CHAPTER 3:

Toolkit

INTRODUCTION

The extensive number of tools available in the *C. elegans* toolkit helps make it an attractive model system for the study of neuroscience. The most obvious is the number of genetic tools available for use. The ease of maintainence, small size, fast generation time, large numbers of identical progeny, and relatively small genome has allowed the characterization of a large mutant library, genetic mapping, and high degree of genetic control over targeting and characterizing specific neurons.[1] The transparent body of *C. elegans* allows live imaging of neuronal activity and development in live, intact animals[2], and electrophysiological methods have been developed to study neuronal activity in the form of graded potentials. In addition, optical stimulation techniques allow temporal control as wells as extent of activation in the intact freely behaving animal.[2,3]

3.1 ELECTROPHYSIOLOGY IN *C. ELEGANS*

The first nematode measured with electrophysiological techniques was *Ascaris lumbrocoides*, a worm with large neurons and graded active synaptic responses.[4] Despite their difference in size, there is striking anatomical similarity between *Ascaris* and *C. elegans*[1], and the studies of the *Ascaris* locomotor circuit provided insight into the excitatory and inhibitory function of specific *C. elegans* motor neurons.

Electrophysiology in *C. elegans* requires puncture of the external cover or cuticle, hence measurement of intact circuits is challenging. The body of *C. elegans* is an unsegmented, tapered cylinder whose structure is maintained by a tube-like arrangement of muscles attached to the hypodermis and a tough cuticle cover. The shape of the body is maintained by its high internal pressure, much like a balloon, and like a balloon, disruption of cuticle integrity results in increased likelihood of rupture. In the case of *C. elegans*, a large enough rupture also means an extrusion of organs and eventual death.

Early electrophysiological measurements in *C. elegans* focused on the neuromuscular functions of the pharynx, and these electropharyngeograms were recordings of extracellular potentials.[5] Eventually, techniques were developed and modified to record synaptic activity at the neuromuscular junction[6] and then to record individual sensory and interneurons[7]. These are high-resolution measurements that allow calculations such as transfer functions of individual synapses.[7] However, because these techniques are so intrusive and disrupt the local environment

of neuropeptides and signaling factors of the recorded neurons, it is not ideal for the study of state modulation. Measurements in the intact worm are necessary and ideal.

3.2 CALCIUM IMAGING IN *C. ELEGANS*

The discovery and use of green fluorescent protein, or GFP, has been most useful in serving as a visual marker for temporal and spatial expression of genes, identification of structure and function, as well as in opening up the field for development of a variety of new tools. Derivatives of the original GFP have been used for a variety of functions, including the fusion to proteins in order to identify compartment-specific localization and dynamics, identify co-localization of different proteins using variants that modify the excitation and emission spectra of the fluorescent protein, and split derivatives serving as detectors for the presence of various molecules or for two molecules' proximity to each other. Of course, the expression of any of these protein and products can serve as buffers and compete with the natural processes that are studied. This should be kept in mind in the context of behavior.

The calcium indicators are fluorescent detectors that increase their fluorescence in the presence of available calcium. Small molecule calcium dyes are an indirect indicator of neuronal activity, but these require injection and lack cell specificity.[8] Genetically encoded calcium indicators allow visualization in an intact animal and availability of a large number of known promoters provide cell specificity. These indicators are based on the protein *calmodulin*, which changes its conformation in response to binding of calcium. The calcium binding region of calmodulin is used to link two proteins, of which one or both are fluorescent molecules, and the changing distance between the fluorescent particles either shifts the spectra or changes the intensity of the wavelengths of light emitted.

GCamP is a calcium indicator that is a fusion of GFP, calmodulin, and M13. In the absence of calcium, the conformation of the circularized GFP allows quenching of the fluorophore, which is altered upon calcium binding.[9] Therefore, there is a dramatic and fast increase in fluorescence in response to calcium. Cameleon uses foster resonance energy transfer (FRET). FRET works through the use of two fluorophores: the donor which is excited with an external light source and an acceptor whose excitation spectra corresponds to the emission spectra of the donor. FRET requires the proximity of the donor and acceptor. Therefore, the ratio of the donor and acceptor emission that can be detected changes with alterations of the calmodulin conformation, and the acceptor to donor ratio increase upon binding of calcium[10].

Ratiometric imaging has its drawbacks and advantages. The non-FRET indicators have better dynamic range, and cameleon often shows a smaller change in response to the same stimulus in a given neuron when compared to GCamP. However, there are also many advantages to ratiometric imaging: movement and lighting artifacts can be detected and cancel out in the ratiometric calculation, altered promoter activity and expression of indicator is not an issue, and it allows for detection of fast events even in the scale of milliseconds.[11] The hardware for ratiometric imaging is a bit more complicated, but worth it. However, I find that the largest drawback to using the FRET indicator in my studies is the effect on behavior.

Light is a potent stimulant and serves a noxious cue in *C. elegans*[12], and it avoids short wavelengths of light by staying beneath the surface of the soil. Animals avoid light and it is shown that illumination with the green to ultraviolet spectra of light is detected by LITE-1 and will induce escape response as well as increase locomotion.[12] We have found that exposure to high intensity light even in the absence of *lite-1*, will induce calcium transients in the ASH nocioceptive sensory neuron of adult animals, as well as those in lethargus. Furthermore, shorter wavelengths of light elicit stronger responses.[12] Therefore, although use of low intensity light for cameleon imaging fails to elicit sensory response in lethargus, it serves as a stimulus in L4 and adult animals which is not ideal and changes sensory response to chemical stimuli.

3.3 THE OPTOGENETIC TOOLBOX

Several techniques have been developed to remotely and optically control neurons in the intact worm. Channelrhodopsin is an algae-derived cation channel that is a microbial-type rhodopsin, a seven transmembrane retinal protein that has no sequence homology to animal rhodopsins. Channelrhodopsin1 is a proton-selective light-activated channel derived from the green algae *Chalmydomonas reinhardtii.*[13] Channelrhodopsin2 is a leaky proton pump that acts as a light-gated nonselective cation channel[14] that has the ability to trigger large currents. A cofactor, *all-trans* retinal, is required for channel activity and the peak of the action spectrum is approximately 460nm. The ChR2 conductance has a large initial transient and decays to a lower steady-state level with continued illumination. The refractory period after activation is shorter at low extracellular pH and at more negative voltages. The estimated conductance of the channel is a low 50 fS. The rise time of the ChR2 current is extremely fast: less than 200 μs; decay is on the order of milliseconds and is pH sensitive.[14]

The first characterizations of ChR2 involved the control of action potential and synaptic transmission in hippocampal neurons.[15] Currently, photoactivatable channels have been used in a range of animal models including worms, flies, fish, rodents and primates.[16-20] In *C. elegans,* ChR2 can be used to drive awake behavioral responses in the presence of the cofactor all-*trans* retinal (ATR).[16] Animals can be fed ATR through the bacteria they eat, and specific promoters can be used to selectively promote ChR2 expression and activation. In addition to study of behavior, it has been used to study release at the neuromuscular junction in worms.[21]

In addition to ChR2, many other types of light-activated channels and molecules exists. Optical inhibition can be induced by using halorhodopsin, and G-protein signaling pathways can be activated by the opto-XRs. Halorhodopsin is a yellow-light activated chloride pump derived from archaebacterium *Natronomas pharaonis* that works with millisecond precision.[22] Use of optical stimulation techniques allow fast, reliable manipulation of neuronal activation and inhibition in specific subsets of neurons. Recent advances provide light-shifted varieties, as well as long-acting and subthreshold channelrhodopsins. In addition, various strategies have been used for cell-specific activation and expression of these light-activated channels.

3.4 MICROFLUIDICS

Microfluidic devices were first developed to miniaturize chemical and biochemical analyses and to make these processes more sensitive, faster, and have higher resolution.[23] Since then, microfluidic devices fabricated in polymers (polymethylsiloxane or PDMS) using soft lithography have been useful in basic and biomedical applications.[24] Microfluidics uses laminar flow and allow precise control and quick manipulation of microenvironments that are not available in conventional macro-scale methods.[25] These devices have been used for cell culture as well as neuronal stimulation[26], and *C. elegans* with its small size of 2-3mm is well-suited for this technology.

Various microfluidic devices exist for immobilization of animals, observation of locomotion, and exposure of animals with either gas or aqueous stimuli. These devices allow simultaneous control of the animals' environment while imaging, and has allowed measurement of sensory response to a variety of stimuli.[27] Manual and automated[28] controls of these devices are possible. And because they are made to conform to the shape of the animals, they allow feeding activity. Additionally, these devices allow fast and reproducible delivery of stimuli, do not rely on the use

of adhesive, and provide more consistent behavior and response than other methods of immobilization.

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