spinal cord is also beginning to be studied (Berkowitz, 2005). In the extreme, a single network can be dynamically reconfigured by neuromodulation to generate multiple rhythms (Nusbaum et al., 2001). While studies of multifunctional networks have demonstrated the remarkable versatility of individual neurons, most have been performed by recording from single neurons during ongoing rhythms. This approach limits the ability to determine the full extent of the multifunctional overlap between networks of neurons. Determining the degree of overlap should provide insight into how the same neurons can be efficiently used to generate multiple behaviors.

An example of two such mutually exclusive behaviors in the medicinal leech is swimming and crawling. The leech coordinates the same sets of longitudinal and circular muscles in fundamentally different ways to achieve these two forms of locomotion, both of which are produced by a CPG (Kristan & Calabrese, 1976; Eisenhart et al., 2000). Importantly, a leech cannot both swim and crawl at the same time. We were interested in whether the two underlying CPGs consist of separate pools of interneurons or, instead,
have overlapping neuronal pools. For example, cell 208, a neuron recently implicated in
decision-making (Briggman et al., 2005), is known to oscillate with both swimming
(Weeks 1982) and crawling (Baader 1997). Since we are ultimately interested in the
behavioral choice between these two behaviors, we also need to understand the interaction
between their CPGs.

The CPG underlying swimming has been extensively studied and it’s mechanism of
operation described (Brodfehrer et al., 1995). Swimming is achieved by alternating dorsal
and ventral contractions of longitudinal muscles that propagate down the length of the
animal (Fig 4.1A; Stent et al., 1978). During this behavior dorsoventral muscles are
tonically activated, helping the animal to flatten. Primarily inhibitory interactions between
CPG interneurons are responsible for driving the oscillatory contractions (Friesen, 1989).
In contrast, although a CPG underlies crawling (Cacciatore et al., 2000; Eisenhart et al.,
2000), none of the interneurons responsible for the rhythm have been identified. Crawling
is achieved by contraction of circular muscles, causing elongation of the body, followed by
simultaneous contraction of dorsal and ventral longitudinal muscles, causing a whole body
contraction (Fig 4.1B). The alternation between contraction and elongation also
propagates down the length of the leech. The switch between these two behaviors can be
rapid, occurring within seconds (Fig 4.6E).

Results

Coherent swimming and crawling oscillations on the dorsal surface

Our strategy for examining the interaction between the two CPGs was to optically
image all of the neurons in a segmental ganglion during the two ongoing motor rhythms.
We used isolated, intact nerve cords and desheathed either the dorsal (Fig 4.1C) or ventral
(Fig 4.3A) side of segmental ganglion 10 (G10) for voltage-sensitive dye (VSD) imaging.
**Fig 4.1.** Swimming and crawling motor patterns in the isolated leech nervous system

A) Swimming is achieved by alternating dorsal and ventral contractions of longitudinal muscles. B) A crawling step is achieved by contraction of circular muscles causing elongation, followed by activation of longitudinal muscles causing contraction. C) The dorsal surface of G10, stained with the VSD (coumarin emission wavelength; Gonzalez et al., 1999). Cell 1 and cell 3 were intracellularly recorded throughout all dorsal surface experiments. D) An example of a crawling episode, defined by alternating contraction (C, green bars) and elongation (E, red bars) that propagates down the nerve cord, recorded in nerves DP(12) and DP(13). Intracellular recordings from cells 1 and 3 in G10 are shown as black traces. The simultaneously recorded raw optical signals are overlaid in red. Cells 1 and 3 oscillated 180° out of phase during crawling. E) An example of a swimming episode, defined by alternating dorsal (D, cyan bars) contractions and ventral (V, blue bars) contractions that propagated down the nerve cord, recorded in nerves DP(12) and DP(13). Intracellular recordings (black traces) and raw optical signals (red traces) for cells 1 and 3 are shown. Cells 1 and 3 oscillated 90° out of phase during swimming. F) The phase (circumferential axis) and magnitude (radial axis) of the coherence during crawling of all optically recorded cells shown in panel C. Color-coded cells (see Fig 4.2 legend) were significantly coherent with cell 3 at the peak crawling frequency (0.09 Hz in this example). The dashed line indicates the threshold for significance (multiple comparisons $\alpha < 0.05$). Error bars for the phase and magnitude represent one standard error. G) The phase and magnitude of the coherence during swimming. Color-coded cells (see Fig 4.2 legend) were significantly coherent with cell 3 at the peak swimming frequency (1.3 Hz in this example).
Figure 4.1D demonstrates an episode of crawling from one such dorsal imaging preparation, and Fig 4.1E shows an episode of swimming from the same preparation. The motor neuron patterns were evident from dorsal posterior (DP) nerve recordings from segments 12 and 13 (DP(12) and DP(13); Figs 4.1D and 4.1E). Each time we recorded optically from the dorsal ganglion surface, we also recorded intracellularly from two motor neurons, cell 1 and cell 3 (Fig 4.1C). Cell 3 excites dorsal longitudinal muscles and cell 1 inhibits cell 3 as well as dorsal longitudinal muscles. The simultaneously recorded raw optical signals from these two cells (red lines; Figs 4.1D and 4.1E) overlay their intracellular recordings (black traces; Figs 4.1D and 4.1E). The phase shift of the optical signals relative to the intracellular recordings (Fig 4.1E) is due to the time constant of the VSD (Cacciatore et al., 1999). To quantify the phase relationships among all cells, we calculated the phase and magnitude of the coherence between the optical signal of cell 3 and every other cell at the dominant frequency for each behavior (Cacciatore et al., 1999; Taylor et al., 2003; also see Materials and Method). Polar plots show the magnitude and phase of those cells that were significantly coherent. Each polar plot was divided into color-coded quadrants (Figs 4.1F and 4.1G). For crawling, we arbitrarily denoted $0^\circ$ as the peak of elongation (red points, Fig 4.1F) and $180^\circ$ as the peak of contraction (green points, Fig 4.1F). For swimming, we denoted $0^\circ$ as the peak dorsal contraction (cyan points, Fig 4.1G) and $180^\circ$ as the peak ventral contraction (blue points, Fig 4.1G). For both behaviors, cells that were not significantly coherent are shown in black.

To visualize the spatial distribution of the phases, we constructed phase maps for crawling and swimming by projecting the color-coded phases onto a map of the individual neurons in the ganglion (Figs 4.2A and 4.2B). Predicted phase maps based on the function of previously identified neurons on the dorsal surface are shown for comparison (Figs 4.2C and 4.2D; Muller et al., 1981; Brodfuehrer et al., 1995; Eisenhart
Fig 4.2. Measured and predicted dorsal surface phase maps A) The locations of coherent neuronal somata during crawling while recording from the dorsal surface (data from Fig 4.1F). The phases were split into four quadrants; red corresponds to elongation, green corresponds to contraction, and orange and yellow lie between contraction and elongation. Black cells were not significantly coherent. B) The locations of coherent neuronal somata during swimming (data from Fig 4.1G). Cyan corresponds to dorsal contraction, blue corresponds to ventral contraction, and pink and purple lie between dorsal and ventral contraction. C,D) The predicted distribution of phases during crawling (C) and swimming (D) based on the known function of neurons on the dorsal surface of segmental ganglia (Muller et al., 1981; Brodfuehrer et al., 1995; Eisenhart et al., 2000) projected onto the canonical dorsal surface map (Muller et al., 1981). The locations of cells 1-4 are indicated in all four panels for comparison.
et al., 2000). We observed neurons in roughly the same positions and phases for all of the neurons in the predicted phase maps for both swimming and crawling. For example, the phases of cells 1-4, prominent longitudinal motor neurons, were identical in both the measured and predicted phase maps. In addition, we measured coherent oscillations during swimming and crawling in many unidentified neurons.

**Coherent swimming and crawling oscillations on the ventral surface**

Similar experiments were performed for neurons on the ventral surface of G10 (Fig 4.3A). During the optical recordings we also made simultaneous intracellular recordings from two different motor neurons, cell AE and cell CV. Cell CV excites circular muscles and is therefore active during the elongation phase of crawling (Fig 4.3B), and cell AE excites the annuli along the body wall of the leech and is depolarized during the contraction phase of crawling (Fig 4.3B). Neither of these two cells is rhythmically active during swimming (Fig 4.3C). The phase and magnitude of the coherence was again calculated for each behavior (Figs 4.3D and 4.3E) using different neurons as the reference (see Materials and Methods). We constructed phase maps of the ventral surface using the same color-coding scheme used for the dorsal surface (Figs 4.4A and 4.4B) and compared them to predicted phase maps based on identified ventral surface neurons (Figs 4.4C and 4.4D; Muller et al., 1981; Brodfuehrer et al., 1995; Baader, 1997; Eisenhart et al., 2000).

**Summary dorsal and ventral phase maps**

To determine the reproducibility of these neuronal maps, we compiled phase maps for 8 dorsal experiments (Figs 4.9 and 4.10) and 6 ventral experiments (Figs 4.11 and 4.12) to construct summary phase maps. A cell was included on the summary phase map if it 1) was observed to have the same phase during swimming and/or crawling, and 2) was roughly the same diameter and located in the same area of a glial packet. Cells meeting
**Fig 4.3. Imaging the ventral surface during crawling and swimming motor patterns**

A) The ventral surface of G10, stained with the VSD (coumarin emission wavelength). For all ventral surface experiments, cell AE and cell CV were intracellularly recorded. 

B) A sample crawling episode with intracellular (black traces) and optical signals (red traces) from cells CV and AE showing that they are 180° out of phase during crawling.

C) A sample swimming episode recorded while imaging the ventral surface. Cells CV and AE were not rhythmically active during swimming, as indicated by both the electrical (black) and optical (red) traces.

D) The phase (circumferential axis) and magnitude (radial axis) of the coherence of cells on the ventral surface in panel A during the crawling motor pattern. Color-coded cells were significantly coherent with cell AE at the peak crawling frequency (0.08 Hz in this example).

E) The phase and magnitude of the coherence of cells on the ventral surface during the swimming motor pattern. For experiments on the ventral surface of the ganglion, a cell with a consistently large oscillation in the anterior lateral glial packet was used as the reference (see Fig 4.4B). Color-coded cells were significantly coherent with this cell at the peak swimming frequency (1.25 Hz in this example). The phases in this plot were rotated so cell 208 was aligned with its known phase (70°) during swimming (Friesen, 1989).
Fig 4.4. Measured and predicted ventral surface phase maps A,B) The locations of coherent neuronal somata during crawling (A) and swimming (B) while imaging the ventral surface (data from Fig 4.3D,E). The phases were split into four quadrants as in Fig 4.2. The location of the reference cell used for swimming is indicated in panel B. C,D) The predicted distribution of phases during crawling (C) and swimming (D) based on the known function of neurons on the ventral surface of segmental ganglia (Muller et al., 1981; Brodfuehrer et al., 1995; Baader, 1997; Eisenhart et al., 2000) projected onto the canonical ventral surface map (Muller et al., 1981). The locations of cells AE, CV, and 208 are indicated in the four panels for comparison. The Retzius cell somata were removed for all ventral surface experiments in order to see smaller cells below them.
these criteria in 4/8 dorsal preparations or 4/6 ventral preparations were included in summary phase maps (Figs 4.5A and 4.5B). We then merged the swimming and crawling phases together for the summary phase maps. Neurons that oscillated with both behaviors are bi-colored; the swimming and crawling phases are denoted above and below the diagonal, respectively. The predicted merged phase maps are shown for comparison (Figs 4.5C and 4.5D). Every neuron in the predicted phase maps was found to correspond to a neuron in the summary phase maps.

**The swimming and crawling networks overlap**

We then analyzed the information contained in the summary phase maps. A histogram of the total number of cells active in each phase of swimming is relatively flat (Fig 4.6A). The greater number of cells in the dorsal and ventral contraction phases reflects the fact that the longitudinal motor neurons are active in one of these two phases. This equal representation of the number of cells in each phase is in agreement with the known phase distribution of the swim CPG (Friesen, 1989). In contrast, we observed more than twice as many neurons active during the elongation phase of crawling compared to the contraction phase (Fig 4.6B), and proportionately fewer neurons in the intermediate phases. In addition, we observed twice as many total neurons oscillating during crawling (188) compared to swimming (90).

There proved to be a high degree of overlap between the swimming and crawling networks. Fully 93% of the cells that oscillated with swimming also oscillated with crawling (Fig 4.6C). We were interested in whether a cell’s phase in one behavior was predictive of its phase in the other behavior. Of the 16 possible phase combinations, we observed examples of cells with 15 different combinations (Fig 4.6D). The phase combinations were randomly distributed (Fisher-Freeman-Halton exact test, p = 0.148). There was therefore no association between a cell’s phase during swimming and crawling. An example of two of the phase combinations during a transition from crawling
Fig 4.5. Summary and predicted merged phase maps A) A summary phase map of 8 dorsal imaging experiments. Cells included in this phase map were observed in at least 4/8 experiments. The swimming and crawling phases have been merged; for cells active during both rhythms, the color on the left indicates the activity phase in swimming, the color on the right indicates the phase in crawling. White cells were not in phase with either oscillation and singly-colored cells were active in only one of the oscillations. B) A summary phase map of 6 ventral imaging experiments. Cells included in this phase map were observed in at least 4/6 experiments. C,D) The predicted merged phase maps for the dorsal (C) and ventral (D) surface based on the function of known neurons.
**Fig 4.6.** Analysis of the summary phase maps  

A) The distribution of cells from the summary phase maps in each phase of swimming. The total number of cells (dorsal surface plus ventral surface) are shown.  

B) The total distribution of cells from the summary phase maps in each phase of crawling.  

C) A Venn diagram showing the total number of cells that oscillated with crawling (188 cells), with swimming (90 cells), and cells that oscillated with both behaviors (84 cells). There are approximately 350 total neurons in G10.  

D) The distribution of phase combinations for cells that oscillated with both crawling and swimming. Black numbers inside each box indicate the number of cells that oscillated for each combination. White numbers indicate the expected counts if the swimming and crawling phases were independent. The distribution of combinations of swimming and crawling phases were not significantly different (p = 0.148).  

E) Intracellular recordings from two cells demonstrating two of the phase combinations (cyan/green and blue/green). The left panel shows a transition from crawling (0 to 50 s.) to swimming (50 to 60 s.). The right panel is an enlargement of the last 15 s. of the recording. Cell 3 (black trace) is an example of a cyan/green cell and cell 4 (gray trace) is an example of a blue/green cell.
to swimming is shown in Fig 4.6E. Cell 3 and cell 4 are synchronously active during crawling (green phase), but 180° out of phase during swimming (blue and cyan phases).

Finally, we estimated what percentage of the total number of neurons in G10 we observed in these experiments. For the 8 dorsal surface experiments, we observed 164 +/- 10 (mean +/- SD) cells and from 6 ventral surface experiments we observed 153 +/- 11 (mean +/- SD) cells. Based on the total number of neurons in G10 (~350), we therefore estimate that we observed, on average, 317 +/- 15 cells, or ~90% of the neurons in G10.

**Identification of neurons using the summary phase maps**

To demonstrate the usefulness of the summary phase maps for identifying circuit elements, we electrophysiologically characterized two previously unidentified neurons on the ventral surface. Their positions and phases are indicated on the ventral summary phase map (Fig 4.5B). We have denoted the first neuron cell 255, based on its position on the canonical map of the leech ganglion (Muller et al., 1981). We confirmed electrophysiologically that, as predicted from the imaging results, cell 255 depolarizes and spikes during the elongation phase of crawling, in phase with cell CV (Fig 4.7B), and hyperpolarizes during contraction. Likewise, dual recordings of cell 208 and cell 255 show that cell 255 oscillates synchronously with cell 208 during swimming (Fig 4.7C). Unlike cell 208, which oscillates above resting potentials, cell 255 oscillates around a hyperpolarized potential relative to rest. Cell 255 was identified in 15 preparations electrophysiologically and filled with a fluorescent dye in 14/15 preparations. In all 14 fills, cell 255 had a bilaterally symmetric arborization and projected a process through a posterior lateral connective nerve (Fig 4.7A). The bilateral symmetry of its arbors is indicative of an unpaired neuron (Weeks & Kristan, 1978; Weeks 1982).

Demonstrating that a neuron oscillates with two behaviors suggests, but does not prove, that it is actively participating in two CPGs. To test this hypothesis, we injected depolarizing and hyperpolarizing current pulses into cell 255 during different phases of
**Fig 4.7.** Cell 255 is a multifunctional interneuron **A)** The morphology of cell 255, located in the posterior medial glial packet on the ventral surface. **B)** Dual intracellular recording of cell CV, a motor neuron to the ventral circular muscles, and cell 255. Cell 255 oscillated in phase with cell CV, during the elongation phase of crawling. **C)** A dual intracellular recording of cell 208 and cell 255. Cell 255 slightly led cell 208’s oscillation and oscillated around a hyperpolarized potential below rest. **D)** Depolarizing current (+2nA for 3-5 s., black horizontal bars) injected into cell 255 during the contraction phase of crawling terminated contraction and started elongation. The cell was held slightly hyperpolarized (-0.1 nA) during this trial. **E)** Depolarizing current (+2nA for 1-2 s., black horizontal bars) injected into cell 255 terminated swimming.
the two ongoing rhythms. Depolarizing current injected during the contraction phase of crawling terminated the contraction and caused an early elongation (Fig 4.7D). Of 120 crawling steps from 8 preparations, similar current injections during contraction reduced the period of crawling steps by 21 +/- 7% (mean +/- SD, ratio of stimulated step periods to unstimulated step periods). This effect was most pronounced when cell 255 was depolarized from a slightly hyperpolarized potential relative to rest (Fig 4.7D). Depolarization during elongation had no effect on the period of crawling steps. Hence, this neuron not only depolarizes during elongation, it also participates in the generation of elongation in the crawling CPG.

Depolarizing cell 255 during swimming either terminated a swimming episode (Fig 4.7E) or slowed the swim period for the duration of the stimulus (not shown). Of 120 attempts in 15 preparations, depolarizing current slowed or stopped swimming 63% of the time. Therefore, cell 255 oscillates with both swimming and crawling and depolarizing current is sufficient to perturb both rhythms. Hyperpolarizing current injections had no consistent effect on either rhythm.

We then focused on a neuron that oscillates during crawling, but not during swimming. We denoted this neuron cell 257, based on its position. Like cell 255, it depolarizes and spikes during the elongation phase of crawling, as shown with a dual recording of cell AE (Fig 4.8B). During swimming, cell 257 is actively hyperpolarized from rest (Fig 4.8C). Cell 257 was identified in 10 preparations electrophysiologically and filled with fluorescent dye in 8/10 preparations. It appears to be a paired neuron that arborizes primarily ipsilateral to the soma location. It projects a process through an anterior lateral connective nerve contralateral to the position of the soma (Fig 4.8A). Depolarizing and hyperpolarizing current injections into a single cell 257 did not consistently perturb either swimming or crawling.
Fig 4.8. Cell 257 is a dedicated interneuron A) The morphology of cell 257, located in the posterior medial glial packet on the ventral surface. B) Dual intracellular recording of cell AE and cell 257. Cell 257 oscillated 180° out of phase with cell AE, in the elongation phase of crawling. C) Dual intracellular recording of cell CV and cell 257. Cell 257 was strongly hyperpolarized during swim episodes.
Discussion

A technological finding of this study is that population imaging can be used to address questions on two different levels when analyzing a system. On the one hand, it allows us to form a global, network level picture of the dynamics of circuits. We used this to learn about the distributed nature of the crawling CPG as well as the overlap between two CPGs. On the other hand, these results can be used to identify circuit elements on a single cell level. We used the phase maps to study multifunctional neurons and also as a guide to begin identifying the individual neurons involved in crawling. These maps reduce the search time involved in identifying unknown circuit structure.

The ability to simultaneously record from large populations of neurons with single cell resolution allowed us to measure the dynamics of networks of neurons. This large scale approach to studying networks has distinct advantages over single cell electrophysiology. In the case of the previously unknown CPG for crawling, we now know twice as many neurons oscillate with crawling versus swimming, indicating that the crawling CPG may be a much more distributed network than the swim CPG. Since we recorded both behaviors in the same preparation, we also explicitly measured the overlap between the two networks. An interpretation of the high degree of overlap between the two circuits may be that the swim CPG is superimposed on top of the crawling CPG. All species of leech can crawl, but only about half of the species swim (Sawyer, 1986). This raises the possibility that crawling may be an evolutionarily more primitive behavior and the swim CPG evolved by sharing part of the existing circuitry used for crawling.

However the two CPGs came to overlap by such a large percentage, we found no correlation between the phases of cells that oscillate with both behaviors (Fig 4.6D). This complicates the understanding of how overlapping networks can drive the same muscle groups in fundamentally different patterns and on very disparate time scales. For the example shown in Fig 4.6E, cells 3 and 4 oscillate synchronously during crawling (an
approximately 0.1 Hz rhythm), but are phase shifted by 180° during swimming (a 1 Hz rhythm). While this could be due to reorganization of premotor circuitry, a recent theoretical study shows that frequency-dependent phase shifts can result from interactions between inhibitory and electrical synapses between neurons as well as intrinsic properties of individual neurons (Pfeuty et al., 2005; Kleinfeld & Mehta, 2005). In other words, a single network driven at two different frequencies could, in principle, generate motor patterns with different phase relationships without recruiting any additional neurons. Neuromodulation may also play an important role in driving the networks as has been shown in the crustacean stomatogastric ganglion (Nusbaum et al., 2001). In the leech, serotonin is known to promote swimming (Mangan et al., 1994) and dopamine has recently been shown to modulate crawling (Krisp & Mesce, 2004). Of primary interest is the mechanism by which the two CPGs generate oscillations. The swim CPG functions by a core of reciprocal inhibitory connections driven by tonic excitation (Friesen, 1989). The connection pattern of the crawling CPG is needed in order to draw conclusions about its mechanism of oscillation.

The summary phase maps we generated not only provide a global picture of the dynamics within a ganglion, they are also practically useful for identifying circuit elements. We noticed a previously unidentified neuron, cell 255, that oscillated in phase with cell 208 during swimming. Cell 255 was of particular interest because it also oscillates with crawling, making it potentially a multifunctional interneuron. Indeed, the ability to perturb both rhythms with intracellular current injection, demonstrates that it is involved in the generation of the two behaviors. To definitively prove its role in the two CPGs, we must identify monosynaptic connections (Taylor et al., 2003) to the swim CPG and the unknown crawl CPG.

We also used the phase maps to identify a neuron, cell 257, active during the elongation phase of crawling. This neuron was of interest because of the large number of
neurons active during this phase. Electrophysiological recordings confirmed why this neuron does not oscillate with swimming, it is actively inhibited during swim episodes. Our inability to perturb either rhythm with cell 257 leaves its role, if any, in the CPGs in doubt. We note, however, that the apparently highly distributed nature of the crawl CPG may reduce the ability of current injections into single paired neurons to significantly perturb ongoing crawling rhythms. Indeed, it has recently been suggested that determining a neuron’s function in a circuit based on its synaptic connections may be more informative than the traditional tests of sufficiency for inclusion in a CPG (Marder et al., 2005).

Multifunctional neurons have been described in many systems (Weimann & Marder, 1994; Popsecu & Frost, 2002; Jing et al., 2004), including in the interaction between swimming and whole-body shortening in the leech (Shaw & Kristan, 1997). Subsets of swim CPG cells are either hyperpolarized or depolarized during whole-body shortening. Cells that are active during one behavior but hyperpolarized during another were termed dedicated neurons; cell 257 is an example of such a neuron dedicated to crawling. While we measured a large overlap between the two networks, it was not 100% overlapping. Identifying more of the dedicated neurons and determining why they are dedicated will help us learn about hybrid dedicate/multifunctional networks.

More interesting than cell 257, from a dynamical systems point of view, is cell 255. This neuron is a multi-rhythmic, multifunctional neuron, it oscillates with two mutually exclusive behaviors on very different timescales and can perturb both behaviors. Therefore, cell 255 must be participating in two different dynamical states of the leech nervous system since swimming and crawling cannot occur concurrently. A key to understanding multifunctional networks will be determining how individual neurons, like cell 255, participate in and drive the dynamical state of nervous systems.
Figure 4.9. Phase maps of coherently active cells during crawling for 8 dorsal surface experiments.
Figure 4.10. Phase maps of coherently active cells during swimming for 8 dorsal surface experiments.
Figure 4.11. Phase maps of coherently active cells during crawling for 6 ventral surface experiments.
Figure 4.12. Phase maps of coherently active cells during swimming for 6 ventral surface experiments.
Material and Methods

Preparation

Animals were adult *Hirudo medicinalis* (2-5 gm), the European medicinal leech, obtained from Leeches USA (Westbury, NY) and maintained in artificial pond water at 15°C. To reliably evoke swimming and crawling, all experiments were performed between the months of December – June. We dissected out the full nerve cord, including the head brain, all 21 segmental ganglia, and the tail brain. We removed the blood sinus around the ganglion to be imaged, ganglion 10 (G10), in all experiments. We dissected away the connective tissue sheath from the ventral or dorsal surface of the exposed ganglion. For ventral surface imaging experiments, we removed the two large Retzius cells in order to see smaller cells below them. We prepared at least two dorsal posterior (DP) nerves, chosen from between G12 and G15, for extracellular recording and stimulation. Cell 3 spikes in this nerve during both the dorsal contractile phase of swimming (at 1 – 1.5 Hz) and the contractile phase of crawling (at 0.04 – 0.1 Hz), so the nerve serves as a monitor of ongoing locomotory behavior. The purpose of recording with two suction electrodes was to confirm the propagation of behaviors down the nerve cord. To elicit swimming, DP12 or DP13 was stimulated with 10 ms electrical pulses delivered at 15Hz, with an amplitude of 2-3 V. We often observed spontaneous swim episodes as well. To elicit crawling, several tail brain nerves were stimulated with a suction electrode using the DP nerve stimulus protocol (Eisenhart et al., 2000). In all experiments, we stabilized the imaged ganglion by pinning small latex strips across the adjacent nerve cord to minimize motion artifact (Cacciatore et al. 1999). The preparation was maintained in a chamber filled with ~10 ml of room temperature leech saline, consisting of (in mM): 115 NaCl, 4KCl, 1.8CaCl$_2$, 1.5 MgCl$_2$, 10 dextrose, 4.6 Tris maleate, and 5.4 Tris base, pH 7.4.
Staining with FRET dyes

The same staining protocol as described in Briggman et al., 2005 was used. We used a FRET-based voltage sensitive dye consisting of a coumarin (donor) molecule and an oxonol (acceptor) molecule (Panvera LLC, Madison, WI). Briefly, we bath loaded a 10 μM concentration of coumarin dye for 30 minutes and a 12.5 μM concentration of oxonol dye for 30 minutes. The bath was replaced with cold leech saline following the staining protocol.

Electrophysiology and cell fills

We recorded intracellularly from cells using 40-60 MΩ glass microelectrodes filled with 1M potassium acetate, using an AxoClamp 2A amplifier (Axon Instruments, Foster City, CA). For each experiment, we monitored two cells intracellularly. For dorsal experiments, we impaled cell 1 and cell 3 and recorded for the duration of the experiment. For ventral experiments, we impaled cell CV and cell AE. These recordings provided additional information about the behavioral state of the preparation as well as a measure of the sensitivity of the optical signals. We recorded extracellularly using suction electrodes and a four-channel differential amplifier (model 1600; A-M Systems, Sequim, WA). We digitized all electrical data at 10 kHz using a 16-bit analog-to-digital board (PCI-6035E; National Instruments, Austin, TX) and custom Matlab (Data Acquisition Toolbox; The Mathworks, Natick, MA) software. We filled neurons using Alexa 488 dextran (10,000 MW; Molecular Probes, Eugene, OR). Microelectrodes were back-filled using concentrations of 25 mg/ml Alexa 488. Cells were then impaled, and dye was iontophoretically injected. Dye was allowed to diffuse for ~ 1 hr, and tissue was fixed overnight, dehydrated, cleared, and mounted on a slide for viewing. Fills were imaged using a confocal microscope (1024ES; Bio-Rad, Hercules, CA).
Optical recording

We used the optical recording system described in Briggman et al., 2005. Briefly, signals were acquired by ratioing alternating frames recording with a cooled CCD camera (NeuroCCD-SM256; RedShirtImaging LLC, Fairfield, CT) at a resolution of 256 x 256 pixels. The frame rate was set according to which behavior was recorded. For swimming, images were acquired at 20 Hz, yielding ratioed signals at 10 Hz. Trials at this frame rate were 10 s. in duration. For crawling, 50 ms. frames were acquired twice per second, yielding 2 Hz ratioed signals. Trials at this frame rate were 60 s. in duration. In the 2 Hz configuration, the excitation light was shuttered off in between frame captures to limit phototoxicity. Imaging data were acquired using custom software written in Matlab (Data Acquisition Toolbox; The Mathworks). We synchronized the optical and electrical recordings by feeding the frame timing signals emitted by the camera into the A-to-D board, along with all of the electrophysiology signals.

Analysis

In a typical experiment, we imaged at least 5 trials each of swimming and crawling. Successful swimming and crawling trials were concatenated, respectively, for further analysis. After acquiring the data, we manually outlined cell bodies and analyzed the resulting signals using Matlab. The phase and magnitude of the coherence was calculated, as described in Taylor et al. 2002, for each cell at the peak oscillation frequency of each behavior. This frequency ranged between 0.04 – 0.1 Hz for crawling and 1 – 1.5 Hz for swimming. For coherence estimates, we used a frequency resolution of 0.67 Hz for swimming, and 0.05 Hz for crawling. The α level for significance was chosen so that the multiple comparisons level for any trial did not exceed 0.05. For dorsal side experiments, the optical signal of a cell 3 was used as the reference for both swimming and crawling. The phase plots were aligned to cell 3’s known phase in swimming and crawling. For ventral side experiments, the optical signal of cell AE was used as the reference for
crawling. Phase plots for ventral crawling were aligned to cell AE’s known phase during crawling. For swimming, a cell in the anterior lateral glial packet with a consistently large oscillation during swimming was used as a reference. Phase plots for ventral swimming were then aligned to cell 208’s known phase during swimming. Cell 208 was observed in each ventral surface imaging experiment. For the calculation of crawling step periods, we followed the procedure outlined in Eisenhart et al., 2000.
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Bibliography

Abarbanel, H, Analysis of Observed Chaotic Data (Spring-Verlag, New York, 1996).


Kaplan D, Glass L, Understanding nonlinear dynamics (Springer-Verlag, New York, 1995).


